

FABRICATION OF A BIOSIMULATED NERVE REPAIR DEVICE FOR GUIDED AXON REGENERATION AND CONTROLLED RELEASE OF BIOACTIVES FOR THE TREATMENT OF PERIPHERAL NERVE INJURY

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A thesis submitted to the Faculty of Health Sciences, University of the
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Doctor of Philosophy



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DECLARATION

I, Poornima Ramburrun, declare that this thesis is my own work. It is being submitted for the degree of Doctor of Philosophy of Pharmacy in the Faculty of Health Sciences at the University of the Witwatersrand, Parktown, Johannesburg, South Africa. It has not been submitted before for any other degree or examination at this or any other university.

.....

Signed this day of October 2017

RESEARCH OUTPUTS

Publications

1. Ramburrun, P., Kumar, P., Choonara, Y. E., Bijukumar, D., du Toit, L. C., and Pillay, V. (2014). A Review of bioactive release from nerve conduits as a neurotherapeutic strategy for neuronal growth in peripheral nerve injury, *Biomed Research International*, **Special Issue: Peripheral Nerve Regeneration: Mechanism, Cell Biology, and Therapies**, 2014, 2-18. (ISI, Impact Factor: 1.579) (Review Article). (Appendix A1).
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3. Ramburrun, P., Kumar, P., Choonara, Y. E., du Toit, L. C., & Pillay, V. Design and Characterization Neurodurable Gellan-Xanthan pH-Responsive Hydrogels for Controlled Drug Delivery. (Published Online: *Expert Opinion on Drug Delivery*, Accepted: November 2016) (ISI, Impact Factor: 5.343) (Research Article). (Appendix A3).
4. Ramburrun, P., Kumar, P., Choonara, Y. E., du Toit, L. C., & Pillay, V. Design, Characterization and Mechanical Evaluation of Neuroprotective PHBV-Magnesium Oleate Aligned Nano-Fibers for Axonal Guidance and Direction. (To be submitted to *Journal of the Mechanical Behaviour of Biomedical Materials*).
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Conference Proceedings

1. Ramburrun, P., Choonara, Y.E., Kumar, P., du Toit, L.C. and Pillay V. Design of Hydrogel Matrices with Intercalated Pristine-Polymer-Particles for Enhanced Porosity and Extended Bioactive Release via In situ pH-Responsive Particulate Leaching, Wits Faculty of Health Sciences Research Day 2015, 8 September 2015. **(Poster)**. (Appendix B1).
2. Ramburrun, P., Choonara, Y.E., Kumar, P., du Toit, L.C. and Pillay V. Intercalation of Hydrogel Tissue-Repair Scaffolds with Pristine-Polymer-Particles for Enhanced Porosity and Extended Bioactive Release, Young Scientists Competition, The Academy of Pharmaceutical Sciences of the Pharmaceutical Society of South Africa (APSSA) Conference 2015, 17-19 September 2015. **(Podium)**. (Appendix B2).
3. Ramburrun, P., Choonara, Y.E., Kumar, P., du Toit, L.C. and Pillay V. Design of Hydrogel Matrices with Intercalated Pristine-Polymer-Particles for Enhanced Porosity and Extended Bioactive Release via In situ pH-Responsive Particulate Leaching, American Association of Pharmaceutical Scientists (AAPS) Annual Meeting and Exposition 2015, 25-29 October 2015, Orange County Convention Centre, Orlando, Florida, USA. **(Poster)**. (Appendix B3).
4. Ramburrun, P., Kumar, P., Choonara, Y.E., du Toit, L.C. and Pillay V. Bioactive-releasing neurodurable porous hydrogel bioscaffolds for tissue-repair, International Conference on BioTribology (ICoBT) 2016 , 11-14 September 2016, Imperial College London, Kensington, London, UK. **(Podium)**. (Appendix B4).

PATENTS

Ramburrun P, Kumar P, Choonara YE, du Toit LC, Pillay V. Pillay. Pristine-polymer intercalated hydrogel drug conduit. Patent P1074PC PCT Filed.

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ABSTRACT

The constant challenges imposed by the clinical procedures in obtaining adequate nerve repair and return of functional recovery to the injured site has buoyed the pursuit for artificial alternatives instead of the utilization of donor tissue grafts for surgical repair. Transected nerve injuries, having tremendous potential to cause permanent paralysis, neuropathies and chronic pain, requires the collective involvement of tissue-engineering and advanced drug delivery applications in combating the perceived challenges associated with its repair. Modern peripheral nerve repair strategies employ the use of polymeric-engineered nerve conduits incorporated with components of a delivery system. This allows for the controlled and sustained release of neurotrophic growth factors and other bioactives for improvements in the innate regenerative capacity of neural tissues. Although currently available synthetic hollow nerve conduits available for clinical use, as an alternative to the nerve autografts, have proven to be successful in the bridging and regeneration of primarily short nerve gap injuries, recent developments entail advancement of nerve conduits to be able to simulate the effectiveness of the autograft which includes, in particular, the ability to deliver growth factors and mimic peripheral nerve structure. A critical factor influencing the level of success of peripheral nerve repair and regeneration is the development of appropriate sustained drug delivery systems for the release of neurotrophic factors and other small molecular weight drugs in addition to the provision of a mechanically stable, biocompatible and preferably biodegradable repair scaffold.

This study, therefore, aimed to investigate and establish the proposed concept of pristine polymer particle intercalation as a novel strategy for achieving controlled and sustained release of bioactives from polymeric implants. An initial study involving an investigation into the drug release mechanisms of indomethacin from swellable sodium tripolyphosphate-crosslinked chitospheres laden with pristine pH-responsive polymethylmethacrylate (PMMA) nanoparticles sought to prove the concept of particle intercalation as a modulator for drug release. Swelling and erosion studies, in conjunction with textural profiling, provided an understanding of the dominant and underlying drug release mechanisms of the chitospheres loaded with the pristine PMMA particles. Drug release studies showed that the pristine particle-intercalated chitospheres extended the release of indomethacin over 144 hours in a first-order compared to the 72 hours release achieved with the control. The study further revealed that in situ porogen leaching leading to porous network and polyelectrolyte complex formation were major mechanisms governing drug release from the chitospheres. Having

successfully proved the concept of pristine polymer particle intercalation, this technique was consequentially applied to the development of a nerve conduit for the dual and controlled-release of proteins and small-molecule drugs.

The design and fabrication of a hollow nerve conduit comprised a physically crosslinked interpenetrating network of a gellan-xanthan hydrogel matrix intercalated with pristine PMMA particles for provision sustained and concurrent release of two model compounds: bovine serum albumin (BSA) and diclofenac sodium. Analysis of a Box-Behnken experimental design demonstrated a near zero-order release of BSA and diclofenac sodium over 20 and 30 days, respectively, modulated via a combination of pH-responsive (pH 7.4) dissolution of the intercalated pristine polymer particles and the unique gelling and erosion properties imparted by the graded addition of xanthan gum to the hydrogel blend. Moreover, the concentration-dependent intercalation of PMMA enhanced matrix resilience while alterations in the gellan-xanthan proportions yielded a means for customizing the mechanical characteristics, particularly matrix rigidity and flexibility, for the attainment of neuro-durable conduits. Optimization of the hydrogel conduit provided an effective means for the sustained delivery of the essential but costly neurotrophic growth factor, Nerve Growth Factor (NGF), over 30 days whilst maintaining its bioactivity. Further advancement of the hollow-lumen gellan-xanthan conduits involved implementation of intraluminal guidance scaffolds in an endeavour for simulating the native structural features of peripheral nerve tissue. An array of electrospun aligned nano-fibrous scaffolds formulated for the inclusion of physical, chemical and therapeutic guidance cues was achieved through the blending and incorporation of polyhydroxy butyric-co-valeric acid base polymer with magnesium-oleate and N-acetylcysteine as potential neuro-active agents as indicated by the enhanced proliferation of PC12 neuronal cells. Integration of the two components, the hydrogel conduit and the nano-fibrous scaffold, heralded the assembly of the final Biosimulated Nerve Repair Device (BNRD) which proved proficient in an *in vivo* rat sciatic nerve model in promoting nerve regeneration and re-establishment of functional recovery compared to the autograft gold standard repair as determined by behavioural assessments and morphometric and histological analysis of muscle and nerve tissues.

The devised BNRD system for the surgical repair and therapeutic treatment of transected peripheral nerve injuries was successful in providing a long-term neuro-durable scaffold with release kinetics appropriate for the adequate promotion of tissue regeneration and healing bringing about restoration of functional recovery through emulating the positive characteristics of the autograft. The results obtained show promise for the rather challenging repair of peripheral nerves.

DEDICATION

This thesis is dedicated to my present and fore families for their immeasurable support and encouragement and their unwavering belief in my capabilities.

ANIMAL ETHICS DECLARATION

I hereby confirm that the following study entitled “An evaluation of the regenerative potential of a polymer-based nerve conduit for the controlled release of bioactives in treating peripheral nerve injury in Sprague Dawley rats” has received approval from the Animal Ethics Screening Committee of the University of the Witwatersrand with ethics clearance number 2014/42/C (Appendix C1).

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LIST OF ABBREVIATIONS

%CrI – % Crystallinity Index
3Ps – Pristine polymer particles
AESC – Animal Ethics Screening Committee
ANOVA – Analysis of Variance
AUC – Area Under the Curve
BDNF – Brain-Derived Neurotrophic Factor
BET – Brunauer-Emmett-Teller
BJH – Barrett, Joyner and Halenda
BNRD – Biosimulated Nerve Repair Device
BSA – Bovine Serum Albumin
CaCl₂ – Calcium chloride
CAS – Central Animal Services
CI – Crystallinity Index
DEE – Drug Entrapment Efficiency
DES – Donor Equine Serum
DF – Deformability Modulus
DHT – Dehydro-thermal Treatment
DMEM – Dulbecco's Modified Eagle Medium
DRG – Dorsal Root Ganglia
DS – Diclofenac sodium
DSC – Differential Scanning Calorimetry
DS-F – Diclofenac sodium-loaded
DS-L – Diclofenac sodium-free
ECM – Extra Cellular Matrix
EDS – Energy Dispersive X-ray Spectroscopy
EngNT – Engineered Neural Tissue
FBS – Foetal Bovine Serum
FE – Fracture Energy
FTIR – Fourier Transform Infrared
GCMs – Gelled-Core-Microenvironments
GDNF – Glial-Derived Neurotrophic Factor
Gel-Xan – Gellan-Xanthan
GG – Gellan gum

HCMs – Hollow-Core-Microenvironments
 IPN – Interpenetrating Network
 MDT – Mean Dissolution Time
 MgOI – Magnesium-Oleate
 MR – Matrix Resilience
 MTT – Methyl-thiazolyl-tetrazolium
 n – Sample number
 NAC – N-Acetyl cysteine
 NAC-F – N-Acetyl cysteine-free
 NAC-L – N-Acetyl cysteine-loaded
 NGF – Nerve Growth Factor
 NT-3 – Neurotrophin-3
 NTFs – Neurotrophic Factors
 P/S/AB – Penicillin/Streptomycin/Amphotericin B
 P⁴[Gel-Xan]IPNs – Polymethylmethacrylate Pristine Polymer Particle Intercalated Gellan-Xanthan Interpenetrating Networks
 PBS – Phosphate buffered saline
 PCL – Poly(caprolactone)
 PDLLA – Poly(D,L-lactic acid)
 PEAD – Poly(ethylene argininy aspartate diglyceride)
 PEC – Polyelectrolyte complex
 PEG – Poly ethylene glycol
 PG – Propylene glycol
 PHAs – Polyhydroxyalkanoates
 PHBV – Poly(3-hydroxybutyric acid-co-3-hydroxyvaleric acid)
 PLGA – Poly(lactic-co-glycolic) acid
 PLLA – Poly(lactide)
 PMMA – Polymethyl methacrylic acid
 PPE – Polyphosphoester
 SC – Schwann cell
 SEM – Scanning Electron Microscopy
 SFI – Sciatic Function Index
 SH3 – Src Homology Domain 3
 SOPs – Standard Operating Procedures
 STPP – Sodium tripolyphosphate
 TA – Texture Analysis
 T_d – Degradation temperature

T_g – Glass transition temperature

TGA – Thermo-Gravimetric Analysis

T_m – Melting temperature

T_{mc} – Melt crystallization temperature

UV – Ultraviolet

XG – Xanthan gum

XRD – X-ray Diffraction

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CHAPTER 1

INTRODUCTION AND MOTIVATION FOR STUDY

1.1. Background to the Study

Peripheral nerve injuries can result in lifelong disability with the potential of diminishing a patient's quality of life. Stressors such as neuropathic pain, poor or absence of functional recovery and muscle atrophy associated with nerve injuries further burden a patient's state of health (Oliveira *et al.*, 2012). Motor vehicle and workplace accidents resulting in bone fractures and deep tissue lacerations are frequently responsible for crush and severed nerve injuries (Ciaramitaro *et al.*, 2010). Much research is focused on the traumatic injuries causing ligation of a peripheral nerve resulting in a gap defect between the severed nerve stumps. The limited innate ability of nerves to regenerate and the inhibitory mechanisms impeding neural tissue regeneration following injury to the nerves intensifies the search for an effective yet simple solution for the promotion of healthy tissue proliferation and reduction in the inflammatory processes hindering the healing capacity of the nervous system (Cao *et al.*, 2009).

The gold standard of treatment for severed peripheral nerve injuries is the nerve autograft which involves the harvesting of donor nerve tissue from elsewhere in a patient's body and implanting it across the gap defect. Although this technique of nerve repair produces effective levels of nerve regeneration and functional recovery, its major drawbacks of multiple surgical incision sites, scarring, lack of donor tissue availability and donor tissue size mismatches restrains its clinical use particularly in long gap nerve injuries exceeding 10mm (Strauch and Strauch, 2013). The difficulties associated with insufficient donor tissue collection and resulting comorbidities necessitates the need for alternative treatment modalities. These alternative treatment techniques comprise artificially constructed materials as a replacement for the harvesting of native tissues. Polymer-engineered materials, preferably biodegradable, are crafted into tube-like devices, nerve conduits, to bridge the gap between severed nerves to assist regeneration. There are a number of commercialised synthetic nerve conduits that have been approved for clinical use in repair of short gap injuries however their success rate is still deemed inferior compared to the nerve autograft (Kehoe *et al.*, 2012; Dinis *et al.*, 2015).

Research studies employing *in vivo* animal models have shown that nerve conduits, synthesised from a range of natural and synthetic polymers, modified to incorporate neurotrophic factors (NTFs), seeded cells or extra-cellular matrix components enhance regeneration in comparison to conduits offering none of the former (Pabari *et al.*, 2011; Kehoe *et al.*, 2012). When considering the inclusion of NTFs within a conduit (Figure 1.1), the release kinetics of the delivery system is of particular importance with regards to initial burst release, the quantity of NTFs released, and the pattern of its sustained release thereafter. Failure of tailoring release kinetics from a conduit affects the potential of axonal regeneration. The release of high doses resulting from uncontrolled burst release may hinder axonal sprouting due to a reduction in the extent of affinity binding for the receptor sites resulting in a lower therapeutic effect achieved in the target tissues (Goodman and Gilman, 2008). Whereas suboptimal doses of NTFs evidently does not elicit an adequate effect on nerve regeneration, large doses are detrimental as they may hinder axonal growth due to the down regulation of TrkA and loss of affinity of TrkA to the growth factors, i.e. nerve growth factor (NGF), in order to initiate a growth response (Allen and Dawbarn, 2006; Ichihara *et al.*, 2008; Kemp *et al.*, 2011; Siniscalco *et al.*, 2011). Therefore, the principal aim of this research project was to investigate a suitable means of controlling the release of bioactives using the intercalation of pristine polymer particles within a scaffold-based matrix system. A crosslinked polysaccharide blend was formulated into a hydrogel nerve conduit for the sustained and simultaneous delivery of NGF and diclofenac sodium via the intercalation of pristine polymer particles. Although the delivery of exogenous NTFs have proven advantageous in attaining an improved quality of peripheral nerve regeneration, its performance on nerve repair lies short in comparison to the nerve autograft.

Currently, increasing efforts are being made to the design of nerve conduits that are able to closely mimic the structure and function of native nerve tissues (Daly *et al.*, 2012; Dinis *et al.*, 2015; Sridharan *et al.*, 2015). This strategy is thought to improve the promotion of nerve regeneration to a level equal to or above that of a nerve autograft (Conti *et al.*, 2004; Allen *et al.*, 2013; Ma *et al.*, 2013; Tavangarian *et al.*, 2013). To achieve this degree of resemblance to native nerve tissues, numerous physical, chemical, and biological factors (Figure 1.2) have to be taken into consideration when incorporating multi-functioning components into a nerve conduit (Shakhbazau *et al.*, 2012; Georgiou *et al.*, 2013).

Peripheral nerve injury initiates processes of inflammation, rapid nerve destruction due to Wallerian degeneration and the onset of pain syndromes (Subramanian *et al.*, 2009). To allow swift recovery and return of physical functioning to the patient, a multifaceted approach is required to target *all* these injury mechanisms. The use of implantable polymeric multifunctional drug delivery technology can aid tissue regeneration, prevent further destruction and alleviate pain syndromes through controlled and targeted drug delivery applications. An additional advancement of this research project intends to equip the previously described hydrogel nerve conduit with multi-functional intraluminal support structures as a strategy for guided axonal regeneration and improved outcomes of functional recovery via artificial simulation of the autograft.

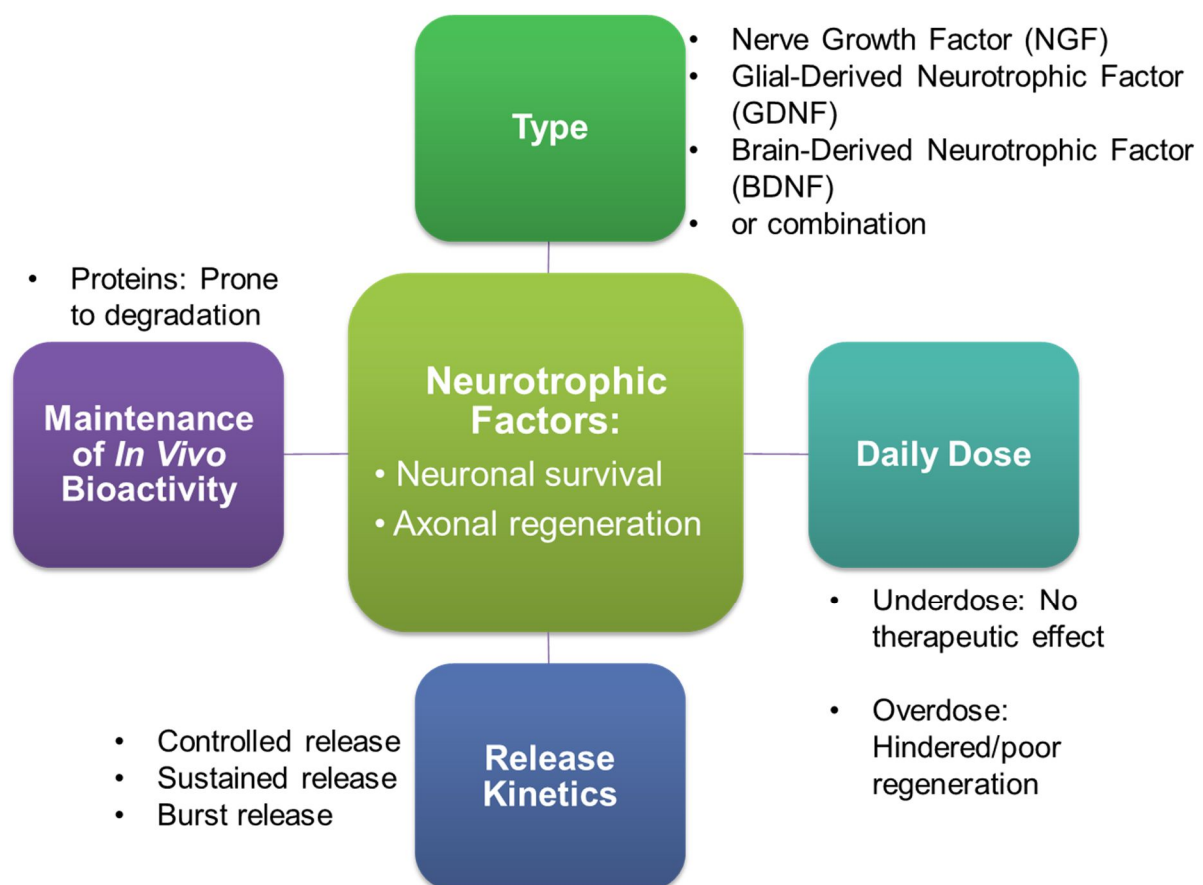


Figure 1.1: Basic consideration aspects of neurotrophic factor delivery.

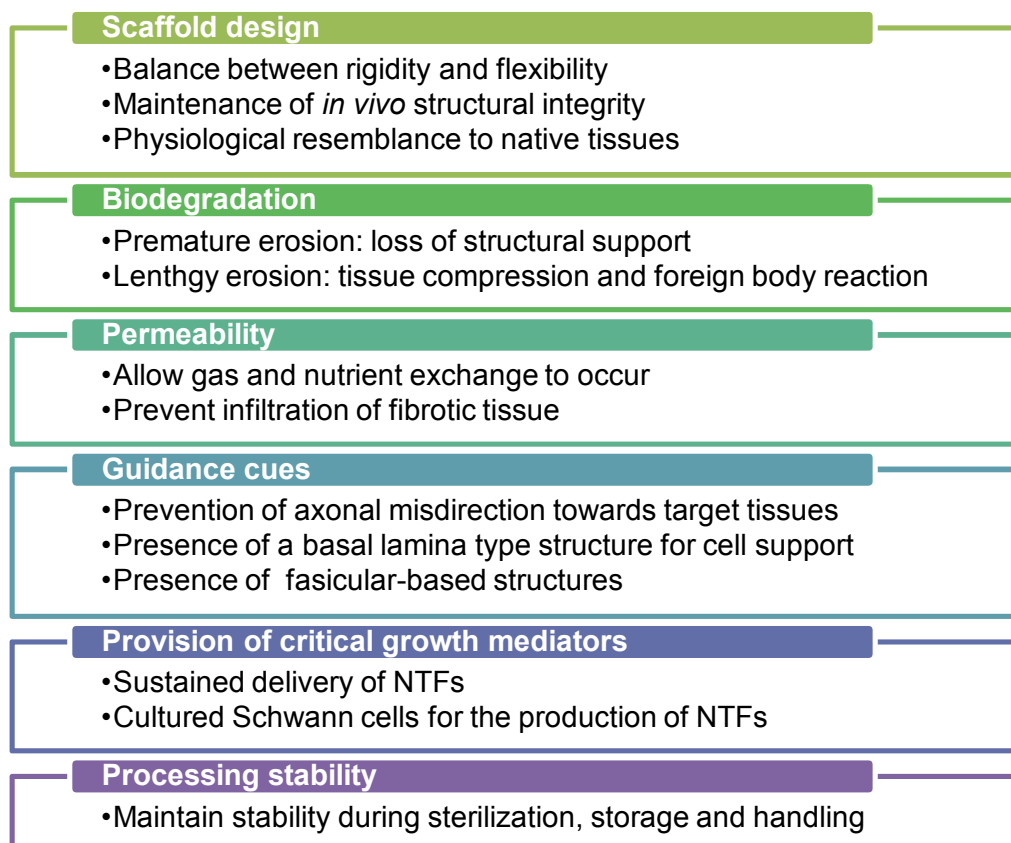


Figure 1.2: Key components of a peripheral nerve regeneration design strategy.

1.2. Rationale and Motivation for the Study

An estimated 2 million people globally suffer from peripheral nerve injuries every year (Sridharan *et al.*, 2015). Approximately 500 000 nerve repair procedures are performed on an annual basis in the USA and Europe collectively with over \$7 billion being incurred due to paralysis resulting from nerve injuries (Belkas *et al.*, 2004; Ichihara *et al.*, 2008). Traumatic peripheral nerve injuries are becoming common in clinical practice due to the increased incidence of road and motor vehicle accidents. The gold standard of peripheral nerve injury treatment, the autograft, presents with many drawbacks such as limited donor tissue availability and secondary deformities resulting from multiple surgery sites. Due to the complications of long-term disability and poor functional recovery of the affected site, peripheral nerve injuries remain a challenge for neurosurgeons and reconstructive surgeons. Although a number of autograft alternatives are commercially available as polymeric bridging devices for the repair of transection nerve injuries, their clinical efficacy is yet to be considered adequate (Kehoe *et al.*, 2012).

Peripheral nerve repair by the body ensue complex stages, of which the mechanisms are not yet completely understood, of neurodegeneration and inflammatory responses before commencement of axonal regeneration. During these physiological processes' attempt at nerve regeneration, site-specific controlled-release bioactive-loaded implants offer effective delivery of neurotrophic growth factors and other therapeutic drugs to alleviate pain and terminate inflammatory responses in addition to provision of a structural support for guided nerve regrowth. This is accomplished through enhanced bioavailability and maintenance of effective therapeutic drug levels over prolonged periods by virtue of a finely controllable drug-release mechanism. Improved systemic side-effect profiles and decreased dosing frequency greatly impacts the level of patient compliance towards a treatment regimen. By providing such features using an implantable device, issues of patient compliance and multiple-dosing regimens, either via the oral route or injectable administration, are eliminated and disease progression decreases.

Bridging devices alone may promote the regeneration of tissues across a nerve gap defect however; functional recovery is often incomplete and poor. For successful nerve regeneration to occur leading to outcomes of improved functional recovery, artificial nerve conduits should possess attributes, as listed in Figure 1.1, that aim to mimic the physiological anatomy of nerve tissue, hence the superior clinical recovery outcomes following nerve autograft procedures.

The aim of this research is to design a nerve conduit with features that may simulate the native architecture of nerve tissue for the improvement of peripheral nerve regeneration and functional recovery by providing the sustained release of neurotrophic factors (nerve growth factor), inflammatory agents for the management of inflammation and pain syndromes (diclofenac sodium) and the inclusion of structural scaffolds for enhanced axonal direction at a cellular level.

1.3. The Mechanism of Action of the Biosimulated Nerve Repair Device

The novel feature of this drug delivery design is the intercalation of pristine polymer particles (3Ps) within the matrix of a polymeric implant to obtain an intercalated network system offering a dual-mechanism controlled-release system using the difference in solubility profiles of the polymers where each polymeric component undergoes dissolution at a different pH and rate. The drug-release mechanism, governed by fluid penetration into the implant, results in swelling of the bioactive-loaded 3P intercalated matrix. The pH-responsive dissolution, at $\text{pH} > 7.0$, of the intercalated 3Ps out of the matrix may result in the formation of core microenvironment pore structures as shown in Figure 1.3. The formation of hollow or

gelled core microenvironments (HCMs or GCMs) acts as drug reservoirs that either promote or inhibit release of bioactive. The formation of HCMs or GCMs is dependent on the gelling and erosion properties of the matrix. In this approach, depending on the polymeric composition of the matrix, a combination of HCMs and GCMs may be formed which control the release of drug thereby offering a dual-mechanism release. GCMs may be able to provide sustained drug-release as the gelled microenvironment further retards the escape of drug molecules compared to hollow microenvironments which impose no barrier to the release of entrapped drug molecules.

The design of this drug delivery system allows the bioactive and HCM or GCM forming 3Ps to be incorporated into the implant in a single step during the fabrication process. Preformed hollow cylindrical conduits incorporate the bioactive and control-release component by injecting a drug-loaded nanoparticle or hydrogel formulation into the lumen of the conduit, either prior or post implantation, involving the need for additional steps during the fabrication and the surgical fixation procedures. Furthermore, axons in transected peripheral nerve injury require some form of a guidance cue, in addition to growth factors, to improve re-innervation of the target organ and eliminate misdirection of the sensory and motor neurons. After peripheral nerve injury, a weak fibrin network is formed by the body to guide regenerating nerves, however, even though the nerve fibers are able to bridge the gap functional recovery still remains poor. Therefore it is thought that incorrect re-innervation of motor and sensory neurons may be occurring. Hence, there is a need for a mechanically stable architecture to guide axons for enhanced motor neuron re-innervation and improved functional recovery of the affected site or limb.

An additional advancement to the project involves the design and inclusion of guidance cues within the hydrogel nerve conduit to enhance the innate regeneration capacity of the injured nerves (Figure 1.4). The incorporation of guidance cues will be achieved via the inclusion of intra-luminal fibers that not only acts as a solid physical guidance cue to direct axon growth but the fibers could be chemically modified and designed to provide the release of a number of therapeutic agents to assist the nerve in regenerating itself (Figure 1.5). Such a design offers an additional “trio” of novel features:

1. **Physical guidance** cues provide directional influence, axon attachment and growth for re-innervation of the target organ/muscle. The presence of a physical substrate and its surface textural properties (rough vs smooth) allows the attachment or alignment of Schwann cells along the intra-luminal fibers and forms a guide for regenerating axons via expression of p75 and TrK receptors in addition to the synthesis of endogenous growth factors.

2. **Chemical guidance** cues via surface modification of the intra-luminal fibers with polymers and molecules containing specific functional groups that serve as guidance cues. Certain functional groups, such as amines, presenting a positive charge could improve the adhesion and attachment of regenerating cells. The delivery of exogenous and the enhanced synthesis of endogenous growth factors from the fibre-adhered Schwann cells serve as chemotactic stimuli for axonal growth and direction.
3. **Therapeutic guidance** cue via incorporation of bioactive agents such as growth factors, anti-inflammatories, corticosteroids and vitamin B12 with the fibers. Different growth factors such as NGF and BDNF or GDNF act as agents for promoting the growth of sensory and motor neurons, respectively. This further allows improved re-innervation and misdirection of motor and sensory neurons. The inclusion of essential nutrients assists with attaining healthy and functional newly regenerated nerve tissues and the release of anti-inflammatory drugs reduce pain, swelling and further inflammation-mediated tissue destruction thus prompting recovery.

Additionally, the alignment of intraluminal fibers within the hydrogel nerve conduit as a luminal filler may enhance the structural integrity of the conduit post implantation and hydration. The force exerted by the intraluminal fibers on the walls of the conduit could prevent the conduit from possible kinks or collapse via the reduction of void space. Moreover, the entrapment of therapeutic agents within the intraluminal fibers prevents extensive escape of these agents out of the conduit and into the surrounding tissues. In this manner any released bioactive molecules will remain for a longer time inside the conduit lumen where it will be readily available to the regenerating axons. This final assembled system would provide a two-step release of bioactives: 1) release from conduit walls during the initial stages of nerve regeneration 2) maintenance release of therapeutic agents from the intraluminal fibers to further facilitate axonal regeneration and target re-innervation.

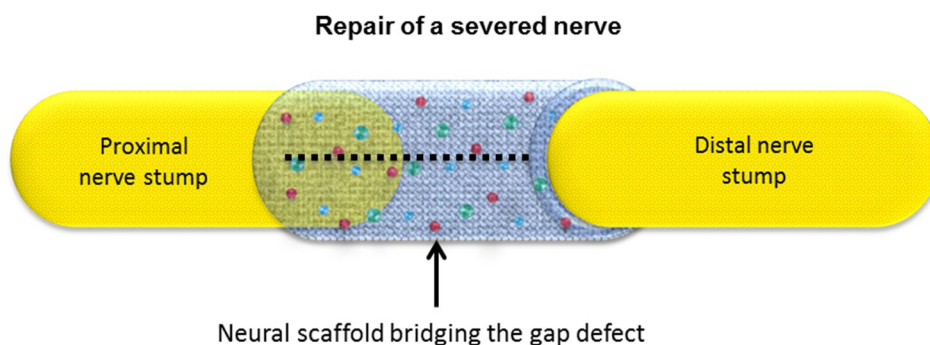
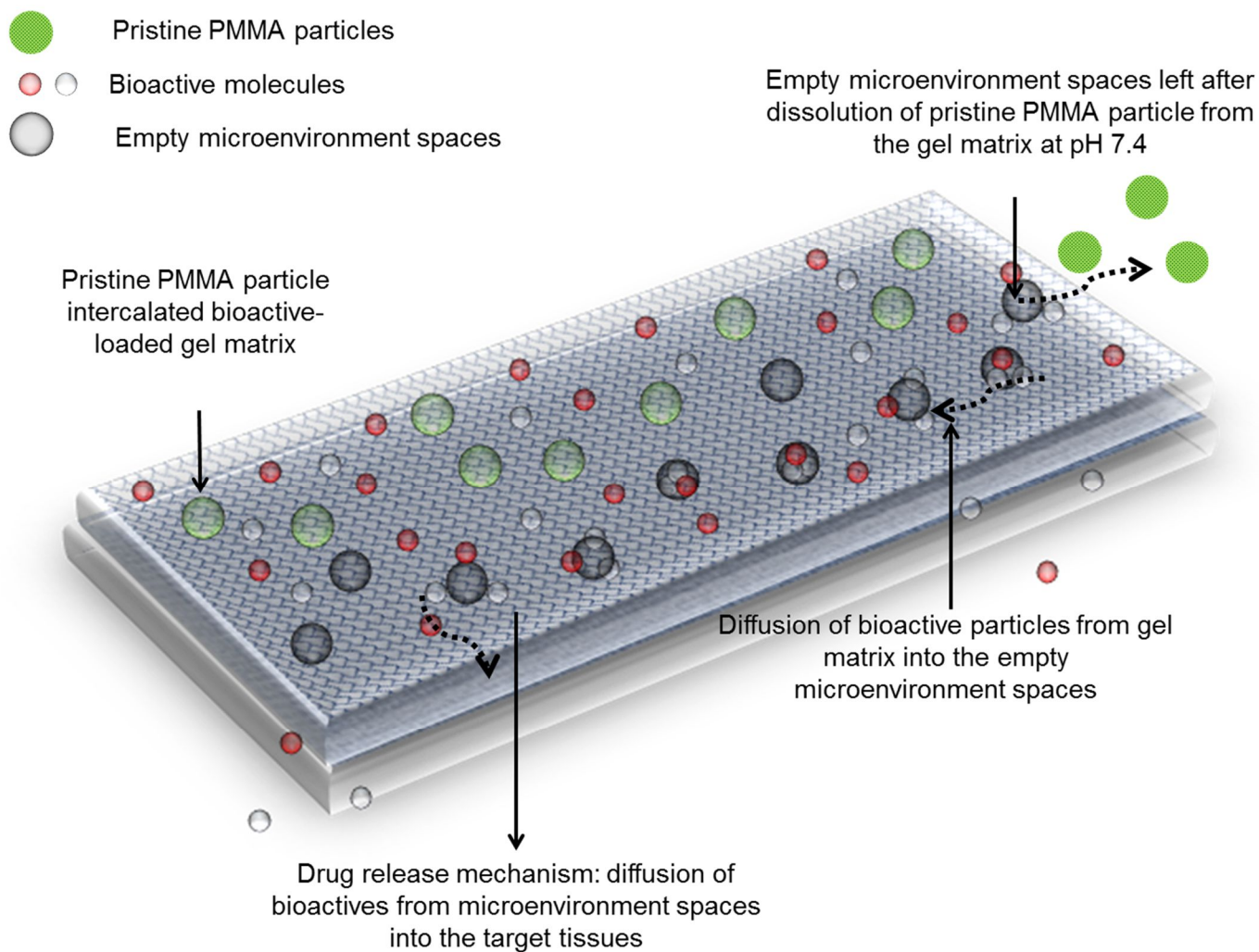


Figure 1.3: Schematic outlining the mechanism of action of pristine-polymer-particle intercalation upon dissolution and bridging repair function of a transected nerve injury.

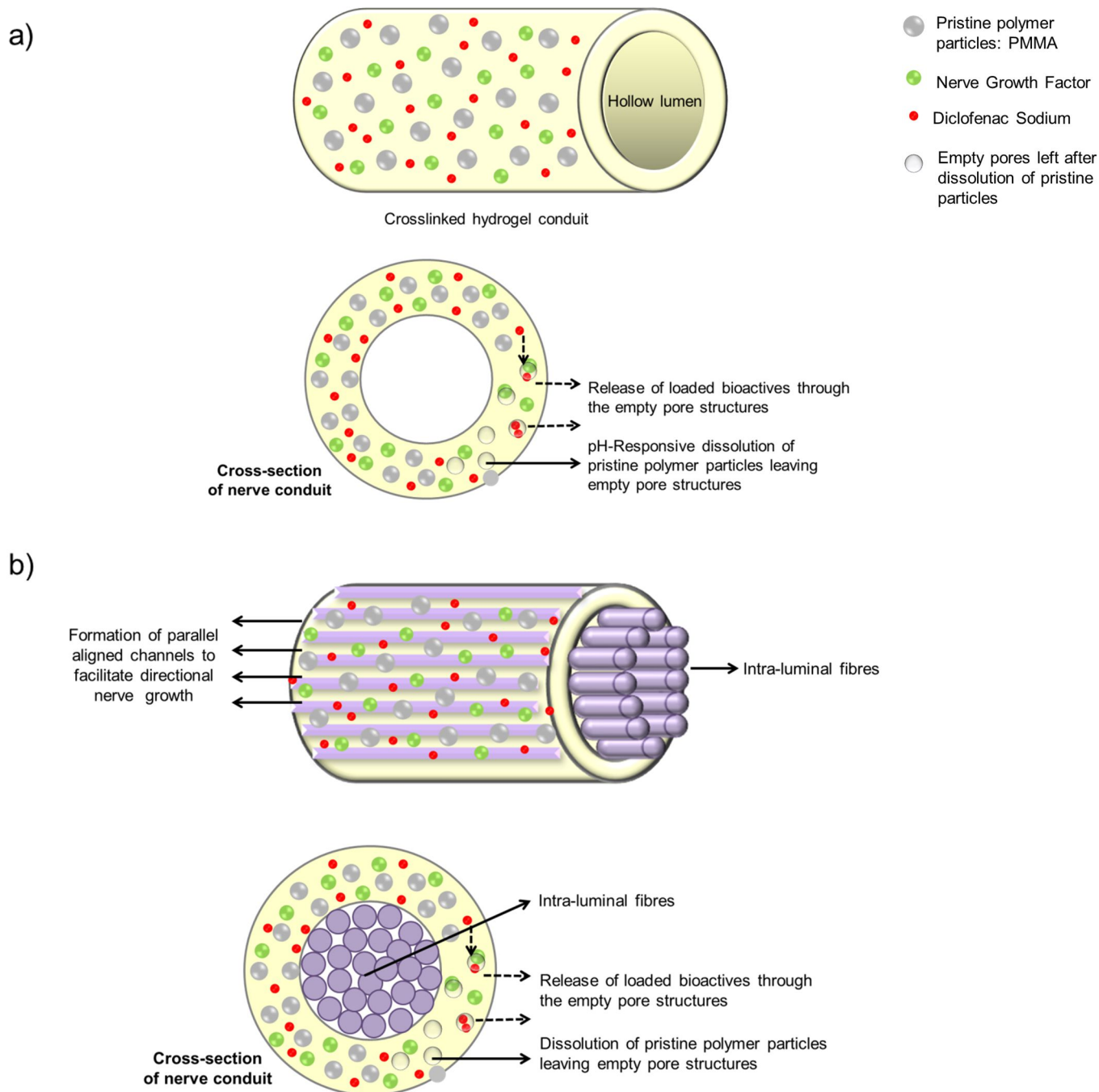


Figure 1.4: Schematic depicting the components and design of a) the independent hydrogel nerve conduit delivery system and b) the incorporation of the intraluminal support scaffolds.

The intraluminal fibers may be able to mimic the natural structure and fascicles of nerves thus improving the regeneration potential. These combined features of the proposed design create a suitable environment within the conduit lumen that cultivates neuronal regeneration (Gu *et al.*, 2014).

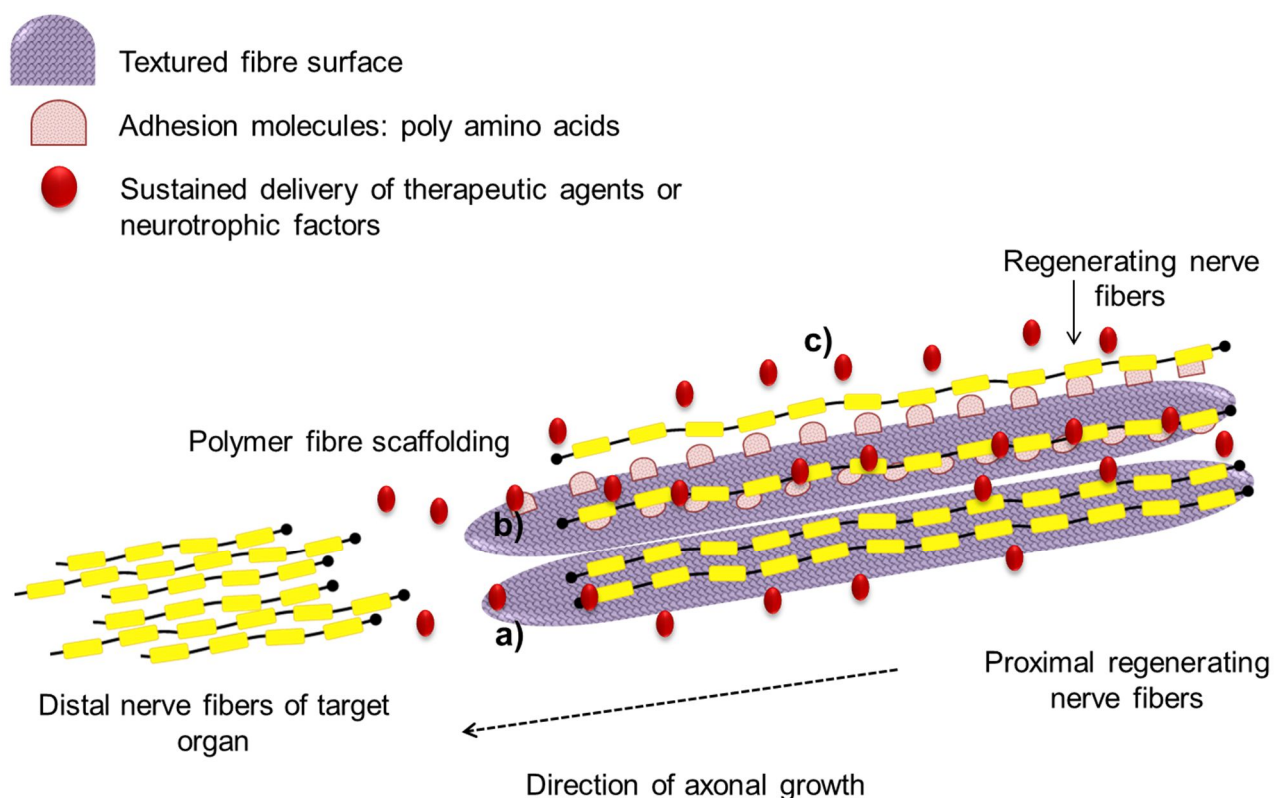


Figure 1.5: Schematic diagram of axonal attachment and growth along the proposed design of intra-luminal fiber structures via the following cues: a) physical b) chemical c) therapeutic.

1.4. Novelty of the Study

The novelty of this study lies in the intercalation of pristine polymer particles within a polymer matrix for the control of drug release and the enhancement of matrix strength and resilience by the formation of interionic complexes and varying degrees of porosity, via its pH-responsive feature, making the matrix a suitable system for prolonged drug delivery and tissue engineering applications. Furthermore, a polysaccharide gel blend comprising two types of naturally synthesised polymers is utilized for the tuning of mechanical strength, flexibility and biodegradability. This novel blend, comprising an interpenetrating gel network system, has the ability to absorb water to simulate the physiological consistency of native tissues while simultaneously providing the release of small molecular therapeutic agents and bio-macromolecules pertinent for tissue healing. Furthermore, the provision of a set of “trio guidance cues” as opposed to only one offers axonal guidance for enhanced re-innervation of target muscles and organs for improved outcomes of functional recovery. Guidance cues are provided by means of longitudinally aligned structural intraluminal fibers modified with topographical cues, chemotactic agents and cell adhesion factors to imitate an environment as offered with the nerve autograft – the gold standard of treatment.

1.5. Possible Therapeutic Applications of the Delivery System

1. Function as conduits for artificial replacement of and drug delivery to segments of damaged arteries, veins, heart valves and ureters.
2. Localised and prolonged drug delivery for the treatment of neuropathic pain and neuralgias.
3. Incorporation of other classes of neurotrophic factors for combined delivery of bioactives from a single device for a synergistic effect on the promotion of tissue regeneration.
4. Enhanced tissue regeneration via the provision of a microenvironment capable of simulating attributes of physiological tissue imperative for the survival of cells including the localised delivery of therapeutic agents and key nutrients.
5. The long-term sustained delivery of small molecular weight and therapeutically potent drugs to injured tissues.

1.6. Aim and Objectives of the Study

The aim of this research was to design and develop a suitable biodegradable and biocompatible nerve repair conduit for the treatment of transected peripheral nerve injuries utilizing a particle-intercalation technique for controlled dual-drug delivery of therapeutic bioactives and modulation of mechanical properties while incorporating the components of independent multifunctional intraluminal structural support scaffolds.

The aforementioned nerve repair device was formulated to meet the following essential design criteria:

1. The hydrogel nerve repair device was formulated to maintain adequate structural and mechanical stability to endure surgical implantation of the device while ensuring tissue textural biocompatibility.
2. The nerve repair device would allow for controlled-release and simultaneous delivery of at least two bioactive agents over a duration of at least 30 days.
3. The intercalation of pH-responsive polymer particles should assist in extending bioactive release rates over the required duration of time while modulating the matrix textural properties at physiological pH.
4. For guided axonal regeneration, the intraluminal structural support scaffolds was designed as to allow sufficient cellular interaction while providing ample pathway for axonal elongation and traverse through the device.
5. The hydrogel matrix component of the device was fabricated in a way as to prevent the degradation of sensitive protein and peptide-based growth factors during loading and entrapment thereby preserving *in vivo* bioactivity of such agents.

For pragmatic fulfilment of the aim, the following objectives will be undertaken:

- i. To select biodegradable polymers, synthetic or natural, for the fabrication of the hydrogel conduit and intra-luminal fibers of suitable mechanical strength, swelling and erosion properties.
- ii. To investigate a suitable and reproducible method of conduit and fibre fabrication utilizing mould-casting and electrospinning techniques.
- iii. To determine device synthesis variables and their upper and lower limits for generation of a 3-factor Box-Behnken experimental design for formulation optimisation purposes.
- iv. To perform physicochemical and physicomachanical characterisation analyses on the formulated device components utilizing Fourier Transform Infrared (FTIR) spectroscopy, Scanning Electron Microscopy (SEM), Differential Scanning Calorimetry (DSC), Textural analysis (TA), Thermogravimetric (TGA) and X-ray diffraction (XRD).
- v. To assess the individual and combined *in vitro* swelling, erosion and bioactive release properties of the conduit and intraluminal fibres.
- vi. To perform cell studies to evaluate the effect of the proposed trio cues on cell biocompatibility and proliferation.
- vii. To assess the *in vivo* performance on nerve regeneration and functional recovery outcomes in a rat sciatic nerve injury model for evaluation of preclinical potential of the device.

1.7. Potential Therapeutic Benefits of this Study

1. The development of an innovative but cost-effective nerve repair device, in terms of appropriate material selection and simple fabrication techniques, for the treatment of short and long gap peripheral nerve injuries through promotion of tissue regeneration and guidance.
2. Enhancement of bioavailability and therapeutic efficacy by delivering prolonged site-specific drug release and improving patient adherence by eliminating the need for frequent oral or injectable drug administrations, particularly at the injury site.
3. The potential of such information being published in peer-reviewed journals and presented at pharmaceutical conferences both local and international.
4. The probability of obtaining a patent and marketing of this drug delivery concept to the industry for potential clinical use.
5. The contribution of noteworthy data pertaining to the regenerative mechanisms employed during physiological repair of damaged neural tissues.

1.8. Overview of this Thesis

Chapter One of this thesis provides an introduction and background to this study, discusses the rationale and novelty and highlights the aim, objectives and potential benefits of this study.

Chapter Two of this thesis provides a comprehensive literature review, focusing on the various controlled-release strategies for the delivery of neurotrophic factors in peripheral nerve repair applications. It discusses the importance of the parameters controlling release kinetics in achieving the delivery of optimal bioactive doses. It further illustrates the physicochemical processes involved in bioactive release and highlights the essential features of an ideal nerve repair conduit.

Chapter Three of this thesis describes the proof-concept study evaluating the ability of the novel pristine-polymer-particle intercalation strategy on extending bioactive released and modulating matrix textural properties using a hydrophilic chitosan model-matrix system. *In situ* particulate leaching and polyelectrolyte complexation as mechanisms underlying the particle-intercalation strategy are discussed.

Chapter Four of this thesis details the formulation and integration of pristine-polymer-particle intercalation into an interpenetrating network hydrogel conduit according to a 3-factor Box-Behnken experimental design. An extensive analysis into the physicochemical, physicomachanical and *in vitro* dissolution characteristics of the system in response to pre-determined variants is discussed following statistical optimization of the experimental design model.

Chapter Five of this thesis describes the formulation and development of electrospun intraluminal guidance scaffolds according to a 3-factor Box-Behnken experimental design. It discusses the physicochemical, physicomachanical and *in vitro* cell biocompatibility properties in response to variations in chemical composition of the formulations.

Chapter Six of this thesis describes the fabrication and *in vitro* analysis of the optimized formulation components, a hydrogel conduit and nanofibrous guidance cues leading to the assembly of the final Biosimulated Nerve Repair Device. This is followed by evaluation of the *in vivo* performance of the device in the rat sciatic nerve model providing comparative evaluations between various experimental groups to the gold standard of treatment. Histopathological and behavioural studies were utilized to assess the level of peripheral nerve regeneration and functional recovery of the operated limb, respectively.

Chapter Seven of this thesis presents the conclusions of the research study and outlines the recommendations for future advancement and use of the device.

1.9. Concluding Remarks

This chapter highlighted the challenges associated with current peripheral nerve repair treatments and outlines mechanical and controlled-release strategies for the potential overall improvement of regeneration and functional recovery of peripheral nerve injuries. It entailed the proposed mechanism of action of the device in terms of its architectural structure and the required implementations for its successful fabrication and functioning. In addition, this chapter presented the rationale, aim, objectives, novelty and potential therapeutic benefits of the research study.

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CHAPTER 2

A REVIEW OF BIOACTIVE RELEASE FROM NERVE CONDUITS AS A NEUROTHERAPEUTIC STRATEGY FOR NEURONAL GROWTH IN PERIPHERAL NERVE INJURY

Abstract

Peripheral nerve regeneration strategies employ the use of polymeric engineered nerve conduits merged with components of a delivery system. This allows for the controlled and sustained release of neurotrophic growth factors for the enhancement of the innate regenerative capacity of the injured nerves. This review focuses on the delivery of neurotrophic factors (NTFs) and the importance of the parameters that control release kinetics in the delivery of optimal quantities of NTFs for improved therapeutic effect and prevention of dose dumping. Studies utilizing various controlled-release strategies, in attempt to obtain ideal release kinetics have been reviewed in this article. Release strategies discussed include affinity-based models, crosslinking techniques and layer-by-layer technologies. Currently available synthetic hollow nerve conduits, an alternative to the nerve autografts, have proven to be successful in the bridging and regeneration of primarily the short transected nerve gaps in several patient cases. However, current research emphasizes on the development of more advanced nerve conduits able to simulate the effectiveness of the autograft which includes, in particular, the ability to deliver growth factors.

2.1. Introduction

The persistence of peripheral nerve injuries as a challenge has over the years stimulated a significant response in the amount of research produced towards the investigation of strategies to overcome the ordeals of this debilitating clinical condition. The peripheral motor and sensory nerves form an extensive and intricate network encompassing a substantially large area of the body. This extensive placement of nerve tissue makes the peripheral nervous system susceptible to trauma inflicted by external forces to any site of the body (Raimondo *et al.*, 2011). Injuries to peripheral nerves are becoming increasingly common due to frequent incidents of trauma resulting from motor vehicle accidents, fractures, lacerations, crush injuries and surgical complications that cause direct or indirect nerve compression from oedema and haematomas (Bian *et al.*, 2009; Dodla *et al.*, 2009; Kemp *et al.*, 2011; Lin and Marra, 2012; Barghash *et al.*, 2013). Peripheral nerve injuries greatly impair the ability to feel normal sensations and exercise muscle movements due to denervation of adjacent tissues and muscles bringing about the loss of sensory and motor function (Tomassoni *et al.*, 2013). This results in paralysis, chronic pain and neuropathies leading to severe disability and a diminished quality of life in those patients who have sustained such injuries (Dodla *et al.*, 2009; Moore *et al.*, 2010; Dubový, 2011; Raimondo *et al.*, 2011; Cobianchi *et al.*, 2013; Jeon *et al.*, 2013; Wu *et al.*, 2013).

Initially, strategies focused on the design of a support structure to perform a similar neuronal function as the gold standard of treatment, a nerve autograft, thereby eradicating the need of obtaining a donor nerve via lengthy or multiple surgical procedures as well as eliminating the concerns of donor tissue availability, resulting morbidities and additional injuries and scarring (Bian *et al.*, 2009; Moore *et al.*, 2009; Pettersson *et al.*, 2010; Cunha *et al.*, 2011; Bozkurt *et al.*, 2012; Cho *et al.*, 2012; Strauch and Strauch, 2013). A nerve autograft involves the transplantation of a donor nerve across a nerve gap defect. In certain cases, a cable graft is required wherein several donor nerves are attached together to create an appropriate sized graft to fit the recipient nerve. Although nerve autografts have a good success rate in the regeneration of nerve across gaps of less than 10mm, it becomes less feasible for longer gap defects largely due to the difficulty in extracting sufficient donor nerve tissue and obtaining nerves of appropriate size in diameter to match that of the damaged nerve (Cunha *et al.*, 2011). Such concerns led to the fabrication and application of artificial synthetic and natural polymeric nerve conduits to span gaps and guide the regrowth of transected nerves by providing a means of structural support and barrier function against the infiltration of scar-forming tissue using hollow polymeric tubing (Pfister *et al.*, 2008; Angius *et al.*, 2012; Lin *et al.*, 2013; Mohammadi *et al.*, 2013). To name a few, commercialised polymer nerve conduits currently on the market and approved for clinical use in humans are NeuroTube, NeuroFlex,

NeuroMatrix, NeuroLac and NeuroGen which are principally composed of collagen with the exception of NeuroTube and NeuroLac which are instead fabricated using polyglycolic acid and poly lactide-co- ϵ -caprolactone, respectively (Pfister *et al.*, 2008; Pabari *et al.*, 2011; Kehoe *et al.*, 2012; Madduri *et al.*, 2012). Although they are able to bridge nerve gaps, provide a regenerative and protective environment for damaged nerves and have been reported to perform therapeutically well in certain cases, their efficacy in nerve regeneration of large nerve gaps does not fully emulate that of an autologous nerve graft. Furthermore, their fairly simple design as a hollow tube does not provide the complete features required for optimal nerve regeneration and functional recovery as listed in Table 2.1 (Pfister *et al.*, 2008; Deumens *et al.*, 2010; Xu *et al.*, 2011; Daly *et al.*, 2013). The deficiency in the success rate of tissue regeneration with these hollow conduits has prompted the development of newer types of nerve conduits (Jiang *et al.* 2010).

This chapter discusses the recent strategies employed for the delivery of growth factors for the application of peripheral nerve regeneration. Characteristics of an ideal nerve conduit necessary for the regeneration of nervous tissue has been briefly mentioned however this article focuses more on the release kinetics of growth factor entrapped polymeric systems. An attempt has been made to highlight the effects of the mechanisms that govern the release of incorporated growth factors from the delivery system as this determines the final dose of growth factor released and its impact on tissue regeneration.

2.2. Modern Development of Nerve Conduits and the Inclusion of Neurotrophic Growth Factors

Nerve conduits under current development have evolved considerably compared to their hollow polymer tube counterparts in terms of the significant modifications in scaffold design and selection of polymer materials. Despite the major advances and various approaches in the design of nerve conduits and repair strategies, the issue of insufficient functional recovery after peripheral nerve injury remains an obstacle that is yet to be overcome (Dalton *et al.*, 2008; Oh *et al.*, 2008; Ma *et al.*, 2013). Research in this field of tissue regeneration has focused immensely on the fabrication of nerve conduits able to provide multiple features that may possibly enhance nerve regrowth and restore functional recovery in a smaller time frame as opposed to previous strategies based solely on bridging nerve gap defects (Yamamoto *et al.*, 2013). Formerly, research was primarily concentrated on the bridging of nerve gaps and evaluation of the results thereafter with little concern for the determination of the extent of functional recovery achieved and degree of similarity to physiological tissues (Ichihara *et al.*, 2008). Currently, escalating focus is being placed on producing nerve conduits that are able to closely mimic the structure and function of native nerve tissues.

This strategy is thought to improve the promotion of nerve regeneration to a level equal to or above that of a nerve autograft (Evans *et al.*, 2002; Huang and Huang, 2006; Tang *et al.*, 2012; Marquardt *et al.*, 2013). To achieve this degree of resemblance to native nerve tissues, numerous physical, chemical and biological factors have to be taken into consideration when incorporating multiple-functioning components into a nerve conduit (Quaglia, 2008; Tang *et al.*, 2013). Table 2.1 lists the pertinent characteristics of a basic peripheral nerve conduit model. The improvement in axonal regeneration when Schwann cells are supplied together with rod-like physical guidance cues in a nerve conduit is shown in Figure 2.1.

Table 2.1: Salient features of an ideal nerve conduit

Conduit Features and Requirements	Importance and Function	References
Mechanical attributes	• Should not bend, buckle or kink post-implantation • Be able to maintain structural integrity to support tissue growth • Overly pliable conduits are unable to withstand pressure from growing and surrounding tissues • Rigid conduits may cause compression and damage to growing and surrounding tissues e.g. blood vessels and nerve stumps • Sufficient flexibility to withstand body movements, particularly at joint sites	Bian <i>et al.</i> , 2009; Cunha <i>et al.</i> , 2011; Kehoe <i>et al.</i> , 2012
Biodegradation rate	• Should degrade at a rate corresponding to tissue regeneration • Pre-mature disintegration removes provision of support structure • Lengthy degradation may cause compression and inflammation of newly generated tissues	Ichihara <i>et al.</i> , 2008; Tavangarian and Li, 2012
Semi-Permeability	• Should allow exchange of oxygen and nutrients and elimination of waste products between internal and external environment of conduit • Prevent infiltration of inflammatory cells and fibrotic tissue • Prevent escape of neurotrophic growth factors secreted by damaged nerve stumps and from system	Ichihara <i>et al.</i> , 2008; Cunha <i>et al.</i> , 2011; Kehoe <i>et al.</i> , 2012
Physical and 3D guidance cues	• Cylindrical and channel-based structures for growth cone guidance • Prevent axonal misdirection during regeneration towards distal nerve	Jiang <i>et al.</i> , 2010; Bozkurt <i>et al.</i> , 2012; Kehoe <i>et al.</i> , 2012
Ability to deliver growth factors or Schwann cells	• Enhancement of functional recovery and axonal regeneration • Important for neuronal survival and	Conti <i>et al.</i> , 2004; Ichihara <i>et al.</i> , 2008, Shakhbazau

	differentiation • Schwann cells for support of axon regeneration and re-myelination	<i>et al.</i> , 2012; Allen <i>et al.</i> , 2013; Marquardt and Sakiyama-Elbert, 2013; Mohammadi <i>et al.</i> , 2013
Processing requirements	• Maintenance of stability during handling, sterilization, storage and surgical procedures	Kehoe <i>et al.</i> , 2012

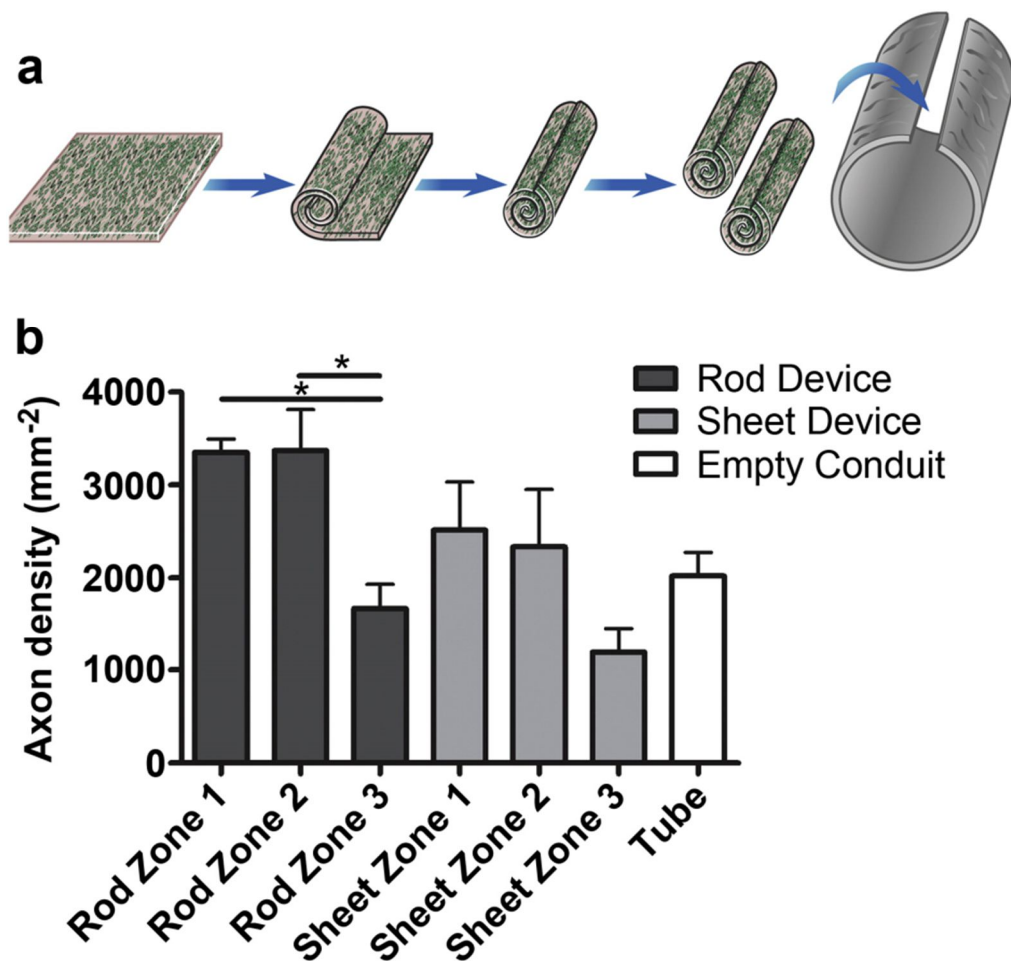


Figure 2.1: Comparison between the inclusion of rods (act as physical guidance cues) and sheets of a collagen-based engineered neural tissue (EngNT) seeded with Schwann cells within conduits in vivo. Flat sheets of EngNT were compared to rods that were made by rolling the sheets, as an approach to delivery within a repair device (a). The different device designs were compared after 4 weeks recovery in a 5 mm rat sciatic nerve gap model, in terms of the distribution of regeneration within and around the EngNT in cross sections (b). Three zones were compared within each device group – the EngNT (zone 1), a region 25 μ m from the EngNT surface (zone 2) and the remaining area within the conduit (zone 3). An empty conduit was also included (Georgiou *et al.*, 2013) (Reproduced with permission from Elsevier B.V. Ltd. © 2013).

2.2.1. Neurotrophic Factors for Improved Peripheral Nerve Regeneration

While peripheral nerves possess the inherent ability to regenerate and sprout new axons, this innate capacity is often insufficient for the regrowth of an adequately healthy and functional nerve (Soller *et al.*, 2012; Cobiainchi *et al.*, 2013). For the promotion of axonal growth and functional recovery many studies have concentrated on the effects of sustained-delivery technologies on the enhancement of nerve regeneration. This includes neurotrophic factors within the nerve conduits, particularly in gap defects of 10mm and larger. The use of neurotrophic factors has been widely studied *in vitro* and *in vivo* and has been proven to enhance nerve regeneration across gaps by enhancing both the rate and quality of nerve regeneration and potentially restoring a marked functional recovery (Markus *et al.*, 2002; Boyd and Gordon, 2003; Willerth and Sakiyama-Elbert, 2007; Moore *et al.*, 2010; Vishnoi and Kumar, 2013). Some leading studies have shown the benefits of utilising combinations of neurotrophic factors that have been incorporated into nerve conduits to determine whether neural regeneration would be further enhanced when compared to the use of single agents (Cao and Shoichet, 2003; Madduri *et al.*, 2010a). Elements which critically influence the regeneration capacity and rate of damaged nerves supplied with exogenous neurotrophic factors (NTFs) are (i) the doses of the neurotrophic factors and its release kinetics at the site of the target tissue; and (ii) the effects of initial burst release and whether the employment of single or multiple neurotrophic factors are used to create a synergistic effect on the promotion of nerve growth.

2.2.2. Impediments Associated with the Inclusion of NTFs

The ability of a scaffold-based delivery system to release therapeutically adequate quantities of NTFs and other bioactives is generally influenced by the type of materials used to fabricate the delivery system and the mechanisms that govern the release of NTFs and bioactives to the target tissue as this determines the rate and the quantity of release of the incorporated bioactives. Furthermore, the selection of materials, the method of incorporation of NTFs and bioactives into the delivery system during fabrication and the end degradation products of the materials used may affect the bioactivity of the NTFs and bioactive agents (Giteau *et al.*, 2008). The delivery of proteins is considered problematic due to their complex nature and stability (Machado *et al.*, 2013; Tobinaga *et al.*, 2014). Maintaining bioactivity of proteins is crucial as they are highly prone to degradation and instability by exposure to light, heat, oxygen, agitation, acidic environments and chemicals (Fu *et al.*, 2000; Lam *et al.*, 2001; Ozkan *et al.*, 2009).

2.3. Requirements for Optimal Peripheral Nerve Regeneration

For the successful peripheral nerve regeneration to occur, the injured tissues require four basic components, namely 1) a scaffold; 2) the inclusion of cells; 3) growth factors; and 4) extra cellular material (ECM) molecules, for the neuronal survival, optimal growth and ultimate regeneration potential (Figure 2.2). Artificial nerve conduits must be designed in a way that is able to provide a scaffold for the support of growing tissues in conjunction with at least one other component to create a basis for the model of a multi-functioning nerve conduit (Bian *et al.*, 2009; Dodla *et al.*, 2003; Johnson and Soucacos, 2008). By utilizing elements such as scaffold design, growth factor delivery, supply of extracellular matrix proteins and a substrate or matrix for the attachment of growing cells, the promotion of axonal regeneration and functional recovery can be significantly improved. These features are required for neural cell proliferation and maintenance of cell shape in addition to guiding and provision of mechanical strength to developing axons (Platt *et al.*, 2003; Leininger *et al.*, 2004; Sohler *et al.*, 2006). The implementation of these components into a nerve conduit delivery system will enable a degree of control over the length and processes of the Wallerian degeneration phase (Pettersson *et al.*, 2010; Shim and Ming, 2010; Kehoe *et al.*, 2012). Wallerian degeneration is a term describing the rapid breakdown of axons and myelin sheaths after injury to neural tissues (Figure 2.3) (Yong and Guoping, 2009). Although it is a process triggered for the creation of a microenvironment supportive for nerve regeneration, it additionally initiates the onset of inflammation and intensification of pain (Wagner *et al.*, 1998; Biondi *et al.*, 2008; Dubový, 2011; Allodi *et al.*, 2012; Kehoe *et al.*, 2012; Li *et al.*, 2013; Tang *et al.*, 2013).

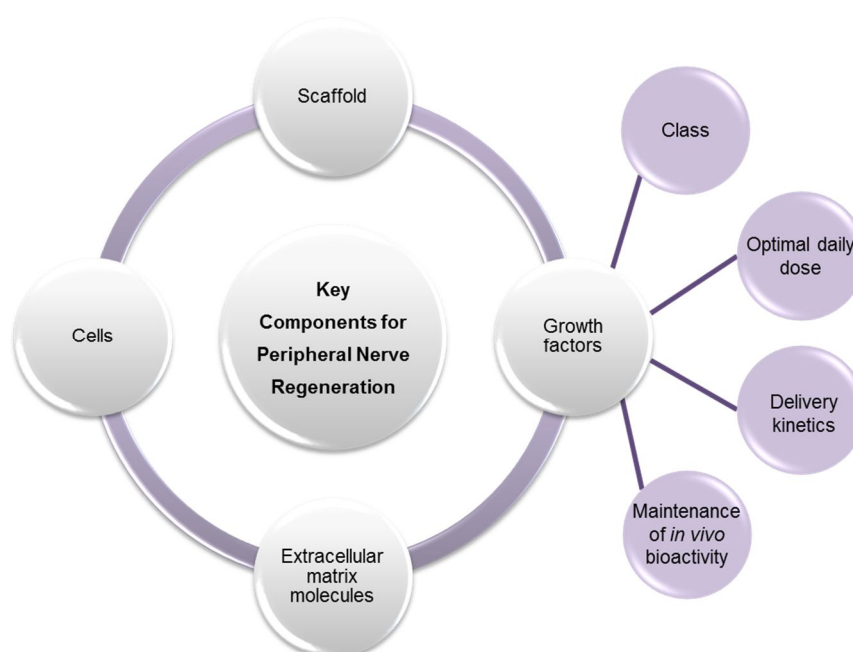


Figure 2.2: Schematic representation of the basic components required for peripheral nerve regeneration and the factors concerned with the delivery of growth factors.

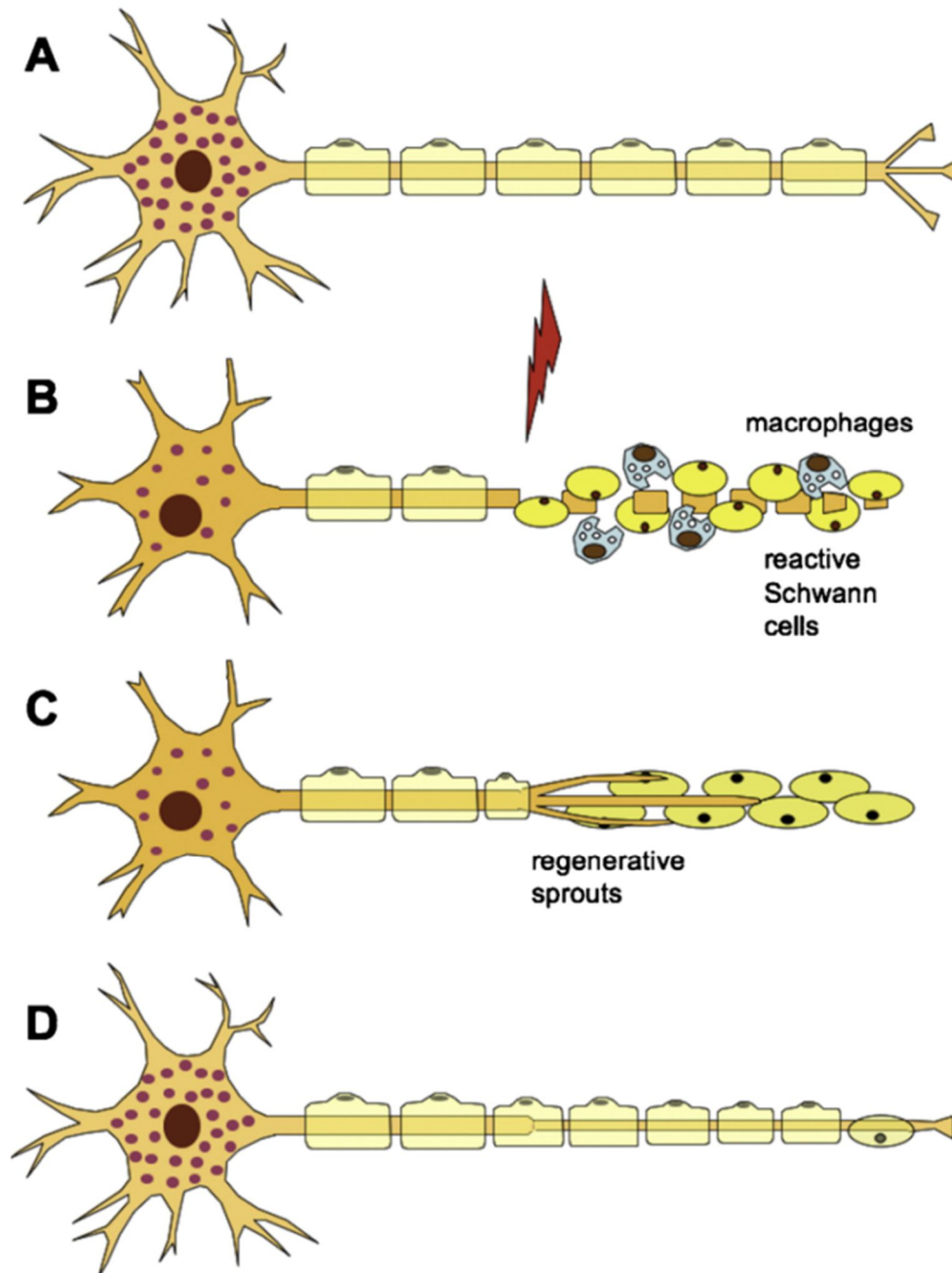


Figure 2.3: Degeneration and regeneration after peripheral nerve injury. (A) Normal neuron and nerve fiber. (B) Wallerian degeneration. The axotomy results in fragmentation of the distal axon and myelin sheaths. Schwann cells proliferate and macrophages invade the distal nerve segment and phagocytose degrading materials. (C) Schwann cells in the distal segment line up in bands of Bungner. Axonal sprouts advance embedded in the Schwann cells and attracted by gradients of neurotrophic factors. (D) Axonal reconnection with end organs and maturation and remyelination of the nerve fiber (Allodi *et al.*, 2012) (Reproduced with permission from Elsevier B.V. Ltd. © 2010).

2.4. Bioactive Release Kinetics and its Effects on Optimal Dosing of Growth Factors

In regards to peripheral nerve regeneration, the release kinetics of the selected delivery system is important particularly with regards to the phenomenon of initial burst release, the quantity of NTFs released and the pattern of its sustained release thereafter. Burst release, defined as a large and immediate release of bioactives and drug from a delivery system within the first 24 hours of placement into dissolution media or biological fluids, can result in overdose effects and dose dumping of the therapeutic agents (Huang and Brazel, 2001). Factors which need to be modified to achieve desirable release kinetics of growth factors are represented in the Figure 2. Inappropriate quantities of NTFs released from a nerve conduit or other incorporated delivery system significantly affects the potential of axonal regeneration. The release of high doses resulting from marked or uncontrolled initial burst release may hinder axonal sprouting due to a reduction in the extent of affinity-binding for the receptor sites since a lower therapeutic effect will then be achieved in the target tissues (Goodman and Gilman, 2008). It has previously been noted that suboptimal doses of NTFs evidently does not elicit an adequate effect on the regeneration of nerves but too-high doses are non-beneficial as it may hinder axonal growth due to the down regulation of TrkA and loss of affinity of TrkA to the growth factors, such as nerve growth factor (NGF), in order to initiate a growth response (Conti *et al.*, 2004; Allen and Dawbarn, 2006; Kemp *et al.*, 2011; Siniscalco *et al.*, 2011).

A study by Conti and co-workers proved that high doses of NGF had an inhibitory effect on the neurite growth in dorsal root ganglia (DRG) explanted from wild-type and knockout mice (Conti *et al.*, 2004). It was noted that DRG cultures exposed to the low dose of a 5ng/ml NGF solution exhibited neurite extension of 482µm in 24 hours whereas an increased NGF solution of 200ng/ml only supported neurite outgrowth to 173µm over 24 hours. However, another study using a heparin-immobilization based delivery system for NGF showed that doses of 20ng/ml and 50ng/ml elicited greater axonal regeneration and fiber density compared to 5ng/ml dose across a 17mm gap in the rat sciatic nerve (Lee *et al.*, 2003). Animal studies conducted on sensorimotor and behavioural recovery by Kemp and co-workers, 2011, showed that NGF concentrations of 800pg/µl provided optimum axonal growth in the early stages of peripheral nerve regeneration compared to animals receiving twice the NGF concentration. However, long-term analysis of peripheral nerve regeneration revealed that animals receiving 80ng/day for three weeks displayed improved behavioural recovery (Kemp *et al.*, 2011).

Further research is required to evaluate the role of delivery systems, release mechanisms and release kinetics on the optimal delivery of NTFs in the most beneficial doses for

enhanced axonal sprouting. Such factors must be tailored to deliver NTF doses that correspond to the regeneration rate of the injured tissues. Furthermore, understanding the mechanisms behind the phenomenon of initial burst release and factors controlling the characteristics of sustained release profiles will allow researchers to gain a deeper insight and knowledge on modification techniques to regulate the delivery of incorporated bioactives. This enables the rational selection of bioactive agents in the correct doses to be employed in the design of nerve conduits and prevent the economical and therapeutic waste of these rather expensive proteins (Huang and Brazel, 2001).

2.4.1. Affinity-Based Delivery Systems for the Sustained Release of GDNF and NGF

Several researchers have focused on *in vitro* and *in vivo* studies utilising heparin-containing affinity-based delivery systems for the sustained release of growth factors in peripheral nerve and spinal cord injuries (Sakiyama-Elbert *et al.*, 2000a, Sakiyama-Elbert *et al.*, 2000b; Lee *et al.*, 2003; Taylor *et al.*, 2004; Maxwell *et al.*, 2005; Wood *et al.*, 2009). The release of growth factors in affinity-based delivery systems is controlled by a cell-based degradation mechanism of the matrix into which the growth factor is immobilised as opposed to passive diffusion-based release from biodegradable polymers (Sakiyama-Elbert *et al.*, 2000; Lee *et al.*, 2003). In a representative study, a fibrin-based matrix incorporated with a heparin-binding peptide, heparin and NGF for potential use in nerve conduits was developed to achieve prolonged release of NGF in addition to protecting the bioactive protein from degradation. The release mechanism of the delivery system was designed to release bioactives in response to cellular activities during regeneration via enzymatic factors using a cross-linked heparin-binding peptide to immobilise heparin into the fibrin matrix (Sakiyama-Elbert and Hubbell, 2000). Interestingly, the immobilised heparin-conjugated protein was able to slow the diffusion-controlled release of NGF from the fibrin matrix providing a sustained release of growth factor and minimised initial burst release (Sakiyama-Elbert and Hubbell, 2000). An earlier study by Sakiyama-Elbert and Hubbell, 2000, involving the development of growth factor-heparin-peptide complexes bound within a fibrin matrix, confirmed satisfactory release of basic fibroblast growth factor through analysis of neurite extension in DRG cultures (Sakiyama-Elbert and Hubbell, 2000b).

An investigation by Wood and co-workers, 2009, on the effectiveness of silicone conduits containing the fibrin immobilized heparin-neurotrophic factor conjugates on nerve regeneration across a 13mm sciatic nerve gap was performed using glial-derived neurotrophic factor (GDNF) and NGF (Figure 2.4) (Wood *et al.*, 2009). The GDNF-containing delivery system showed better results in terms of the myelinated fiber count and nerve fiber density compared to the NGF-containing delivery system, however, neither was superior in

performance to the nerve isograft. Using the same heparin affinity-based delivery system, the release of NGF exceeded the nerve regenerative effects of the isograft *in vivo* across a 13mm gap. In another study, Taylor and co-workers, 2004, demonstrated that the ratio of heparin to growth factor can be modified by increasing the heparin content to achieve a sustained and well-defined zero-order release of neurotrophin-3 (NT-3) over 14 days for the repair of spinal cord injuries (Taylor *et al.*, 2004). Changing the concentration of heparin varies the number of available binding sites to the incorporated growth factors and as a result the release rate of growth factors can be modulated by consequently increasing its retention time within the fibrin matrix (Willerth and Sakiyama-Elbert, 2007).

The studies discussed above confirm that affinity-based delivery systems are efficient in promoting neural regeneration as the release rate is allowed to be modulated by the regenerating cells. The neurotrophic factors complexed to peptide-bound heparin form a non-diffusible protein complex allowing it to be restricted to and diffuse out of the matrix system at a slowed rate (Wood *et al.*, 2009). In addition, the proteolytic cleavage of the growth factors can be prevented in this manner thereby providing a method for reducing the potential loss of as well as increasing the bioavailability of the bioactives at the site of injury (Wood *et al.*, 2009; Madduri and Gander, 2012). Affinity-based delivery systems can be used to immobilise a number of growth factors into scaffolds fabricated from a variety of natural and synthetic polymers making it suitable for many tissue engineering applications (Sakiyama-Elbert, 2011). Chu and co-workers, 2011, designed a polycation-heparin complex to control the delivery of growth factors over 30 days. The polycation, poly (ethylene argininy l aspartate diglyceride) (PEAD) improved loading efficiency and bioactivity of the growth factors while providing a linear mode of release kinetics (Chu *et al.*, 2011). Further modification to the initial affinity-based approach have resulted in the development of photocrosslinked heparin-alginate, methylcellulose-SH3 and PEG-heparin hydrogels for the controlled and prolonged release of growth factors for bone repair, spinal cord injuries, wound-healing and vascular therapies (Nie *et al.*, 2007; Jeon *et al.*, 2011; Pakulska *et al.*, 2013).

Minor burst release effects, reported in these studies, could be attributed to a passive diffusion mechanism. The degree of burst release is dependent on the concentration gradient that exists between the NGF rich matrix and the regenerating tissues. As this concentration gradient is brought into equilibrium by the slowly diffusing NGF from the fibrin matrix, the amount of NGF released is reduced and the release kinetics of the system becomes more uniform until a near zero-order release behaviour is observed. Although passive diffusion may be minimally controlled by external factors, the diffusion rate can be

substantially decreased by the crosslinking action of the peptide and immobilization of the heparin into the fibrin matrix which may be considered as the rate-limiting step for the passive release process of the growth factors (Sakiyama-Elbert and Hubbell, 2000a; Moore *et al.*, 2010). Hence, the affinity-based technique of protein delivery has been shown to provide a sustained delivery of bioactives by remarkably slowing down the bioactive release processes which can be tailored by increasing the ratio of bound heparin to NTFs to delay the release of the heparin-binding NTFs (Hubbell, 2008).

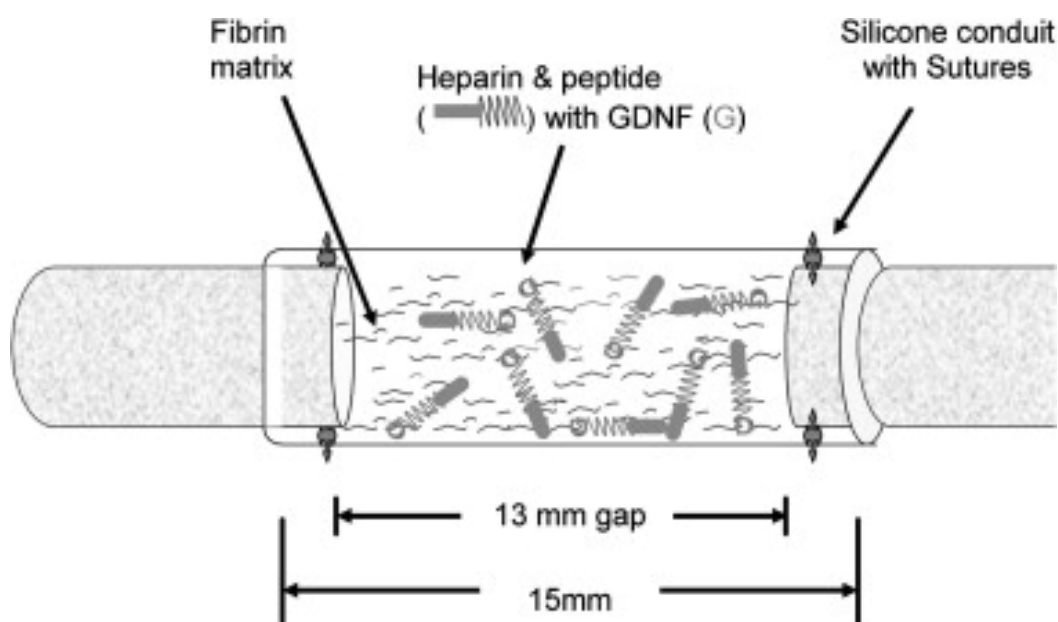


Figure 2.4: Schematic representation of surgical implantation of nerve guidance conduit containing the affinity-based delivery system. A 13 mm nerve gap was repaired with a 15 mm silicone conduit containing fibrin matrices with or without delivery system and growth factor and sutured to the transected proximal and distal stumps, incorporating 1 mm of nerve on either end. The delivery system consisted of a bi-domain peptide cross-linked into the fibrin matrix at one domain while the other binds heparin by electrostatic interactions. The growth factor can then bind to the bound heparin, creating a matrix-bound, non-diffusible complex, which can be retained for cell mediated degradation of the fibrin matrix (Wood *et al.*, 2009) (Reproduced with permission from Elsevier B.V. Ltd. © 2009).

2.4.2. NTF-loaded Crosslinked Polymer Conduits

Crosslinked polymer conduits offer a relatively simple approach for the entrapment of NTFs. The nature of crosslinking, either physical or chemical, affects the release behaviour of the incorporated bioactive molecules by influencing the water-uptake, swelling and erosion characteristics of the polymer material. Crosslinking not only affects the polymer but can have an effect on the entrapment of NTFs depending on the crosslinking agent used, its mechanism of action and the chemical structure of the NTFs. Effects of crosslinking on NTFs comprise of mainly interaction of specific chemical moieties between the crosslinker and the NTF or via the differences in the electrostatic charge. A few studies have shown the effects

of ionic and covalent crosslinking on the release of NTFs from nerve conduits and microsphere-loaded NTFs (Chan *et al.*, 2008; Chang, 2009; Yang *et al.*, 2011). In a study by Madduri and co-workers, 2010, poly (lactic-co-glycolic) acid (PLGA)-coated crosslinked and non-crosslinked collagen tubes were evaluated for *in vitro* release as potential sustained-release peripheral nerve conduits (Madduri *et al.*, 2010b). Collagen tubes, physically crosslinked using a dehydro-thermal treatment (DHT), were loaded with equal doses of GDNF and NGF. Exterior coating of the collagen tubes restricted the incorporated NTFs within the collagen tube lumen preventing the escape of the NTFs through the walls of the tube. Reducing the chance of NTF loss via escape or leakage from the conduit ensures that the bioavailability of the loaded dose is not compromised and that the full intact dose becomes available to the damaged tissues over the entire regeneration period (Xu *et al.*, 2002; Xu *et al.*, 2003; Xu *et al.*, 2011).

Mechanical strength, one of the factors that determine the degradation properties of the scaffold, was controlled by the induction of crosslinking and PLGA-coatings. Non-crosslinked collagen tubes released high quantities of NTFs within the first 2-3 days whereas the crosslinked collagen tubes were able to minimise burst release to a level that was indistinct. The crosslinking process, achieved through a reaction between the free amino and carboxylic acid groups in collagen, was able to retard the degradation of the collagen tubes in the presence of collagenase (Madduri *et al.*, 2010b). This allowed crosslinked tubes to deliver a sustained-release of NTFs over 30 days whereas non-crosslinked tubes rapidly degraded within 2 days. Regarding the dose of NTFs used, 80ng of GDNF alone was sufficient for the regeneration of the sciatic nerve across a 10mm gap within 14 days of implantation of the PLGA-coated crosslinked collagen nerve conduits as opposed to tissue growth in conduits, spanning a similar gap length, without the inclusion of NTFs being noted after 30 days (Ahmed *et al.*, 2005; Chen *et al.*, 2005; Madduri *et al.*, 2010b; Huang *et al.*, 2012). Similarly, genipin-crosslinked chitosan nerve conduits immobilized with NGF developed by Yang and co-workers, 2011, provided a sustained release of NGF over 60 days. A burst release of 2.1ng/day of NGF was reported during the first 3 days progressing to a steady decrease in NGF release over the following 20 days. The release kinetics stabilized to exhibit zero-order release over the next 40 days with a consistent quantity of 0.22-0.25ng of NGF being released daily. Likewise, poly caprolactone (PCL) tubes internally lined with a coat of genipin-crosslinked gelatin were able to deliver NGF over 60 days in a zero-order manner delivering less than 1ng daily. *In vivo* studies showed that the crosslinked tubes produced the regeneration of more axons compared to non-crosslinked tubes and the autografts (Chang, 2009).

The mechanism of genipin crosslinking involves a reaction with amine groups which in this case was present in both the polymer as well as the NTF. The use of genipin as a crosslinker enhanced the ability of the delivery system in mitigating burst release and obtaining a zero-order release profile by altering the swelling and degradation properties of chitosan in addition to effectively trapping NGF molecules within the polymer network via its reaction with $-NH_2$ groups (Butler *et al.*, 2003; Jin and Hourston, 2004; Chang, 2009; Yang *et al.*, 2011). In the studies described, the primary mechanism controlling the release of NTFs is polymer degradation along with passive-diffusion during the initial release phase. Crosslinking is effective in modifying water-uptake ability, swelling and degradation processes to control the release of bioactives and minimise burst release to achieve sustained and prolonged delivery. Burst release results from swelling of the conduit walls initiated by water-uptake into the polymer chains. Swelling produces the formation of a hydration layer which facilitates the transport of bioactives via diffusion which occurs spontaneously and over which little control can be exerted (Figure 2.5). This hydration layer creates a diffusion pathway for the entrapped bioactives. With time the hydration layers increase as the polymer swells thereby lengthening the pathway of diffusion that the bioactive molecule must cross before it is finally released into the conduit lumen. This mechanism brings about a decrease in release rates over time providing zero-order release kinetics (Conti *et al.*, 2007). Combined with slower degradation rates, smaller quantities of bioactives can be delivered over a prolonged time whereas rapid degradation releases large amounts of bioactives in a shorter time.

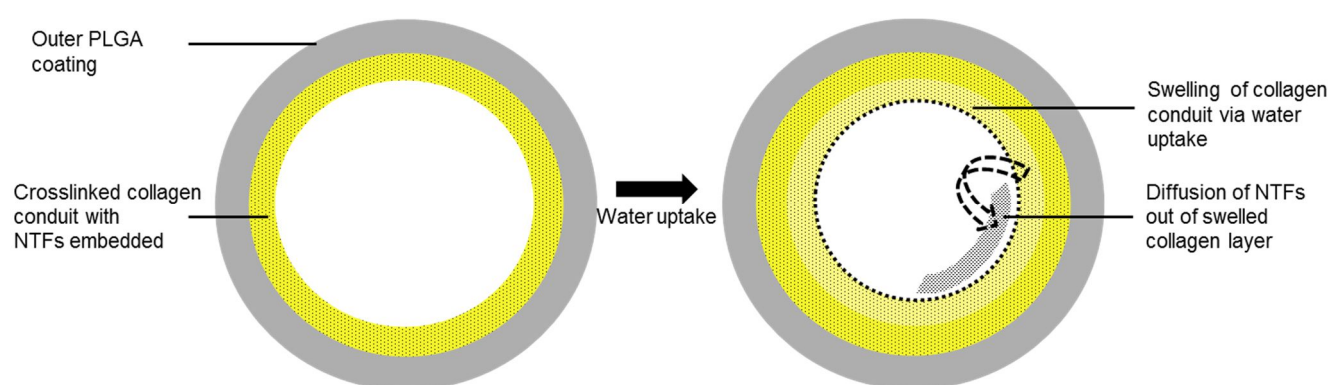


Figure 2.5: Representation of the design and release from the collagen crosslinked conduits via water-uptake and swelling initiated diffusion.

2.4.3. Microsphere Technologies for the Delivery of NTFs

Microspheres, usually fabricated from polymeric materials, are spherically shaped particles with diameters in the micrometre range and well-known for their capability to deliver drugs and bioactive molecules. Microspheres are frequently used for NTF encapsulation employing different methods of fabrication involving the use of ionic and covalent crosslinkers, double emulsion methods and spray drying to evaluate the varying degrees of crosslinking, effects on swelling, encapsulation efficiencies and degradation characteristics as the factors controlling the release of incorporated proteins (Aral and Akbuğa, 1998; Shu and Zhu, 2000; Mi *et al.*, 2003; Buranachai *et al.*, 2010; Ye *et al.*, 2010; Yang *et al.*, 2011). Several studies have shown promising results in the ability of microspheres to deliver sustained and constant quantities of bioactives as they can effectively be combined with more than one delivery system to control the release kinetics (Vishnoi and Kumar, 2013; Silva *et al.*, 2009; Park *et al.*, 2010). Careful selection of a suitable method for microsphere preparation is required as the different techniques affect the final outcome on encapsulation efficiency, release kinetics and preservation of bioactivity of the growth factors (Figure 2.6) (Ye *et al.*, 2010).

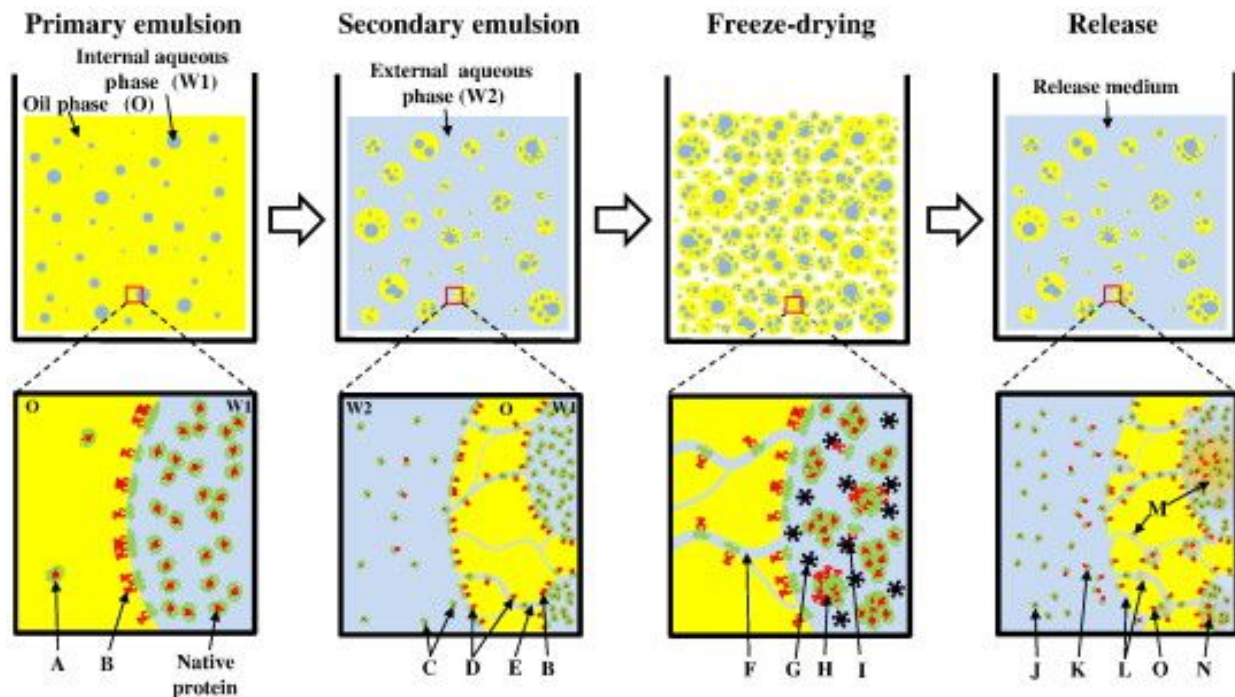


Figure 2.6: Typical W/O/W double emulsion method to prepare microspheres containing protein drug (upper panel) and microscopic events during fabrication process (lower panel). The sequence of fabrication is primary emulsion, secondary emulsion, solvent extraction/evaporation (not shown), (freeze-)drying, and drug release test. With negligible partition of protein into oil phase (A), the organic solvent–water interface during W1/O emulsion results in protein denaturation (B). During generation of secondary emulsion, water channels connecting internal (W1) and external (W2) aqueous phases (E) allow proteins to escape from droplets (C), and provide more chances of protein denaturation by increased surface area of the oil–water interface (D). The water channels become pores (F) of microspheres hardened by freeze-drying. Ice crystal (G) is known to provide a hazardous condition inducing protein denaturation (I). Irreversible aggregation (H) between protein molecules can be formed if stabilizer or cryoprotectant is not added. Normally, microspheres made by double emulsion have a broad range of particle size distribution as well as different protein amount in each microparticle. In a release test, a burst release of protein at the initial period (< 24 h) is mostly due to the protein release (K) from the proteinaceous film on the particle surface (D). With time, proteins are release from particles (J) by diffusion and degradation (L) of polymer (e.g., PLGA). Microparticle degradation cumulates acidic products inside particles (M), which further facilitates protein denaturation (N). Protein adsorption on hydrophobic polymer surface (O) often leads to incomplete release of protein drugs (Ye *et al.*, 2010) (Reproduced with permission from Elsevier B.V. Ltd. © 2010).

2.4.4. Crosslinked Chitosan Microspheres

Chitosan microspheres have been widely applied for protein delivery applications with various degrees of success in achieving sustained zero-order release kinetics for periods ranging from 3 days to over 2 months using growth factors, hormones and bovine serum albumin as model proteins (Yuan *et al.*, 2007; Karnchanajindanun *et al.*, 2011; Wu *et al.*, 2011; Zhang *et al.*, 2011; Jose *et al.*, 2012). Sinha and co-workers, 2004, extensively

reviewed the applications of chitosan microspheres for various categories of drugs while stating the favourable degradation, biocompatibility and hydrophilic characteristics and simpler processing procedures that chitosan offers (Sinha *et al.*, 2004).

For the application of peripheral nerve injuries, chitosan-PCL microspheres for the delivery of GDNF were expected to release therapeutically sufficient quantities of GDNF in a controlled manner. Having a brush-like chain structure, the authors proposed that the PCL side-chains could act as hooked branches for the entrapment of GDNF by increasing the potential of protein entanglement onto the PCL chains. Depending on the quantities of chitosan, PCL and the crosslinker used, release profiles varied from near first-order to zero-order kinetics over 49 days. Reduced burst release and zero-order kinetics resulted from the release mechanisms, swelling, diffusion and degradation, being primarily governed by the increasing amounts of PCL and genipin (crosslinker) used in the microsphere formulations (Wu *et al.*, 2011).

A study by Zeng and co-workers, 2011, investigated the effects of crosslinking on bioactive release and encapsulation efficiency of NGF-loaded microspheres and obtained similar results to studies described above. Chitosan microspheres were ionically crosslinked using various concentrations of sodium tripolyphosphate (STPP) to control burst release and provide a slow and sustained release of NTF over 7 days. Increase in STPP concentration resulted in a lower NGF encapsulation efficiency but showed a reduction in the initial burst release and offered slow and sustained release of NGF. This is attributed to the high crosslinking density obtained, when STPP concentrations are increased, which prevented swelling of the microspheres and inhibited the rate and reduced the quantity at which NGF is released. STPP of concentrations 1%, 5% and 10% produced microspheres that exhibited burst release of 45.5%, 24.6% and 18.4%, respectively of the total encapsulated dose within 12 hours. The release of NGF from the crosslinked chitosan microspheres occurred in 3 stages: 1) a rapid burst release triggered by swelling, 2) a slower diffusion-based release through pores and channels within the microspheres and 3) a further slowed release phase dominated by erosion-based release through chitosan biodegradation or in combination with diffusion of NGF (Zeng *et al.*, 2011).

Multichannel chitosan-PCL nerve conduits were fabricated to house the microsphere delivery system (Liao *et al.*, 2013). The design of the conduit was such that it resulted in longitudinal arrays of hollow cylindrical channels running across the entire length of the tube (Figure 2.7). In addition to the delivery of NGF the provision of physical guidance cues in the form of these longitudinal channels furthers the promotion of axonal regeneration and accurate direction of growth (Bozkurt *et al.*, 2012; Ke *et al.*, 2014). The NGF-loaded microspheres embedded into these channels were able to significantly retard the release of NGF in comparison to microspheres alone. The authors proposed that the release behaviour and quantity of the microsphere-encapsulated NGF can be independently controlled by changing the initial loading dose of NGF in the microspheres. To explore this concept, crosslinked chitosan microspheres loaded with initial NGF doses of 5 and 10ng/mg microsphere were studied *in vitro* over 60 days for differences in release kinetics and quantity of NGF released (Liao *et al.*, 2013). There was no significant difference in the burst release and cumulative release of NGF between the high and low loading doses. Both types of microspheres exhibited burst release of approximately 10% and over 60 day study period almost all of the loaded NGF had been released. Despite similar release profiles in terms of percentage cumulative release, it is clear that the higher NGF-loaded microspheres will release a greater amount of growth factor compared to the microspheres loaded with a low initial dose as amount of growth factor present is increased (Liao *et al.*, 2013).

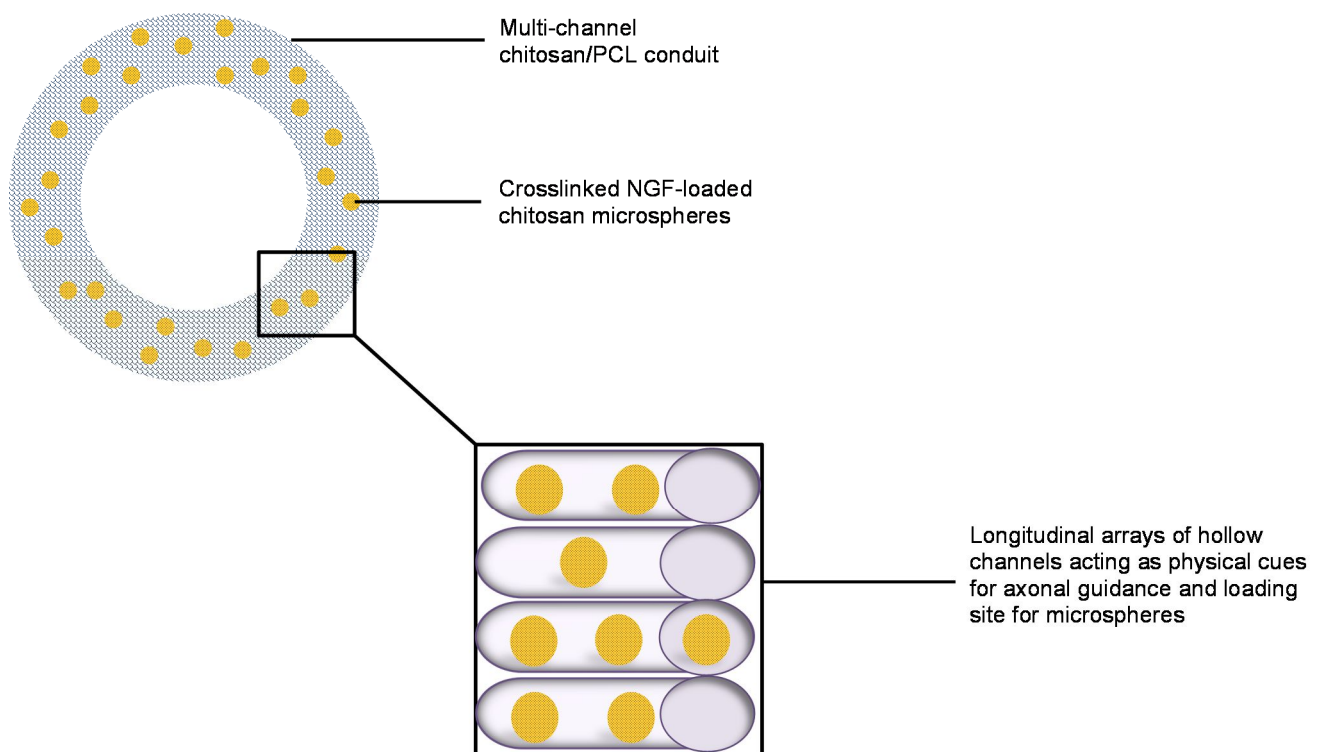


Figure 2.7: Representation of the internal design and configuration of the multi-channel conduits providing physical cues for enhanced regeneration.

Crosslinking effects, similar to those described in the study by Zeng and co-workers (Zeng *et al.*, 2011) were observed by Liao and co-workers, (Liao *et al.*, 2013). Increasing STPP ratios ranging from 1 to 6 of crosslinker:chitosan solution resulted in significantly inhibited swelling of the microspheres by 116% to 70% which controlled swelling to restrain diffusion-based burst release. However, in contrast to the previous reports (Zeng *et al.*, 2011; Liao *et al.*, 2013) the encapsulation efficiency was directly proportional to the crosslinker ratios.

2.4.5. NTF-loaded PLGA Microspheres

PLGA microspheres have been extensively investigated for the encapsulation and controlled release of various proteins (Morlock *et al.*, 1997; Péan *et al.*, 1999; Lam *et al.*, 2000; Takada *et al.*, 2003; Jaklenec *et al.*, 2008; Ye *et al.*, 2010; Rui *et al.*, 2012). NGF-loaded PLGA microspheres fabricated by Péan and co-workers, 1998, for injectable administration to the brain also exhibited first-order release kinetics over 12 weeks. Burst release was attributed to the highly porous structure of the microspheres allowing easy penetration of water into the matrix thereby allowing rapid release of NGF via diffusion osmotic pumping (Figure 2.8) (Péan *et al.*, 1998; Fredenberg *et al.*, 2011). The prolonged release period was thought to arise from adsorption and entanglement of the protein onto the polymer chains (Péan *et al.*, 1998). NGF-encapsulated PLGA microspheres prepared by spray freeze-drying technique achieved a biphasic release over 30 days. The release pattern consisted of an inclining rapid-release phase gradually stabilizing to a zero-order release after medication of the microspheres with zinc carbonate to reduce burst release and enhance sustained release (Lam *et al.*, 2001). PLGA microspheres loaded into chitin tubes manufactured by the double emulsion/solvent evaporation technique, releasing BSA as the model protein, provided a first-order release profile over 84 days as investigated by Goraltchouk and co-workers (Goraltchouk *et al.*, 2006).

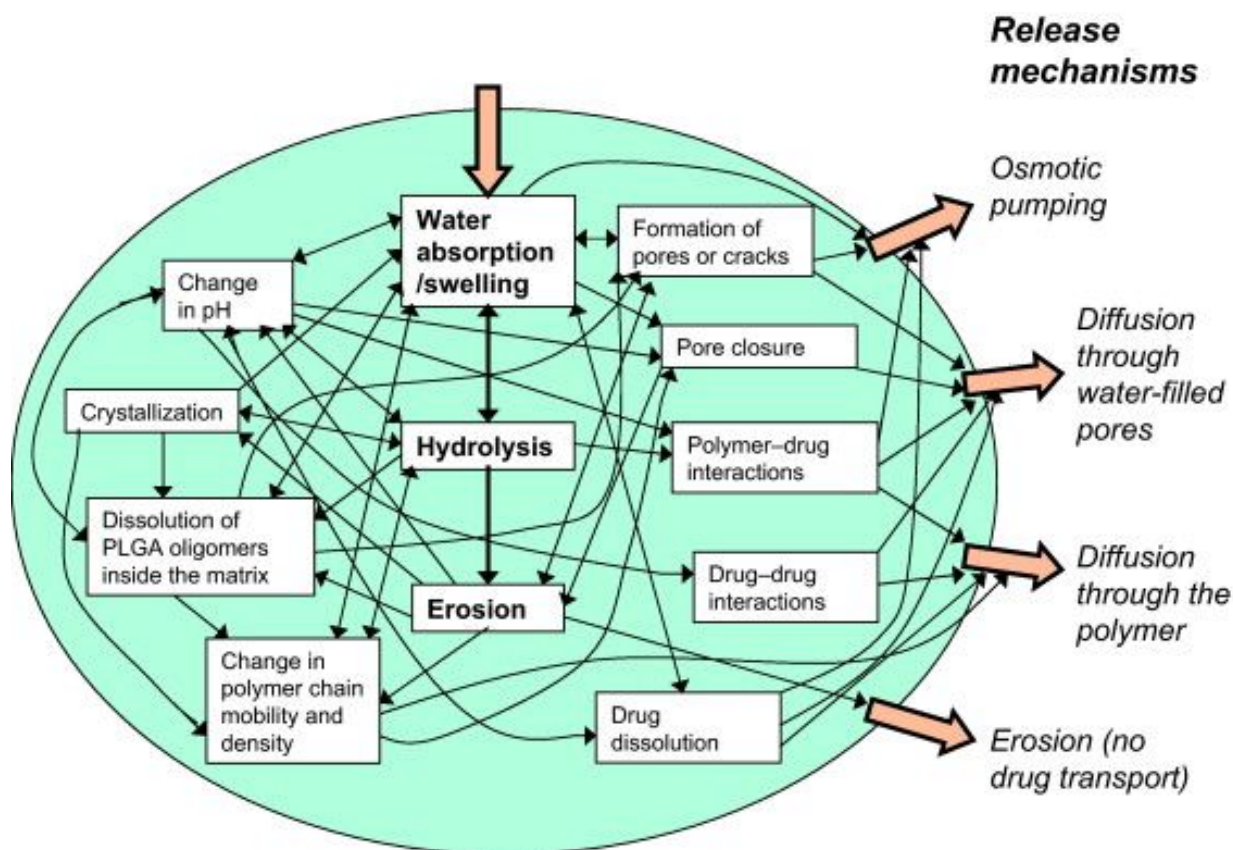


Figure 2.8: The complex picture of physicochemical processes taking place within PLGA matrices, leading to drug release. The influence of processes on drug release and on other processes is illustrated by arrows. Note that some arrows point in both directions (Fredenberg *et al.*, 2011) (Reproduced with permission from Elsevier B.V. Ltd. © 2011).

Concerning peripheral nerve regeneration, double-walled PLGA-PLLA microspheres loaded with GDNF into the inner walls of a bi-layered PCL nerve conduit to achieve sustained delivery for a minimum period of 50 days was developed (Kokai *et al.*, 2010). The efficacy of the delivery system was evaluated in a rat sciatic nerve model of gap 15mm over a study period of 6 weeks. Each nerve conduit was loaded with an approximate dose of 95ng GDNF. The microspheres were embedded into the walls of a PCL conduit which then received another porous layer of pure PCL. This assembly of polymers and microspheres exhibited an initial burst release during the first day followed by a slower near zero-order release over the next 64 days. The release kinetics of the GDNF-loaded microsphere-embedded nerve conduits was able to support the regeneration of blood vessels, intercellular fibers and Schwann cells, *in vivo*, compared to the negative controls (Kokai *et al.*, 2010). NTF release can further be controlled by a combination of polymer degradation and diffusion processes (Figure 2.9). The additional layer of PLLA surrounding the GDNF-loaded PLGA microspheres not only increased the time of NTF release by slowing the process of diffusion but also provided a strategy for protection of the GDNF protein by containment of the growth

factor within the core of the two-layered microsphere structure. Furthermore, this double-wall of polymers may assist in the prevention of excessive NTF loss as it remains confined within the microsphere core. The inner solid PCL layer, into which the microspheres are embedded, further delay the ultimate release of GDNF as this layer has to erode in order for microspheres to be released into the lumen of the conduit where the microspheres can undergo biodegradation for the diffusion and release of the entrapped GDNF. The outer porous PCL layer of the nerve conduit allows for cellular infiltration and exchange of metabolic substances and nutrients which are vital for the proliferation of physiological tissues (Kokai *et al.*, 2009; Kehoe *et al.*, 2012).

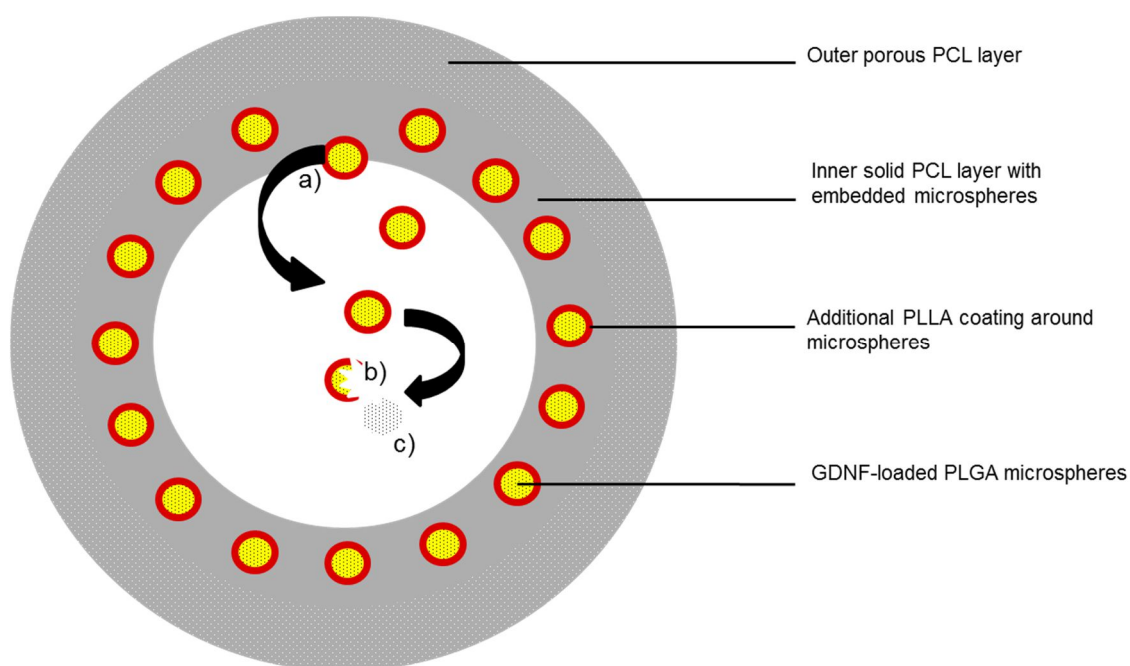


Figure 2.9: Representation of the double-walled PLGA-PLLA microspheres, a) release of microspheres from solid PCL layer via degradation of PCL, b) degradation of microspheres and subsequent release of GDNF, c) release of GDNF by diffusion through degradation-formed pores and channels in the microsphere matrix.

Researchers have proposed that microsphere delivery systems for NGF composed of PLGA exhibited significant initial burst release phases followed by a slow continuous release pattern where the subsequent release of growth factor in the slowed-release phase could be lower than the initial burst release (Xu *et al.*, 2002). Furthermore, it is thought that the acidic degradation products of PLGA may cause inactivation of the protein-based NGF creating a concern for the maintenance of NGF stability and ultimate bioactivity (Xu *et al.*, 2002; Luan *et al.*, 2006; Giteau *et al.*, 2008). The erosion behaviour of PLGA dominates its ability in controlling release kinetics and inhibiting initial burst release. The interior of PLGA

microspheres degrade at a faster rate than the polymer surface on the outside and this bulk-degradation coupled with its autocatalytic mechanism increases the potential for large amounts of entrapped bioactives to be released over a short time frame (Sackett and Narasimhan, 2011; Versypt *et al.*, 2012).

2.4.6. Miscellaneous Microsphere Delivery Systems for NTF Delivery

Xu and co-workers (Xu *et al.*, 2002) fabricated polyphosphoester (PPE) microspheres for the delivery of NGF. The PPE polymer, P(DAPG-EOP), used for synthesizing the NGF-loaded microspheres has a phosphate backbone composed of oligomeric D,L-lactide blocks and has been previously shown to exhibit near zero-order release kinetics of drugs used in delivery systems for the application of other disease conditions, by virtue of its collective mechanisms of surface erosion and bulk degradation (Nair and Laurencin, 2007). PPEs are degraded via hydrolytic and enzymatic cleavage of the phosphate bonds at physiological conditions resulting in the formation of phosphates, alcohols and diols as the ultimate breakdown products. Hence it is advantageous in tissue engineering applications as compared to the hydrolysis-induced degradation of PLGA resulting in acidic compounds which may further lower the pH of the entire delivery system and the local surrounding tissues leading to inflammation and inactivation of acid-labile bioactives (Nair and Laurencin, 2006 and 2007; Sokolsky-Papkov *et al.*, 2007 and Fredenberg *et al.*, 2011). During the first week 45% of the loaded NGF was released with an initial burst release of approximately 4%. A slower release phase exhibiting sustained near zero-order release kinetics was observed over the subsequent weeks with an average NGF release rate of 0.5ng/mg microspheres per day. At the end of the 10 week study period 60% of the total encapsulated NGF was released (Xu *et al.*, 2002).

In vivo studies were performed using the rat sciatic nerve regeneration model where a silicone nerve conduit containing the PPE microspheres was implanted across a 10mm nerve gap followed by a subsequent study utilizing P(BHET-EOP/TC) conduits as the microsphere carrier. Each conduit, loaded with an NGF dose of 100ng, was able to within 2 weeks promote a positive growth of nerve fibers compared animals received a conduits filled with a 50ng/ml NGF saline solution. After 3 months, all animals receiving NGF-loaded microspheres showed a positive muscle reflex, an epineurium-surrounded regenerated nerve cable bridging the 10mm nerve gap containing myelinated axons, thicker myelin sheaths and higher fiber densities compared to the controls. The silicone nerve conduits with the NGF-releasing microspheres generated a higher population of axons and fiber density though PPE nerve conduits which prevailed in the regeneration of larger axons and thicker myelin. The authors proposed that the investigated strategy of NGF delivery would be

favourable compared to the nerve autograft and its associated issues of loss function at donor sites, insufficient collection of grafting materials and the formation of neuromas (Xu *et al.*, 2002).

GDNF-loaded silk microspheres dispersed throughout a silk conduit were successful in releasing GDNF for Schwann cell migration and nerve tissue proliferation over six weeks (Lin *et al.*, 2011). The aqueous solubility of silk offered ideal processing characteristics for the incorporation of growth factors unable to resist harsh fabrication procedures when using polymers requiring acidic and organic solvents (Wang *et al.*, 2007; Uebersax *et al.*, 2007; Wen *et al.*, 201). Crystalline beta-sheet formation in silk forms physical crosslinks which reinforces the structure of silk imparting enhanced mechanical strength and degradation properties (Wang *et al.*, 2007; Wang *et al.*, 2010). In another study, photochemically crosslinked collagen microspheres were utilised to preserve NGF bioactivity and with the addition of tween 20, collagen microspheres significantly improved zero-order release of BSA while simultaneously eliminating burst release (Chan *et al.*, 2008).

2.5. Multiple Layer Strategies and Polyelectrolyte Complexes for the Controlled Release of NTFs

Another strategy for controlling the release of NTFs is the deposition of bioactive molecules between layers of polymers varying in size, number, material selection and its ionic charge. In this manner, release kinetics of NTFs can be regulated by changing the quantity of polymeric material in the following ways: 1) by delaying water penetration to hydrate the consequent polymer layers before reaching the NTF layer interface, 2) by increasing the diffusion pathway that must be crossed by the NTF molecules before becoming available to the target tissues and 3) by extending polymer erosion or degradation before exposure of the NTF layer (Conti *et al.*, 2007; Kokai *et al.*, 2010). As noted in a study by Yang and co-workers, 2011, electrostatic properties can assist the enhanced entrapment of NTFs via ionic interaction between NTF and crosslinker or NTF and polymer. With layer-by layer deposition or blending of oppositely charged polymers, various polyelectrolyte complexes (PECs) can be formed for immobilizing NTFs and controlling its release (Luo and Wang, 2014; Tsai *et al.*, 2014). The outcome on release profiles utilising PECs can be seen in a study conducted by Pfister and co-workers, 2008, using a multiple-layered PLGA-coated system. Hollow nerve conduits were fabricated to control the release of NGF by altering the position of NGF between various concentric layers of PLGA and an alginate-chitosan PEC (Figure 2.10).

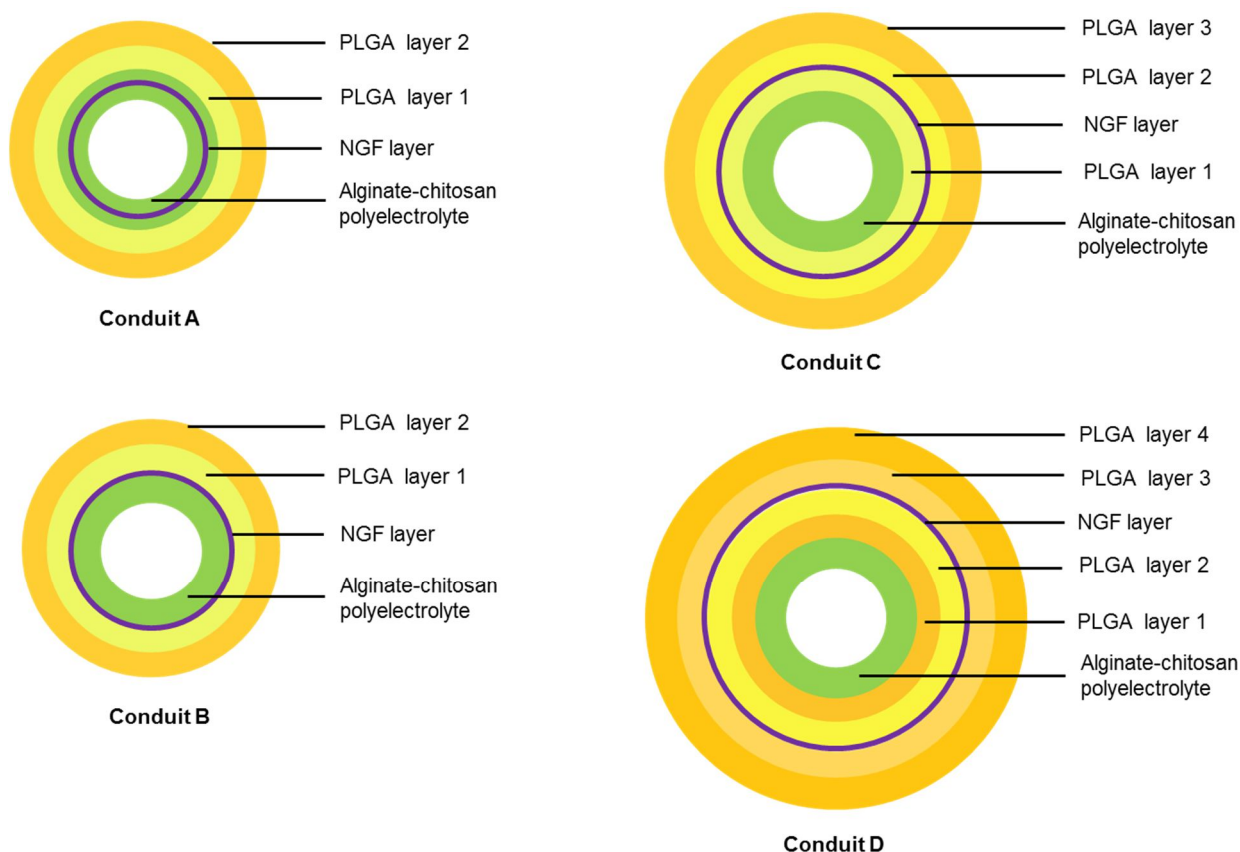


Figure 2.10: Representation of the different conduits and the various positioning of NGF, alginate-chitosan, and PLGA layers.

Conduit A, consisting of NGF centrally placed within the alginate-chitosan PEC was capable of providing a single-phase near-perfect zero-order release kinetics with an indistinct initial burst release. It is thought that the firm entrapment of NGF within the PEC layer was due to electrostatic interaction between the positively charged NGF and the negatively charged alginate (Chaudhari *et al.*, 2013). Unlike conduit A, a biphasic release pattern was reported for the 15-day study period with conduits B, C and D. An escalating rapid-release phase was noted during the first week followed by a plateaued effect achieving a near zero-order release over the final week. Conduit B, having the NGF layer deposited just outside the PEC layer had the highest burst release, particularly during the rapid-release phase. Conduit D, having the NGF layers interposed between additional layers of PLGA was more efficient in minimising burst release and significantly prolonging the delivery of NGF at the end of 15 days (Pfister *et al.*, 2008). Using a similar concept of PEC formation, Xu and co-workers, 2011, developed a multi-layered nerve conduit using a layer-by-layer electrostatic self-assembly technique to create a PDLLA/chondroitin sulphate/chitosan PEC of varying layers. NGF was immobilised onto the conduit via carbodiimide crosslinking. The nerve conduits were evaluated for their *in vitro* release kinetics of NGF and its effect on the potential of nerve regeneration across a 10mm gap in the rat sciatic nerve model (Xu *et al.*, 2011).

Despite a high initial burst release over the first day followed by a sharply plummeting release of NGF over the next day, the multi-layered PEC system was successful in providing a zero-order profile with a sustained release of 1ng NGF daily for over 50 days. Implantation of the conduit across a 10mm sciatic nerve gap in the rat proved the conduit to be closely matched to the outcomes of the autograft when comparing the two in terms of myelination, axon diameter and nerve conduction velocity over a period of 3 to 6 months (Xu *et al.*, 2011). PECs, particularly those fabricated from multi-layered techniques, are being extensively investigated for the delivery of proteins and their use in tissue engineering applications (Cooper *et al.*, 2005; Gucht *et al.*, 2011; Luo and Wang, 2014; Tsai *et al.*, 2014). Besides presenting a milder method of immobilisation for delicate proteins, PECs offer improved physicochemical strength with a minimised swelling and erosion propensity thereby prolonging bioactive release (Cooper *et al.*, 2005; Li *et al.*, 2009). To obtain various release kinetics, proteins can be deposited onto the surface of a pre-formed PEC layer, in between PEC multi-layers or distributed within a PEC layer for a steadier release (Cooper *et al.*, 2005). PECs have been used for the delivery of proteins and peptides for several controlled release applications and should be further studied for their use in delivering NTFs in peripheral nerve injury.

2.6. Combined Delivery of Neurotrophic Factors: Effects on Neuronal Regeneration

The simultaneous delivery of two or more NTFs from nerve conduits may be more beneficial in peripheral nerve regeneration than the delivery of a single NTF. Each NTF has a unique therapeutic mechanism of promoting regeneration of and sustaining neuronal cells. The co-delivery of NTFs may enhance nerve regeneration by targeting and promoting various different pathways of neuronal growth and survival in addition to reducing the initial loading and daily doses of NTFs thereby providing perhaps a more cost-effective route in utilising NTFs. Schwann cells (SCs), forming the predominant component of glial cells in the peripheral nervous system, are known to express a number of receptors for ECM molecules and NTFs (Chernousov and Carey, 2000; Evans *et al.*, 2002). It is proposed that NTFs exhibit their actions by binding to two receptor types, namely the p75NTR receptor and the Trk class of receptors comprising TrkA (NGF), TrkB (BDNF) and TrkC (NT-3) located on SCs and neurons throughout the nervous system (Patapoutian and Reichardt, 2001; Allen *et al.*, 2013; Morcuende *et al.*, 2013). SCs have been reported to particularly express high levels of the p75NTR receptor which NTFs act on to modulate the migration, proliferation and myelination capacities of SCs in peripheral nerves (Johnson *et al.*, 1988; Taniuchi *et al.*, 1988; Cosgaya *et al.*, 2002). The binding of NTFs to the p75NTR receptor is thought to occur with a similar low affinity across all NTFs whereas binding activity to the Trk class of receptors is more specific (Carter *et al.*, 1996; Syroid *et al.*, 2000). NGF, having particular

affinity for TrkA, functions to promote the survival and maintenance of sensory neurons whereas binding to p75NTR receptor sites on SCs enhances myelination of axons (Piirsoo *et al.*, 2010; Allen *et al.*, 2013). Similarly, the neurotrophin BDNF is reported to be a potent promoter of myelination via activation of the p75NTR receptors located on SCs whereas activation of the same receptors by NT-3 enhances SC migration. It was noted by researchers that although NT-3 had positive effects on SC migration, activation of SC-TrkC receptors inhibited myelination (Cosgaya *et al.*, 2002; Yamauchi *et al.*, 2004; Piirsoo *et al.*, 2010). GDNF has been investigated for its actions in the promotion of motor neuron survival, SC migration and the induction of myelination of small axons via activation of the Ret tyrosine kinase (Höke *et al.*, 2003; Iwase *et al.*, 2005; Piirsoo *et al.*, 2010; Catrina *et al.*, 2013). In this regard, cautious NTF selection for combined therapy is necessary as the delivery of more than one NTF may prove to confer either synergistic or opposing biological activity. Furthermore, the activation of specific receptors and its resulting effects on the different cell types found in peripheral nerves must be taken into account.

In an interesting study by Madduri and co-workers, 2010, it was shown that GDNF-releasing nerve conduits promoted only axonal elongation in chicken embryonic DRG whereas a combined-release of GDNF and NGF enhanced axonal elongation in addition to the promotion of branching of the nerve tissue. It was noted, in DRG assays, that optimal growth occurred at doses of 1-10ng/ml of GDNF or NGF but combined GDNF and NGF required a total reduced dose range of 0.1-1ng/ml for an optimal growth response. Furthermore, *in vivo* studies across a 10mm nerve gap showed significantly improved axonal outgrowth when using a combination of 40ng GDNF and NGF each in comparison to 80ng GDNF alone (Madduri *et al.*, 2010a). To enhance the synergistic effect of the GDNF/NGF combination, a variety of collagen and silk fibroin nerve conduits were designed for the extended and simultaneous release of the growth factors in a zero-order fashion (Catrina *et al.*, 2013).

The selection of NTFs for combined delivery to achieve a synergistic response in the enhancement of axonal regeneration must be carefully determined as not all NTFs may work synergistically for a heightened effect. Another investigation, using concentration gradients of NTFs, showed that combined concentrations of NGF and BDNF had no significant effect of synergism when evaluated for axonal growth response in DRG however a combination of NGF and NT-3 was shown to be successful in achieving synergism at concentrations of 80ng/ml/mm each. The NGF/NT-3 combination of growth factors was more effective in axonal regeneration compared to a single dose of NGF of 133ng/ml/mm concentration and capable of guiding axons over 12.5mm distance as opposed to that of 7.5mm, respectively (Cao and Shoichet, 2003).

2.7. Cell-Engineered Applications for Peripheral Nerve Regeneration

Another technique of delivering neurotrophic factors to injured peripheral nerves involves the exogenous culturing of support cells into the matrix of natural or synthetic-based conduits. Such cells, Schwann cells (SCs), mesenchymal, neural and embryonic stem cells, interact via various molecular pathways to bring about peripheral nerve regeneration by providing substrates and molecules that partake a pivotal regulatory function in axonal migration and proliferation (Evans *et al.*, 2002; Gu *et al.*, 2011). The purported positive interactions between support cells and peripheral nerves led to the inclusion of these cells into nerve conduits—a growing field of interest as an alternative to the nerve autograft.

Schwann cells, being the principal support cells of the peripheral nervous system, are capable of secreting neurotrophic factors in addition to providing an ECM-scaffold system that fulfils the fundamental requirements for an environment sustainable of neural cell growth (Goto *et al.*, 2010). Several studies have investigated methods of SC transplantation and over-expression of genes encoding specific proteins as a promising outlook for peripheral nerve regeneration (Hadlock *et al.*, 2000; Evans *et al.*, 2002; Mosahebi *et al.*, 2003; Goto *et al.*, 2010; Shakhbazau *et al.*, 2012). Studies have shown the regenerative potential of genetically modified SCs over-expressing fibroblast growth factor, in short and long gap sciatic nerve defects of 5mm and 15mm, respectively (Haastert *et al.*, 2006; Jungnickel *et al.*, 2006). A group of investigators designed isogenic NGF-transduced SCs for the supply of NGF immediately after nerve injury and during the early stages of nerve regeneration when endogenous neurotrophin levels are particularly low (Shakhbazau *et al.*, 2012). The over-expression of NGF from the transplanted SCs was able to provide long-term and increased delivery of the NTF for at least two weeks after induction of sciatic nerve injury. In the presence of proliferating SCs, bands of Büngner are formed which guide axonal regeneration and target tissue innervation from the proximal to the distal stump of the transected nerve thus enhancing nerve regeneration potential (Evans *et al.*, 2002; Goto *et al.*, 2010). Since, it is thought that SCs secrete a number of NTFs comprising of NFG, BDNF and GDNF which act through different mechanisms to enhance and accomplish complete nerve regeneration, the incorporation of SCs may be considered a valuable addition in enhancing the functionality of nerve conduits (Goto *et al.*, 2010; Madduri and Gander, 2010; Gu *et al.*, 2011). The same group of researchers later developed a method for the regulation of GDNF expression using dendrimers and lentiviral transduction for the modification SCs paired with the administration of doxycycline for improved functional recovery in a rat nerve model (Shakhbazau *et al.*, 2013). Similarly upcoming research has focused on the ability of modified SCs to deliver GDNF for axonal regeneration (May *et al.*, 2013; Santosa *et al.*, 2013).

Although SCs are popular for the investigation of cell and gene-based therapy in neural regeneration strategies, neural and mesenchymal-derived stem cells have likewise shown significance in the ability to deliver NTFs such as BDNF, GDNF and NGF (García *et al.*, 2004; Nourizadeh *et al.*, 2011; Ma *et al.*, 2012; Wang *et al.*, 2012). Such cell-based delivery applications may offer several advantages in terms of providing suitable release kinetics for the delivery of multiple NTFs in therapeutically adequate quantities where concerns of protein degradation and inactivation from manufacturing processes may be eradicated thereby ensuring complete bioactivity of these physiologically delivered NTFs (Dove, 2002; Madduri and Gander, 2010).

2.8. Concluding Remarks

The therapeutic benefits offered by the use of neurotrophic growth factors for the application in enhancing regeneration and healing of transected and damaged peripheral nerves are noteworthy. The full potential of such potent growth factors can only be harvested if employed in a delivery system capable of precisely releasing adequate quantities of growth factor for a sufficient amount of time following the most desirable release kinetics, preferably zero-order release or a via a delivery system that offers a gradient-based release of NTFs. Burst release mechanisms must be further investigated so that minimal quantities of bioactives are released upon *in vivo* implantation of the nerve conduit resulting in potential hindrance of nerve growth. Furthermore, bioactivity of entrapped NTFs must be ascertained as fabrication procedures influence their ultimate therapeutic potency. In conjunction with *in vitro* release studies, animal models are valuable in assessing the effects and differences of the observed release kinetic profiles as similar release kinetics cannot be simply assumed to occur *in vivo*. *In vitro* studies cannot fully emulate the biological and physiological conditions present in the body especially after subsection of internal organs to injury. Hence different studies on different types of nerve conduits may present with similar release kinetics compared to each other but have different regeneration effects on the regeneration potential on tissues. Although numerous new polymeric nerve conduits delivery systems have been proposed with several designs suggesting great promise in the significant improvement of peripheral nerve regeneration, further work must be carried out to smooth out concerns associated with release kinetics, ideal NTF selection and dosing in accordance to tissue requirements, the length of treatment and restoration of functional recovery.

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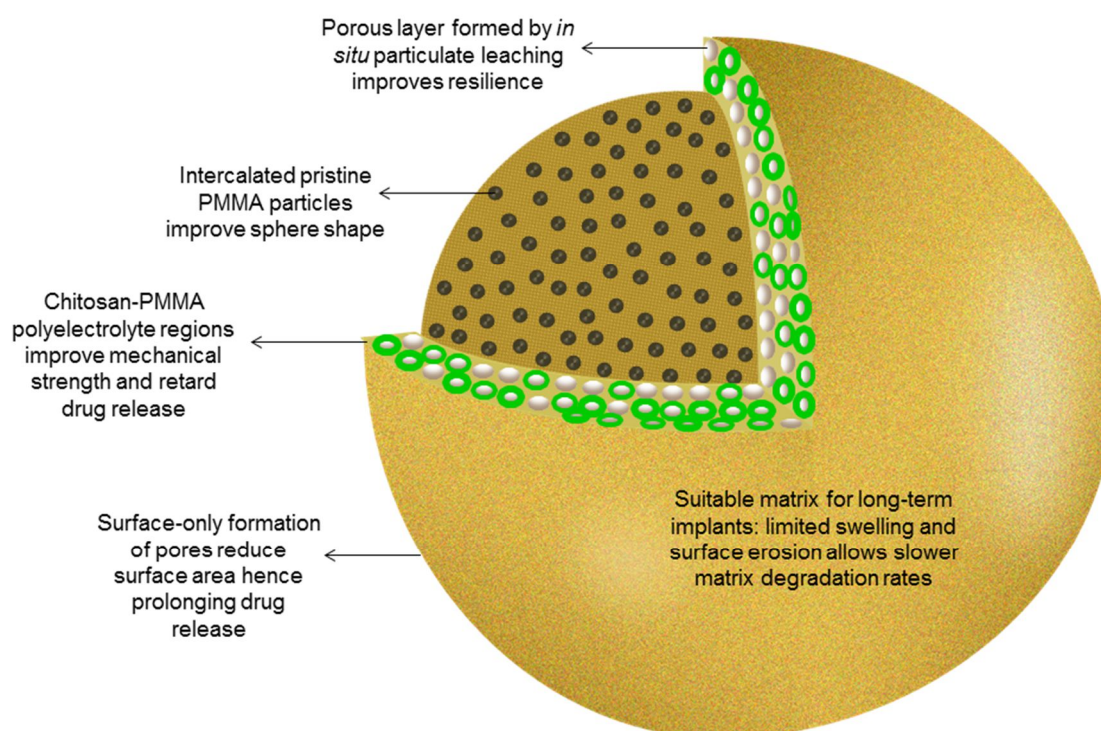
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CHAPTER 3

PROOF OF CONCEPT: DESIGN OF CHITOSPHERES LOADED WITH PRISTINE POLYMER PARTICLES FOR EXTENDED DRUG DELIVERY VIA POLYELECTROLYTE COMPLEXATION AND PARTICULATE LEACHING

Abstract

The aim of this study was to investigate the drug release from swellable chitospheres laden with pristine polymethylmethacrylate (PMMA) nanoparticles. Chitosan matrices were prepared by sodium tripolyphosphate crosslinking from a chitosan suspension containing the model BCS class II drug, indomethacin. PMMA particles were added to the chitospheres as the modulator for drug release. Swelling and erosion studies in conjunction with textural profiling provided an understanding of the dominant and underlying drug release mechanisms of the ionically crosslinked chitospheres loaded with the pristine PMMA particles. A series of drug release studies performed in PBS pH 7.4 showed that the pristine particle-loaded chitospheres released indomethacin over 144 hours in a first-order manner with 50% drug release occurring over 48 hours. The study also revealed that *in situ* porogen leaching for pore creation and polyelectrolyte complex formation were the main mechanisms of release from the chitospheres. The results of this study may be utilized for the development of neuro-implants for controlled delivery of bioactives to the brain where scaffolds of superior mechanical strength and reduced swelling properties are required.



3.1. Introduction

Chitosan matrices, particularly spheres, formed from ionotropic and covalent crosslinking have long been used as a three-dimensional matrix for the study of release profiles of numerous drugs with the intent to modulate drug release profiles in a manner that provides drug release of appropriate kinetic order and therapeutic effectiveness (Gupta and Kumar, 2000; Mi *et al.*, 2003; Sarkar *et al.*, 2013). These multiparticulate spherical matrices (Ko *et al.*, 2002; Lin *et al.*, 2005; Pasparakis *et al.*, 2006; Srinatha *et al.*, 2008; Kotadia *et al.*, 2009; Harris *et al.*, 2010; Yang *et al.*, 2011; Jonassen *et al.*, 2012; Rampino *et al.*, 2013) comprise chitosan alone or blended with other property-modifying natural or synthetic polymers that have been widely used for the oral delivery of therapeutic agents and for long-term sustained delivery of proteins, peptides and other essential growth factors for tissue regeneration applications and gene delivery technologies (Anal *et al.*, 2003; Vimal *et al.*, 2013).

Chitosan is readily soluble in acidic pH, with the resultant protonated amine group available for ionic interaction with multivalent ions for immediate gelation (Jonassen *et al.*, 2012; Shenvi *et al.*, 2014). The cationic nature of chitosan also makes it simple for producing physically crosslinked gelling matrices and furnishes the ability to electrostatically interact with counter ionic polymers to form polyelectrolyte complexes (PECs) (Cerf *et al.*, 2014; Nath *et al.*, 2015). Previously reviewed PEC applications show that PECs have unique physicochemical and mechanical properties to that of the parent polymers and offer an additional dimension to the delivery system and enhanced properties for tailoring drug release patterns to the desired site (Hamman, 2010). Sodium tripolyphosphate (STPP) has been frequently used as an ionic crosslinking agent for chitosan matrices due to its rapid crosslinking action and safety compared to glutaraldehyde, epichlorohydrin and ethylene glycol diglycidyl ether that have proven physiological toxicity (Mi *et al.*, 1999; Shu and Zhu, 2002; Li and Huang, 2012; Shenvi *et al.*, 2014). Chitosan-based multiparticulates can instantly be synthesised using STPP which forms either intermolecular or intramolecular linkages that stabilize the positively charged amino groups of chitosan resulting in crosslinking (Mi *et al.*, 1999, 2003). Previous studies, aiming to extend the release of indomethacin from crosslinked chitosan and other multiparticulate systems over 12-24 hours have been reported (Mi *et al.*, 2002; Yuksel *et al.* 2011). However, the poor mechanical strength of these matrices limits its use in the pharmaceutical industry and the prolonged release of drug and loading efficiency still needs to be improved (Shu and Zhu, 2000). Strategies aimed at improving mechanical strength of and more particularly the controlled release of drug from chitosan beads includes the use of genipin as a covalent crosslinker as opposed to ionic crosslinkers. Yang and co-workers (2011) investigated the mechanical properties of STPP/genipin co-crosslinked chitosan beads whereas Harris and co-workers

(2010) employed a series of chitosan hydrochloride genipin crosslinking reactions for the controlled release of drugs and proteins. Another widespread method employed for enhancing the drug delivery properties of chitosan beads involve the polyelectrolyte complexation of chitosan with anionic polymers either via blending of polymers prior to bead formation or coating of the beads employing a series of layering techniques using mainly alginates (Wang *et al.*, 2011; Gong *et al.*, 2011; Zhang *et al.*, 2011; Torelli-Souze *et al.*, 2012; Zhou *et al.*, 2013) and pectins (Maestrelli *et al.*, 2012; Ribeiro *et al.*, 2014).

Therefore, the present study makes use of chitospheres to investigate drug release profiles when pristine (previously unmodified and drug-free) polymer nanoparticles of a polymethyl methacrylate (PMMA) polymer are distributed throughout the matrix. The electrostatic interaction between the native polymers producing the PEC can significantly improve the mechanical properties, particularly resilience and hardness, in addition to prolonging drug release.

The use of PMMA is to control drug release from the chitospheres. PMMA (as the pristine nanoparticles) is an anionic copolymer of polymethacrylic acid and methyl methacrylate (Eudragit S100, Evonik, Midrand, Gauteng, South Africa). PMMA is soluble at a pH value of > 7.0 and electrostatic interactions between anionic PMMA and cationic chitosan may assist in prolonging drug release. Incorporating it as pristine particles within the chitospheres allows the PMMA to function as a porogen and simultaneously form a PEC after the pH-responsive dissolution of the particles. This technique of inducing porosity and PEC formation holds promise for improving matrix resilience and extending drug release as the matrix undergoes hydration. Such PECs between chitosan and polymethacrylate copolymers are possible and have been previously confirmed where polymethacrylates, in solubilised solution form, were used as surface coating agents for pH-controlled release of entrapped drugs (Lorenzo-Lamosa *et al.*, 1998; Moustafine *et al.*, 2008; Hamman, 2010). A PEC was inadvertently formed at the PMMA film-chitosan bead interface upon coating, however the primary use of the PMMA coating was for its gastro-protective and enteric drug-releasing properties. To our knowledge, this is the first report of in situ polyelectrolyte synthesis in addition to in situ porogen leaching. The anti-inflammatory drug, indomethacin, is a BCS Class II drug with good permeability and poor aqueous dissolution that limits its bioavailability (Obitte *et al.*, 2014). Transition changes of indomethacin from crystalline to amorphous states within the loaded chitospheres may potentially improve its solubility and hence enhance bioavailability (Yuksel *et al.*, 2011; Priemel *et al.*, 2013). Such changes induced within the chitospheres by addition of PMMA and its pH-responsive dissolution may significantly prolong drug release.

The aim of this study was to determine the mechanistic interactions of PMMA as pristine particles with chitospheres and evaluate its modulating ability to prolong drug release in comparison to conventional chitosan spheres. This work involved the preparation of the chitospheres via ionotropic gelation in order to investigate the shift in drug release profiles induced by the incorporation of PMMA pristine particles in varying ratios. Analysis of the drug release profiles using non-linear kinetic modelling and the corroboration thereof with further characterisation such as SEM imaging, textural profiling, swelling and erosion studies were undertaken in order to determine the mechanisms governing drug release from the modified chitospheres.

3.2. Materials and Methods

3.2.1. Materials

Chitosan (Poly D-glucosamine, deacetylated chitin, medium molecular weight), indomethacin (>99% TLC), sodium tripolyphosphate (technical grade, 85%) and dialysis tubing cellulose membrane (molecular weight cut-off = 14 000Da) were purchased from Sigma Aldrich Co (St. Louis, MO, USA). Eudragit S100 was purchased from Evonik (Midrand, Johannesburg, South Africa) and was used without further modifications as the pristine PMMA particles. Acetic acid glacial (98.5%) and ethanol (99% absolute) were purchased from AceChem (Johannesburg, Gauteng, South Africa) and LabChem (Edenvale, Gauteng, South Africa) respectively. Millipore water was used for all the preparations.

3.2.2. Synthesis of chitospheres

Chitospheres were prepared by the conventional method of ionotropic gelation, adapted from Bodmeier, *et al.* (1989) and Shu and Zhu, (2000), using STPP as the ionic crosslinking agent. A 2%^{w/v} chitosan solution was prepared by dissolving chitosan in 1M acetic acid at room temperature until homogenous. The model drug, indomethacin (1%^{w/v}) was dissolved in pure ethanol and added to the chitosan solution. After evaporation of ethanol, PMMA (as the pristine PMMA nanoparticles), was added to the chitosan-indomethacin solutions in the following ratios of chitosan to pristine PMMA particles: 2:1, 1:1 and 1:2 (Table 3.1). This produced a homogenous distribution of drug throughout the polymer solution. Multiparticulates from a chitosan-indomethacin solution were prepared without the addition of pristine PMMA particles as a control formulation. The indomethacin loaded chitosan-PMMA particle suspensions were then dropped through a 21 gauge hypodermic needle at a flowrate of 0.4mL/min into STPP crosslinking solutions of 1%, 2% and 3% ^{w/v} to form chitospheres. The chitospheres were left to cure in the crosslinking solutions for 30 minutes (Mi *et al.*, 1999; Shu and Zhu, 2000 and 2002) before being separated, washed and collected. The chitospheres were washed with 1M acetic acid to remove excess drug and

any uncrosslinked chitosan from the surface. The chitospheres were then dried at 50°C for 48 hours and stored in a desiccator before further physicochemical characterization and drug-release profiling.

Table 3.1: List of chitospheres prepared with different concentration ratios of chitosan and PMMA.

STPP crosslinking	Chitosan : pristine PMMA particles ratio ^{w/w}
1%	Control (chitosan only)
	2:1
	1:1
	1:2
2%	Control
	2:1
	1:1
	1:2
3%	Control
	2:1
	1:1
	1:2

3.2.3. Characterization of chitospheres

3.2.3.1. Evaluation of size, surface morphology and pore formation

The chitosphere size was assessed using a digital Vernier calliper (Krafft, DV150GW, Schoellerstr Düren, Germany). The mean diameter was measured on multiple chitospheres (n=12). Diameter measurements were taken at four different angles of individual chitospheres. Measurement of height, for irregularly shaped or flat-based chitospheres, was taken for the calculation of volume. For the evaluation of the surface morphology, dry chitospheres were fixed to aluminium stubs using double-sided adhesive carbon tape and then sputter coated with gold-palladium for 40 seconds using an EPI sputter coater (SPI Module TM sputter-coater and control unit, West Chester, PA, USA). Samples were viewed using a FEI Quanta 400 FEG-SEM scanning electron microscope (FEI, Hillsboro, OR, USA) and images were taken of the chitosphere surface and microstructure.

3.2.3.2. Porosimetric characterization of chitospheres

The surface area and pore size of hydrated chitospheres were evaluated using a Porosimetric Analyzer (Micromeritics ASAP 2020, Norcross, GA, USA). After 24 hours exposure to dissolution medium of pH 7.4, hydrated chitosphere samples of 100mg were frozen at -85°C and lyophilized (Freezone 12, Labcono, Kansas City, USA) before degassing samples for elimination of residual moisture and contaminants. A glass filler rod inserted into the sample tube reduced the total free space volume in the tube thus hastening the

degassing stage involving an evacuation and heating phase. Thereafter, the sample tube was transferred to the analysis port for determination of surface area, pore size and pore volume in accordance with the BET and BJH models. An evacuation rate of 50.0mmHg/s with a temperature ramp rate of 10°C/min, target temperature of 40°C and a hold temperature of 30°C for 900min was used. The BET linear equation (Equation 3.1) was used to determine the monolayer capacity.

$$p/n^a(p^0-p) = 1/n_m^a \times C + (C-1)p/n_m^a \times Cp^0 \quad \text{Equation 3.1}$$

Where, n^a refers to the quantity of N₂ adsorbed at the relative pressure p/p^0 , n_m^a was the monolayer capacity and C was exponentially related to the enthalpy of adsorption in the first adsorbed layer.

Using the molecular cross-sectional value a^m , the surface area was determined from the monolayer capacity employing Equations 3.2 and 3.3.

$$A_s(BET) = n_m^a \times L \times a_m \quad \text{Equation 3.2}$$

$$a_s(BET) = A_s(BET)/m \quad \text{Equation 3.3}$$

Where $A_s(BET)$ and $a_s(BET)$ are the total and specific surface areas, respectively, of the adsorbent of mass m and L is the Avogadro constant.

3.2.3.3. Swelling and matrix erosion determination

Swelling and degradation studies were performed simultaneously. Samples of 50mg drug-free chitospheres were placed in a cellulose dialysis membrane and immersed in 100ml of PBS pH 7.4 in an orbital shaking incubator (LM-530-2, MRC Laboratory Instruments Ltd., Hahistadrut, Holon, Israel) maintained at 37°C and set at 50rpm for sample agitation. Samples were removed at predetermined time intervals and chitosphere size and mass were measured for the determination of change in volume and swelling ratio, respectively, lightly blotted with tissue paper to remove excess water. The wet chitospheres were then left to air-dry for 72 hours to ensure complete removal of residual moisture before being weighed to determine the eroded mass.

3.2.3.4. Determination of drug entrapment efficiency

For the determination of indomethacin entrapment, drug-loaded chitospheres prepared from 2ml aliquots of the polymer-drug solution (n=3) were ground into a fine powder and dispersed in 50mL of PBS pH 7.4 for 72 hours under agitation to allow complete dissolution

of the drug. Thereafter 5mL samples were removed, filtered through a 0.22µm filter and assayed for drug content using UV-spectroscopy (Implen NanoPhotometer, Implen GbmH, München, Germany) at a wavelength of 319nm. For additional validation of drug entrapment efficiency (DEE), drug loss resulting from leaching processes while curing of chitospheres in the crosslinking solution was determined using UV-spectroscopy by measuring the absorbance of the STPP medium samples at 319nm after formation of the chitospheres. The %DEE was calculated from the actual and theoretical drug loading of the chitospheres. All tests were performed and analysed in triplicate.

3.2.3.5. Determination of molecular vibrational transitions and analysis of PEC formation

Samples of chitospheres (both drug-loaded and drug-free) from each formulation (listed in Table 3.1) were analysed for the degree of crosslinking and interactions between the native polymers (chitosan and PMMA) for PEC formation. Samples were analysed over an FTIR spectra of wavelength between 4000-600cm⁻¹ using a PerkinElmer Spectrum 2000 ATR-FTIR (PerkinElmer 100, Llantrisant, Wales, UK) spectrometer fitted with a single-reflection diamond MIRTGS detector.

3.2.3.6. Determination of thermophysical properties of the chitospheres and native polymers

The thermodynamic properties of the chitospheres were determined using a Temperature Modulated Differential Scanning Calorimeter (TMDSC) (Mettler Toledo, DSC, STAReSystem, Swchwerzenback, ZH, Switzerland). Powdered chitospheres samples of 15mg were sealed in aluminium crucibles and heated over a temperature range between 25-300°C at a heating rate of 10°C/min. TMDSC curves were plotted as sample size against temperature and the integral value, onset and endset of melting was determined. The determination of change in sample weight as a function of temperature was performed using a Thermogravimetric analyser (TGA) (PerkinElmer, TGA 4000, Llantrisant, Wales, UK). Samples were heated at a rate of 10°C/min from 0-900°C under continuous nitrogen purging. Thermograms were generated as percentage weight vs. temperature and analysed using PyrisTM software (PerkinElmer, Llantrisant, Wales, UK).

3.2.3.7. Textural profiling to determine physicomechanical properties of chitospheres

Textural analysis characterization using a Texture Analyser (TAXT.*plus* StableMicrosystems, Surrey, UK) was undertaken on hydrated chitospheres. The Matrix Resilience (MR), Deformability Modulus (DF) and Fracture Energy (FE) were determined. MR (N.sec) was determined by the ratio of the areas under the compression and decompression curves

generated by Force-Time textural profiles. This represented the ability of the chitospheres to recover to its original form after application of an external deforming stress. The Area under the Curve (AUC) from a Force-Distance textural profile allowed the determination of the FE (N.m) which is the energy required to rupture a hydrated swollen chitospheres. The DM (N/mm) is an indication of the chitosphere matrix hardness and was computed from the gradient of the Force-Distance profile from zero to the point of peak force. The textural analysis parameters utilized include a pre-test and post-test speed of 1.00mm/sec, a test speed of 0.5mm/sec, a trigger force of 0.5N and a load cell of 5kg. The target mode was set to 50% strain for MR and force for DF and FE.

3.2.3.8. *In vitro* drug release studies on chitosphere formulations and kinetic modelling of release data

In vitro release studies were performed using chitospheres prepared from 2mL samples of the chitosan-pristine PMMA particle suspension so that each sample had approximately 20mg of indomethacin. The average mass of chitospheres prepared from a 2mL sample is listed in Table 3.2. After drying at 50°C the chitospheres were placed into jars containing 100mL PBS of pH 7.4 (n=3). Samples were kept in an orbital shaking incubator maintained at 37°C and set at 50rpm for sample agitation. At predetermined time intervals 5mL of release medium were sampled and replaced with a fresh, equal volume of release medium to maintain sink conditions. Samples were filtered using 0.22µm PVDF syringe filters and analysed for the drug quantity by UV absorbance at wavelength 319nm. To gain further insight into the drug-release mechanisms, the drug-release profiles were kinetically modelled using the models listed in Table 3.3. Furthermore, the difference and similarity between the various dissolution profiles of the chitospheres and that of the Control were determined using fit factors of F1 as the difference factor and F2 as the similarity factor using Equations 3.4 and 3.5, respectively (Yuksel *et al.*, 2000; Shah and Deshpande, 2014).

$$F1 = [(\sum_{n=1}^n(Rt - Tt) / (\sum_{n=1}^n Rt))] \times 100 \quad \text{Equation 3.4}$$

$$F2 = 50 \times \log [(1 + (1 + (1/n) \sum_{n=1}^n(Rt - Tt)^2)^{-0.5} \times 100] \quad \text{Equation 3.5}$$

Where *Rt* and *Tt* refers to the percentage of drug released at time *t* of the control and the test formulations, respectively, and *n* indicates the number of sampling points.

Table 3.2: The average mass of chitospheres containing approximately 20mg of indomethacin (n=3).

Formulation	Average Weight (mg)
Control (No pristine particles) 1% STPP	101.30 ±0.294
Control (No pristine particles) 2% STPP	139.60 ±0.989
Control (No pristine particles) 3% STPP	139.97 ±0.188
2:1 1% STPP	115.50 ±0.081
2:1 2% STPP	132.73 ±0.205
2:1 3% STPP	158.90 ±1.358
1:1 1% STPP	127.46 ±0.047
1:1 2% STPP	141.8 ± 0.489
1:1 3% STPP	189.76 ±1.681
1:2 1% STPP	159.53 ±0.094
1:2 2% STPP	174.27 ±0.531
1:2 3% STPP	228.97 ±1.114

Table 3.3: Models and parameters utilized for kinetic modelling of release data.

Model Name	Parameters	Significance	Reference
Zero-order $C = K_0t$	K_0 = zero-order rate constant t = time	Describes drug dissolution from dosage forms that do not disintegrate but release drug slowly	Dash <i>et al.</i> , 2010
First-order $\text{Log}C = \text{Log}C_0 - kt/2.303$	C_0 = initial concentration of drug K = first-order constant	Describes drug dissolution from porous matrices	Dash <i>et al.</i> , 2010
Higuchi $Q = Kt^{1/2}$	Q = % drug release K = design variable of the system t = time	Describes drug release occurring via diffusion in one dimension	Dash <i>et al.</i> , 2010 Singhvi and Singh, 2011
Hixson-Crowell $Q_0^{1/3} - Q_t^{1/3} = K_{HC}t$	Q_0 = initial amount of drug loaded Q_t = amount of drug released in time t K_{HC} = rate constant t = time	Describes drug release from matrix systems undergoing a change in surface area and diameter	Dash <i>et al.</i> , 2010 Singhvi and Singh, 2011

Korsmeyer-Peppas $M_t / M_\infty = Kt^n$	$M_t / M_\infty =$ fractional drug release at time t K = rate constant t = time n = release exponent	Employed to find the mechanism of drug release by calculating the value of n. If $n < 0.45$ it corresponds to Fickian diffusion, $0.45 < n < 0.89$ to non-Fickian anomalous transport, $n = 0.89$ to Case II transport and $n >$ 0.89 to Super case II transport	Dash <i>et al.</i> , 2010 Singhvi and Singh, 2011
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3.3. Results

3.3.1. Analysis of volume, surface morphology, erosion and pore formation as per SEM imaging

It was noted that chitospheres made from chitosan alone had a more flattened morphology and were lower in volume compared to the chitospheres made from the formulations containing increased polymer mass contributed by the addition of the PMMA pristine polymer particles. Furthermore, increase in concentration of the STPP crosslinking solution produced chitospheres more spherical in shape and with larger volumes (Figure 3.1). The equation used to calculate the volume of the chitospheres was that of a sphere cap due to its shape. Chitospheres were not completely spherical but rather had a small flattened base due to the drying process while resting on the base of a dish as seen in Figure 3.2. Thus, the equation employed offered a more accurate determination of the chitospheres volume. Chitospheres rounder in shape had a height closer to the value of its diameter, hence, having a larger volume capacity compared to those with a flattened base.

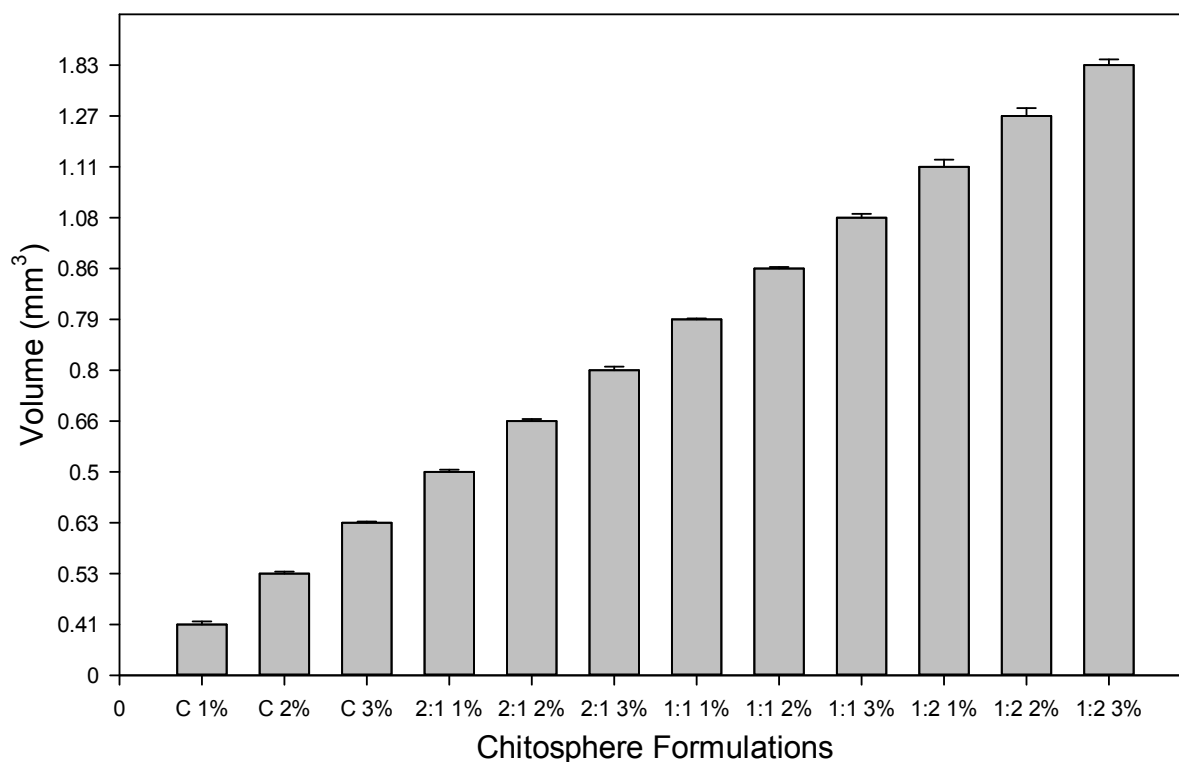


Figure 3.1: Depiction of the comparison of volume across the different formulations used to fabricate PMMA-loaded chitospheres (n=12).

The difference in volume between chitospheres containing PMMA particles and the Controls may be possibly due to the formation of larger droplets at the end of the needle as a result of increased viscosity of the chitosan/PMMA suspension imparted by the addition of a large volume of powdered particles also increasing the total polymer mass content dispensed in each droplet (Kulkarni *et al.*, 2011). The surface morphology of the Control chitospheres were found to be fairly smooth in comparison to those containing pristine PMMA particles with slight folds occurring around the middle of the chitosphere surface whereas closer inspection of the surface of PMMA-containing chitospheres revealed a rough surface where the presence of PMMA particles is clearly noted on the chitosphere surface. SEM images of the degraded chitospheres samples were taken at 1, 2 and 5 days after exposure to PBS. Chitospheres containing pristine PMMA particles maintained their spherical shape and most of the structural integrity compared to the Control samples which appeared cracked and fragmented and the 2:1 chitospheres samples, which underwent a slight change in shape. This may suggest that not only does the addition of pristine PMMA particles enhance the mechanical strength of the chitospheres but a polyelectrolyte complex (PEC) may be formed upon hydration between the cationic chitosan and anionic PMMA producing chitospheres that are robust in their structural shape. An array of pores is evident on the surface of chitospheres containing larger quantities of pristine PMMA particles, namely the 1:1 and the

1:2 chitospheres. These pores are probably formed from the dissolution and leaching out of the intercalated pristine PMMA particles; as such pore structures cannot be identified on the chitosan-only chitospheres. Furthermore, it is noted that the 2:1 chitospheres and, upon closer inspection, the 1:1 and 1:2 chitospheres underwent a process of detachment of the outermost layer. This suggests the occurrence of initial surface erosion whereas the Control chitospheres, which does not exhibit this trait of surface flaking, may degrade by bulk degradation of the matrix (Zhang *et al.*, 2002).

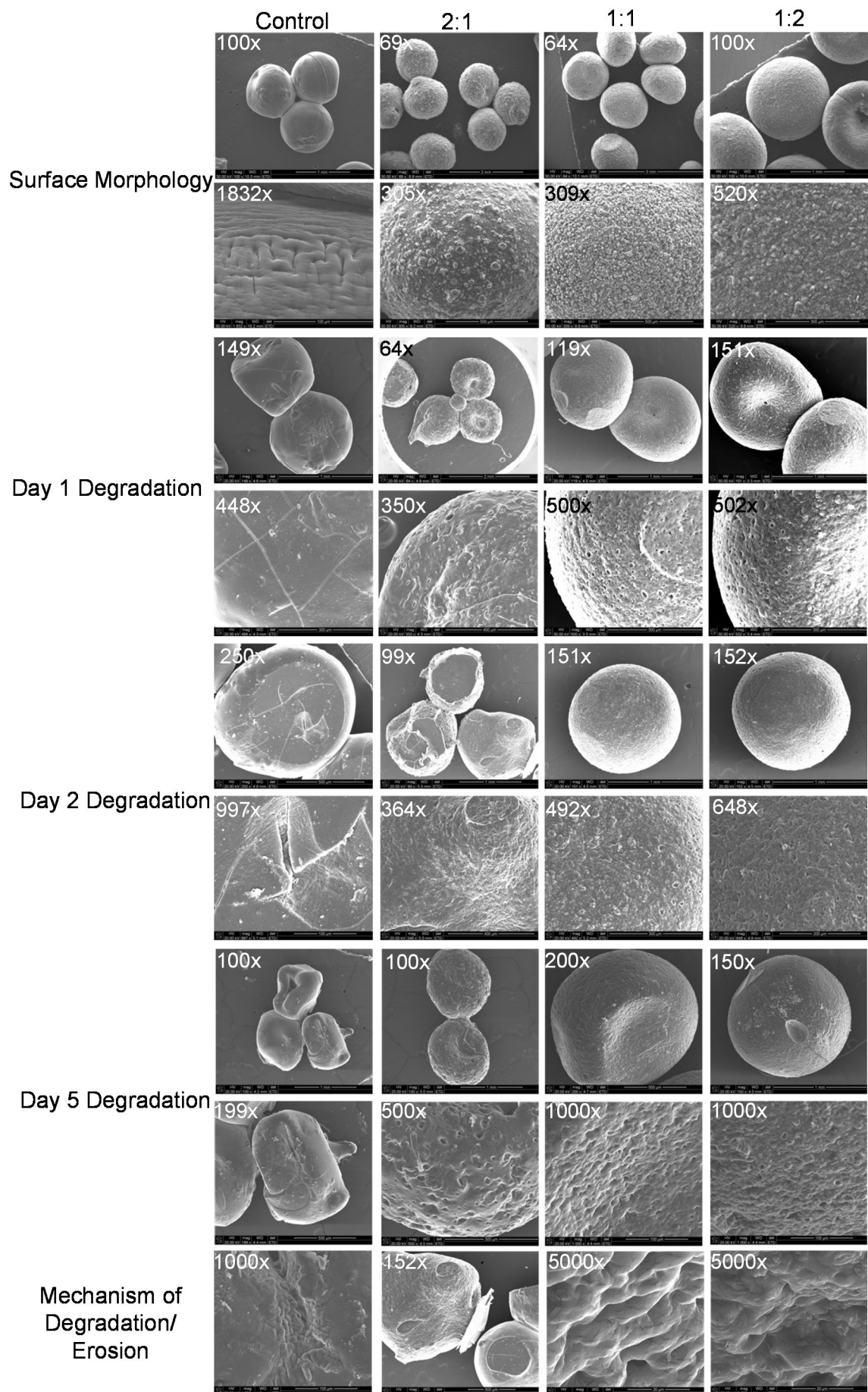


Figure 3.2: SEM images depicting the surface morphology of the 2%w/v STPP crosslinked chitospheres and their appearance at Days 1, 2 and 5 after degradation studies (n=3).

3.3.2. Porositometric analysis of chitospheres post hydration

Porositometric analysis of chitospheres hydrated in pH 7.4 dissolution medium revealed the differences in surface area, pore size and pore volume when chitospheres are prepared with varying concentrations of PMMA particles. The results listed in Table 3.4 show that the Controls (PMMA-particle free matrices) exhibited a high surface area but a smaller pore size and cumulative pore surface area compared to the chitospheres. The 1:1 chitospheres contained the largest pores and smallest surface area. Increasing the PMMA-particle content decreases the total surface area and increases pore size in terms of pore width, diameter and volume. The pore size and volume reflects the cumulative surface area of pores on the sample where pores larger in diameter and depth contribute to a larger pore surface area but smaller BET total surface area. However, further increase in PMMA-particle concentration (1:2 chitospheres) relative to chitosan results in a slight decline in pore size with a BET surface area similar to that of the 2:1 chitospheres. The lower BET surface areas of the chitospheres compared to that of the Controls, despite formation of pores, may further suggest that pore formation occurs only on the surface of sphere (as visualised by SEM) and not throughout the entire sphere which would result in an apparent increase in the total surface area of the sample. Although exhibiting a lack of pores, the significantly larger surface area of the Control multiparticulates, $25.5898\text{m}^2/\text{g}$, may be contributed by the formation of cracks on the surface as imaged by SEM in Figure 3.2. The technique of nitrogen gas adsorption over a range of relative pressures for the determination of pore characteristics produce adsorption isotherms (Figure 3.3) which provide insightful information regarding the pore size distribution in terms of pore width categorised as micro- (<2nm), meso- (2-50nm) and macropore (>50nm) ranges (Sing *et al.*, 1985; Groen *et al.*, 2003). According to this classification, the pore size distribution of the chitospheres lies within the mesopore range. The control matrices and the 2:1 chitospheres presented a Type II isotherm with a H2 hysteresis loop indicating an unrestricted monolayer-multilayer adsorption of a nonporous adsorbent. The 1:1 and 1:2 chitospheres exhibit a Type IV isotherm, indicating existence of a multilayer material, with a H3 hysteresis loop suggestive of complete pore filling including capillary condensation which occurs in the presence of mesopores (Sing *et al.*, 1985; Groen *et al.*, 2003). The presence of a H2 and H3 hysteresis loop denotes an interconnected pore system of random distribution within the chitosphere matrix (Groen *et al.*, 2003). The initiation of the shift from a Type II isotherm and H2 hysteresis towards a Type IV isotherm and H3 hysteresis can be seen in Figure 3.3b, corresponding to the isotherm generated for the 2:1 chitospheres where the PMMA particles, in the lowest concentration, are introduced.

Table 3.4: Porosity analysis of chitospheres after hydration in PBS pH 7.4 for 24 hours.

Porositometric parameters	Control	2:1	1:1	1:2
Surface area				
Single point surface area (m ² /g)	4.3590	8.7846	7.0496	11.3063
BET surface area (m ² /g)	25.5898	12.6663	7.5959	12.4776
BJH Adsorption cumulative surface area of pores between 17.000 Å and 3000.000 Å diameter (m ² /g)	8.5990	10.940	7.572	14.690
BJH Desorption cumulative surface area of pores between 17.000 Å and 3000.000 Å diameter (m ² /g)	57.6271	25.3956	10.3718	16.1719
Pore volume				
Single point adsorption total pore volume (cm ³ /g)	0.0192	0.0194	0.0278	0.0451
BJH Adsorption cumulative volume of pores between 17.000 Å and 3000.000 Å diameter (cm ³ /g)	0.0285	0.0524	0.0549	0.0883
BJH Desorption cumulative volume of pores between 17.000 Å and 3000.000 Å diameter (cm ³ /g)	0.0591	0.0526	0.0547	0.0879
Pore size				
Adsorption average pore width (4V/A by BET) (nm)	2.9945	6.1330	14.6279	14.4678
BJH Adsorption average pore diameter (4V/A) (nm)	13.2699	19.1567	28.9953	24.0334
BJH Desorption average pore diameter (4V/A) (nm)	4.0991	8.2890	21.1114	21.7308

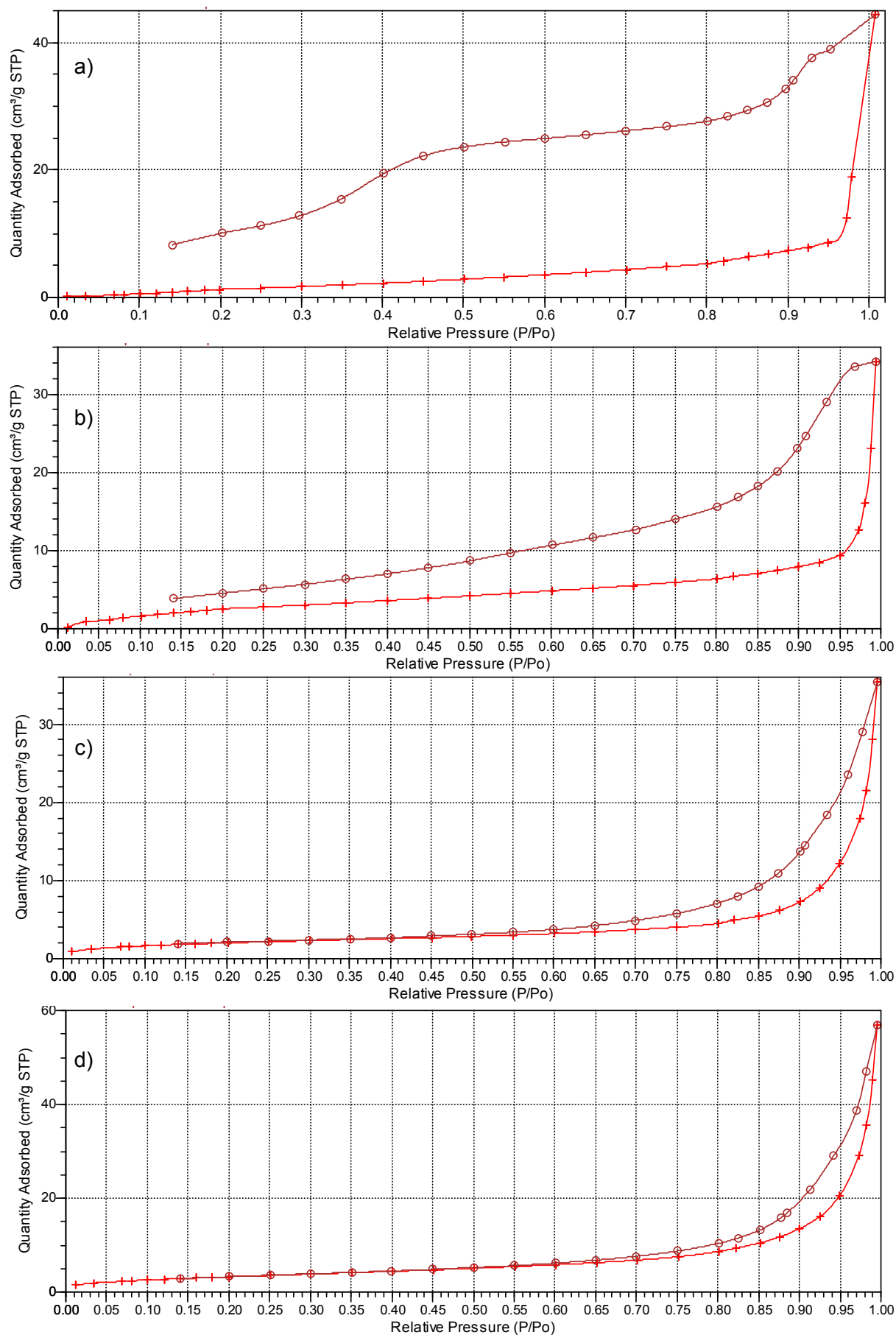


Figure 3.3: Isotherm linear plots of the 2%^{w/v} STPP crosslinked chitosphere STPP formulations a) Control b) 2:1 c) 1:1 d) 1:2 (n=1).

3.3.3. Determination of drug entrapment and drug loss

The concentration of the STPP crosslinking solution had insignificant effects ($p < 0.05$) on the drug loss and drug entrapment efficiency (DEE). The quantity of drug lost during the crosslinking curing period and that entrapped within the chitospheres were very similar across the varying crosslinking concentrations. Generally, it was noted that increasing the quantity of pristine PMMA particles slightly enhanced DEE by minimising the percentage of drug lost into the crosslinking solution during the curing process (Table 3.5). Being prepared from a more viscous polymer suspension, the pristine PMMA particles may prevent the diffusion of drug molecules out of the chitospheres. Drug loss may be attributed to leaching of indomethacin out of the chitospheres and into the surrounding solution by a passive diffusion process across a high-to-low concentration gradient. However, the percentage of indomethacin retained within the chitospheres was relatively high for all chitospheres ranging from 97-99%.

Table 3.5: Drug loss and %DEE for the various formulations of indomethacin-loaded chitospheres (n=6).

Chitosan:PMMA Formulation Ratio	% DEE
Control (No pristine PMMA particles)	97.75 $\pm$ 0.028
2:1	98.10 $\pm$ 0.0307
1:1	98.61 $\pm$ 0.026
1:2	98.74 $\pm$ 0.041

3.3.4. Analysis of PEC formation within PMMA-loaded chitospheres upon hydration in PBS pH 7.4

FTIR spectra of 24-hour hydrated chitosan-only multiparticulates and chitospheres were obtained to determine the molecular composition and crosslinking degree of the chitospheres as shown Figure 3.4. Crosslinking is evident from the P=O vibrations occurring at 1150cm^{-1} as this indicates the quantity of bound STPP ions which form interchain links between the $-\text{NH}_3^+$ protonated groups of chitosan (Mi *et al.*, 1999). The molecular integrity of the native polymer compounds, chitosan and PMMA (Eudragit® S100), can be discerned in the FTIR spectra of the PMMA-loaded chitospheres. Chitosan maintains its OH stretching at 3200cm^{-1} and the peak at 1630cm^{-1} is indicative of its amino ($-\text{NH}_2$) bending vibration. The presence of PMMA is indicated by the C=O vibrations of the esterified carboxyl groups characterised by the strong peaks at 1700cm^{-1} . The broad peak of chitosan at 3600cm^{-1} and the peak representing the amine bending at 1630cm^{-1} are evident in all of the formulations however with increase in the concentration of PMMA the intensity of these peaks decrease. On the contrary, the carboxylic acid peak of PMMA at 1700cm^{-1} is strongly detected in

formulations 1:1 and 1:2, as those chitospheres contained the highest quantity of pristine PMMA particles, whereas the signal produced in the 2:1 formulation was very weak but evident. Chitospheres containing pristine PMMA particles showed the appearance of a new peak at 1540cm^{-1} . This peak is not evident in the Control formulation; however, it increases in intensity as the concentration of pristine PMMA particles increase indicating increased formation of this particular bond representing PEC.

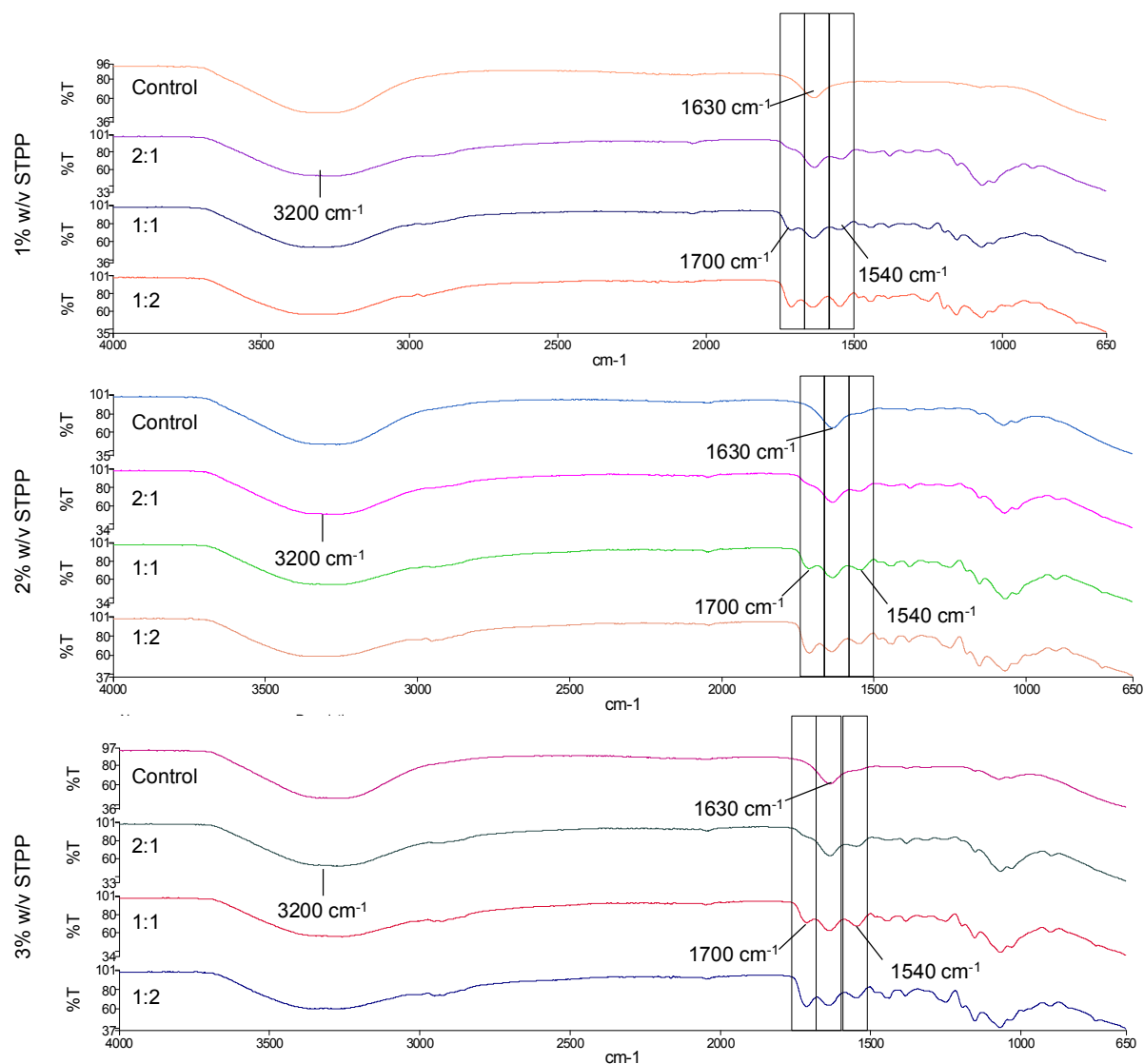


Figure 3.4: Spectra showing the FTIR analysis of the various chitospheres formulations, after 24 hours of hydration in PBS pH 7.4, categorised according to the % w/v of STPP used.

3.3.5. Changes in thermodynamic properties upon PMMA addition to chitospheres

The DSC curves (Figure 3.5) show that chitospheres cured in stronger STPP solutions required more heat to initiate melting. The melting points of the chitospheres increased when a 2:1 ratio of chitosan:PMMA was used. However, a further increase in the PMMA

concentration caused a decrease in the melting points with the exception of chitospheres prepared in a 1% w/v STPP solution. PMMA (Eudragit® S100) has a melting point of 200°C whereas chitosan exhibits the onset of melting at about 270°C corresponding to values reported in literature (Ruiz-Caro and Veiga-Ochchoa, 2009; Ramachandra and Nandhakumar, 2011). When the concentration of PMMA exceeds that of chitosan, the onset of melting decreases since PMMA is abundantly present in the chitosphere matrix. Regardless of the concentration of STPP used, 1:2 chitospheres exhibited similar melting points.

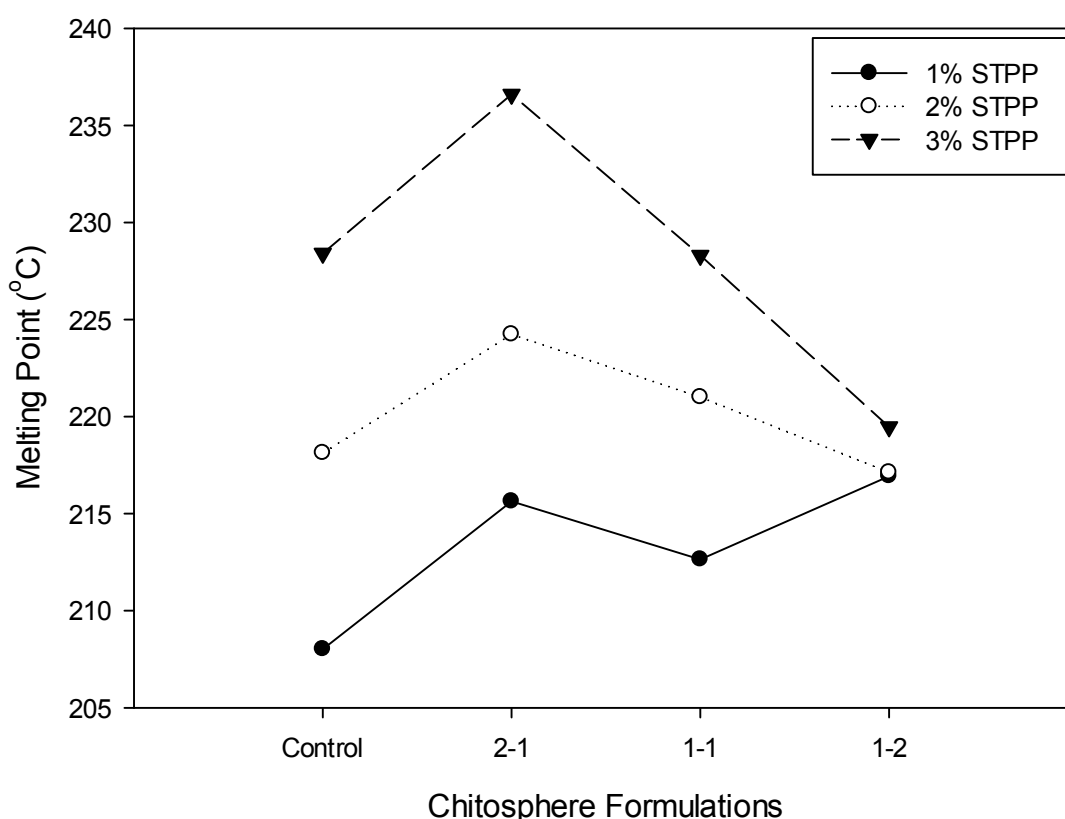


Figure 3.5: Profile depicting the influence of pristine PMMA particles concentration on the melting point of the chitospheres (n=2).

The TGA thermograms showed the degradation of chitospheres in 3 steps, whereas the degradation of the Control multiparticulates occurred in 2 steps. Between 264.45°C and 900°C chitosan exhibited a decrease in weight from 11.02% to 90% with 10% of the weight remaining at 900°C. The degradation of PMMA started at 284°C where 94.035% of the sample remained but this quickly declined to 7.984% at 448.08°C, and at 540°C complete degradation took place. The first step in the thermal degradation of the chitospheres, ranging from 100-190°C, was probably due to the loss of residual water as only a 2% loss in weight was observed. The second stage of degradation was noted in the temperature range of 266-

380°C where 30-40% weight loss was noted. This may be due to the decomposition of chitosan in the matrix, whereas a combination of chitosan and PMMA decomposition may have occurred in matrices comprising pristine PMMA particles. The final stage of degradation occurring after 450°C pertains only to the pristine particle-loaded chitospheres and demonstrates a further weight loss of 50-60% of the initial weight. This most likely indicated the decomposition of PMMA particles as a decrease in weight was particularly noted in samples containing higher ratios of pristine PMMA particles to chitosan. At each thermal degradation stage outlined, an increase in STPP crosslinker enhanced the thermal stability of the chitospheres. This can be seen in Figure 3.6, which shows the % weight remaining at 900°C, signifying the end of the run. The chitosan-only multiparticulates demonstrated the least loss in weight denoting that it has a higher thermal stability compared to those chitospheres containing pristine PMMA particles which apparently weaken the matrices' thermal stability and accelerates its heat degradation.

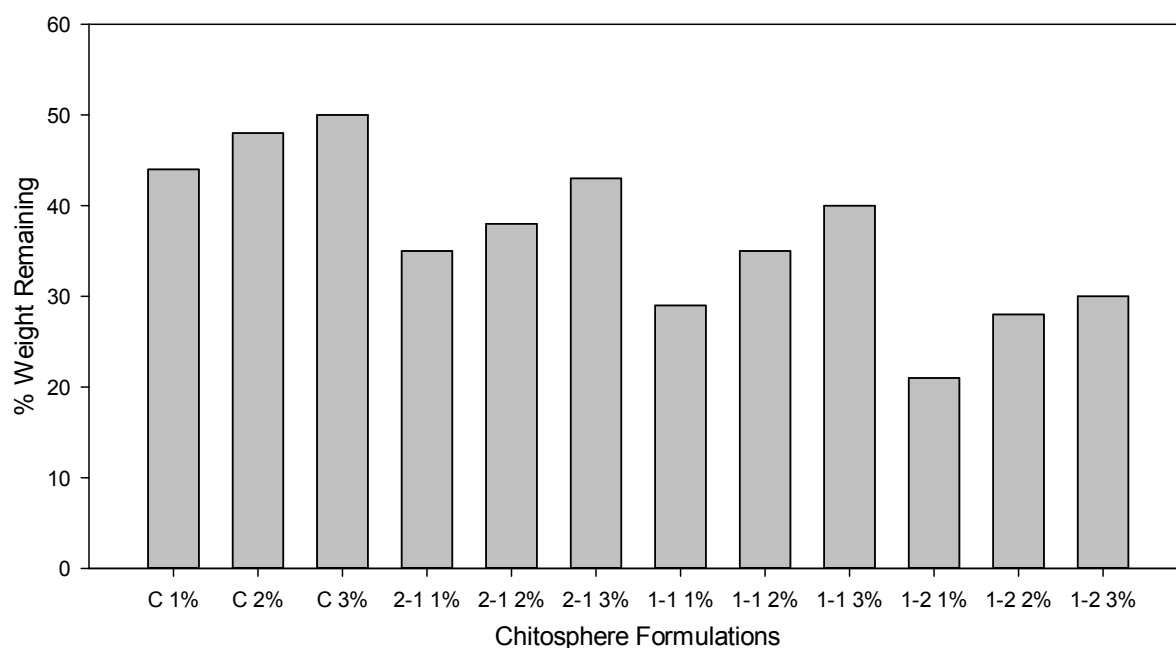


Figure 3.6: Graphic representation of the % weight remaining after thermal degradation of the various chitosphere formulations and Controls at a temperature of 900°C.

The decline in the properties of thermal stability corresponds to the data obtained from DSC studies which show decreased melting points of chitosan matrices containing a higher PMMA content. Similarly, as noted in both TGA and DSC, the amount of crosslinker influences the thermal stability via its effects in the degree of the matrix density and the strength of the polymer inter-chain linkages formed between the polymer and crosslinking ions. Chitospheres containing high ratios of pristine polymer particles have lower thermal

stability as the native PMMA polymer itself is easily melted in addition to the insufficient ionic linkages between the crosslinker and chitosan and the absence of such bonds between the crosslinker and pristine PMMA particles.

3.3.6. Gravimetric water-uptake and matrix erosion of PMMA-loaded chitospheres

Determining the degree of hydration and swelling is essential for understanding the transport of drug molecules through the polymer matrix. Likewise, studying the pattern of erosion is important in determining the drug release mechanisms since the release of entrapped drug from a polymer matrix often occurs via more than one mechanism (Varma *et al.*, 2004). Factors such as water-uptake, swelling and erosion play a role in influencing the diffusion, cleavage and ultimate release of the drug molecule from within the polymer chains to the surrounding dissolution media (Varma *et al.*, 2004; Alexis, 2005). In addition to this, swelling and erosion studies provide insight into the effect of the different pristine PMMA particle concentrations on crosslinking density and drug release.

Analysis of the % Swelling Ratio (%SR) and % Volume Ratio (%VR) showed that increasing the concentration of pristine PMMA particles within the chitosan matrices resulted in decreased water-uptake and hence less changes in the volume of the chitospheres with the exception of formulation 2:1. Chitospheres formulated with a 2:1 ratio of chitosan:PMMA particles showed the highest degree of swelling, which exceeded the swelling propensity of the Control when crosslinked in a 1%^{w/v} solution of STPP (Figure 3.7a-c). As the percentage of the crosslinking solution increased to 3%^{w/v}, the swelling ratio of formulation 2:1 approached similar values to that of the control formulation. Formulation 1:2 exhibited the least swelling and change in volume. The change in volume indicated by %VR showed that the Control and 2:1 chitospheres exhibited swelling to such an extent that the %VR was 4.5 and 4.8 times higher than that of the 1:2 chitospheres, respectively.

On the contrary, degradation data showed a somewhat reversed trend compared to the observations noted in the swelling studies (Figure 3.7d-f). Regardless of the strength of STPP crosslinker used, the Control multiparticulates degraded the fastest over the 30-day study period. Chitospheres containing the highest PMMA particle content degraded faster than those made from a lower PMMA particle concentration. Chitospheres made from a 2:1 suspension of chitosan:PMMA pristine particles underwent the least loss of bulk mass, however, when a 3%^{w/v} crosslinking solution is utilized the 1:1 formulation exhibited the slowest and steadiest degradation pattern. The 2:1 formulation degraded the least, hence, suggesting that increasing pristine PMMA particle concentration hastens the degradation rate of the matrix. Increasing the pristine particles concentration suggests an increase in the

number of pores created by the pH-responsive dissolution of PMMA out of the chitosan matrix in a time-dependent manner. Chitospheres with a higher PMMA content were observed to undergo a substantial loss of mass as compared to the matrices without PMMA particles. This may be due to the pristine polymer particles, which form the bulk of the chitospheres weight, dissolving and leaching out of the chitosan matrix upon exposure to PBS of pH 7.4 which leaves behind an array of porous microenvironments along the surface of the polymer matrix remnants. Furthermore, such chitospheres presented with reduced swelling as there was less chitosan content for the absorption of water into its polysaccharide chains. Figure 3.8 depicts the concentration of pristine PMMA particles and its effect on the number of pores created. However, as mentioned elsewhere in this investigation, the formation of pores is possibly a surface phenomenon only as seen in the SEM images of the degradation samples in Figure 3.2. During the first 2 days the mass loss of the chitospheres were less for the 1:1 and the 1:2 chitospheres, but as the penetration of water proceeded, these chitospheres underwent a rapid incline in degradation.

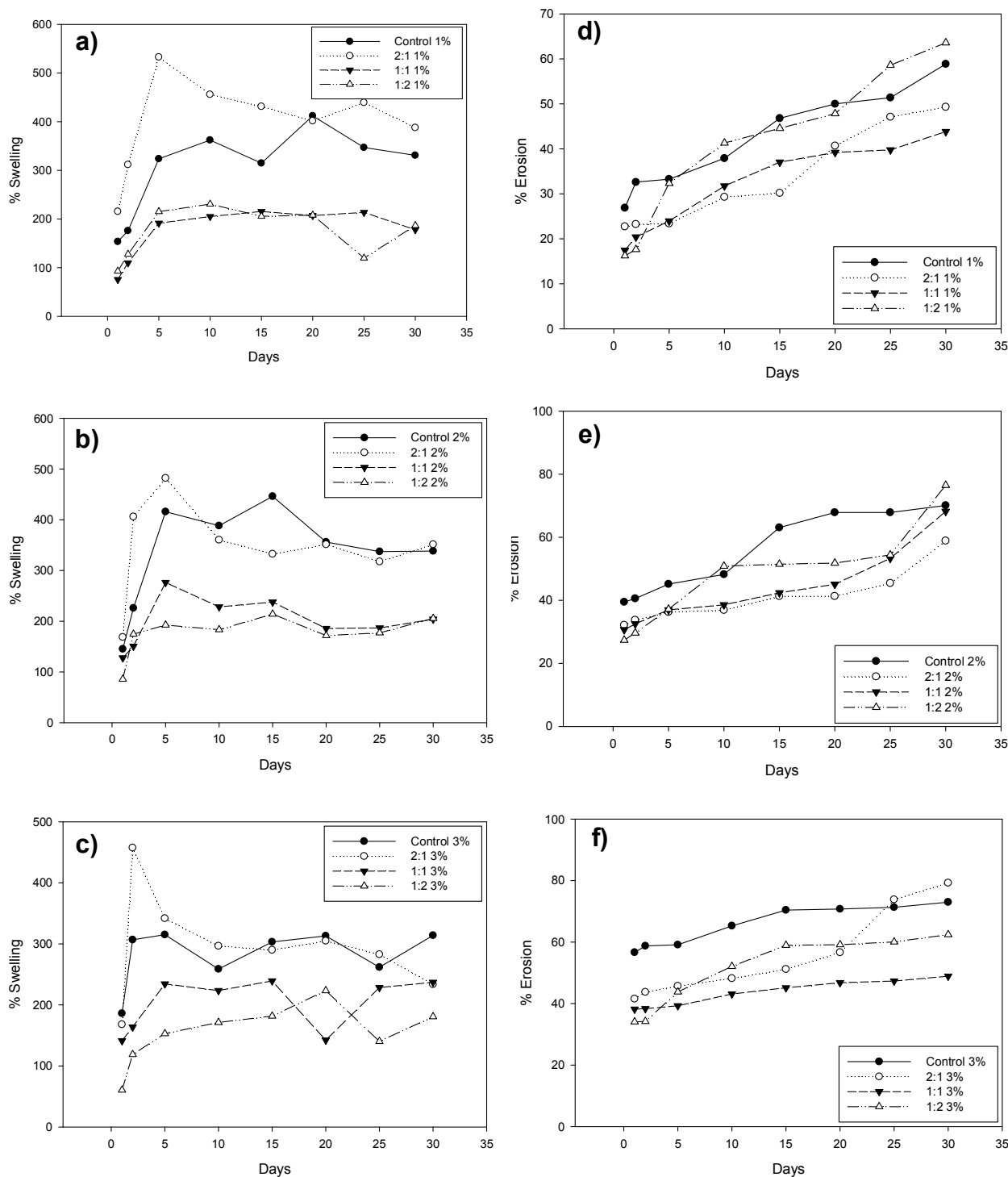


Figure 3.7: Profiles depicting % SR (a-c) a) 1% w/v STPP b) 2% w/v STPP c) 3% w/v STPP and % degradation (d-f) d) 1% w/v STPP e) 2% w/v STPP f) 3% w/v STPP of the different chitosphere formulations according to crosslinking percentage ($n=3$).

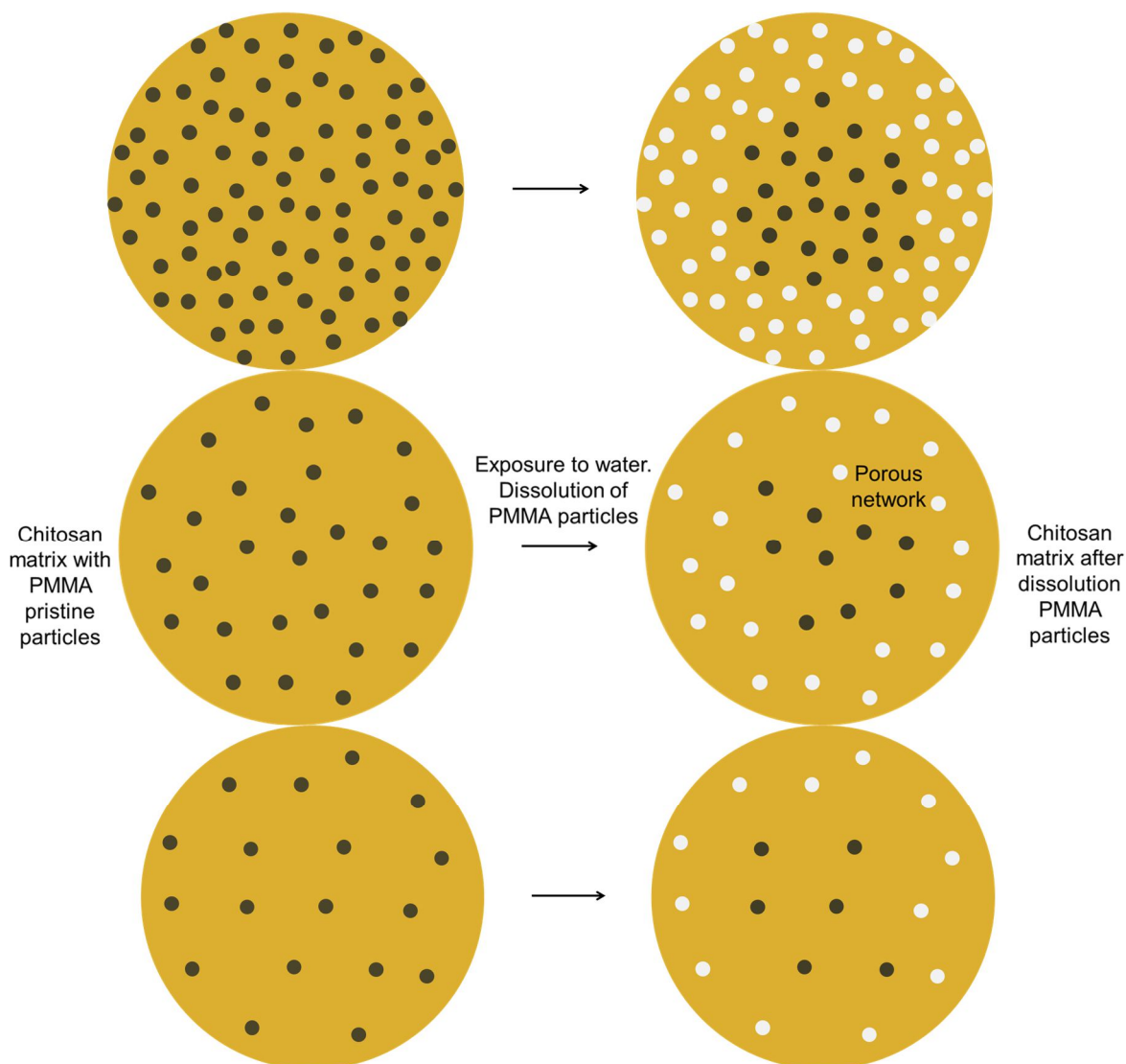


Figure 3.8: Representation of the effect of the concentration of pristine PMMA particles on the mass loss with regards to the quantity of chitosan left after PMMA particle dissolution.

3.3.7. Elucidation of mechanical properties of chitospheres

Textural analysis on chitospheres exposed to PBS provides insight into understanding the mechanism of swelling, degradation and drug release. Upon absorption of water, the chitosan matrices undergo various changes in mechanical properties depending on the concentration of crosslinker used and the total content of PMMA particles dispersed throughout the matrix. The penetration of water into the chitospheres matrix initiates disentanglement of the chitosan polysaccharide chains resulting in polymer relaxation. Polymer disentanglement and chain relaxation allows the further influx of water into the core of the matrix. The movement of water in and out of the matrix allows the dissolution of the pristine PMMA particles thus creating a series of pore structures via an *in situ* leaching out process.

MR refers to the ability of an object to recover to its original state after application of a force. It is also used as an indication of the presence of a porous matrix structure where the pores confer a sponge-like attribute to the matrix thereby allowing it easily return to its original shape. Porous matrix structures have a higher MR compared to less porous and more compact matrices. The term 'compact' refers to a densely packed matrix network where minimal spaces are left between the polymer chains. Densely packed matrices are often brittle in nature and generally display low MR and high deformability moduli (DF) (matrix rigidity and flexibility) as they tend to resist any applied mechanical stress as opposed to dissipating the energy throughout the matrix to undergo deformation as with pliable matrix systems (Singh, 2007; Bawa *et al.*, 2011).

The MR and DF values, as represented in Figure 3.9, show that as the concentration of pristine PMMA particles increase it produces chitospheres of higher MR and lower DF. This shows that matrices containing pristine PMMA particles are more resilient due to the formation of a porous matrix network hence its ability to recover after application of stress and undergo plastic deformation indicated by the low DF values. This increase in MR due to pore formation is validated by the SEM imaging and the porositometric analysis. Chitospheres containing more PMMA particles presented with larger pores of a greater cumulative pore surface area and improved MR. Such a matrix, being more flexible, is able to absorb energy to undergo deformation and hence require more energy to rupture the chitospheres as opposed to compact less resilient matrices which resists the force applied until it breaks as indicated by the fracture energy (FE). FE was obtained as the amount of work done to initiate fracture of an object when a compressive stress is applied (Tanaka *et al.*, 2005). The chitosan-only matrices exhibiting low a MR and high DF suggest that these multiparticulates are brittle. The FE, equating to the amount of energy in Joules required to rupture a hydrated chitosphere, showed that the 1:1 chitospheres displayed superior strength however a trend was noted where an increase in PMMA content improved FE with such chitospheres requiring more energy than the Controls to rupture the matrix. FE decreases as the chitosphere matrix undergoes degradation over time.

Initially, during the first 2-5 days, the Controls and 2:1 chitospheres exhibited the highest MR compared to the 1:1 and 1:2 chitospheres, however, upon further exposure to PBS MR rapidly increases whereas that of the Control and 2:1 chitospheres gradually decreases. This increase in MR corresponds to the proposed theory of pristine PMMA particles leaching resulting in the formation of pores which enhance the resilience of the chitospheres matrix.

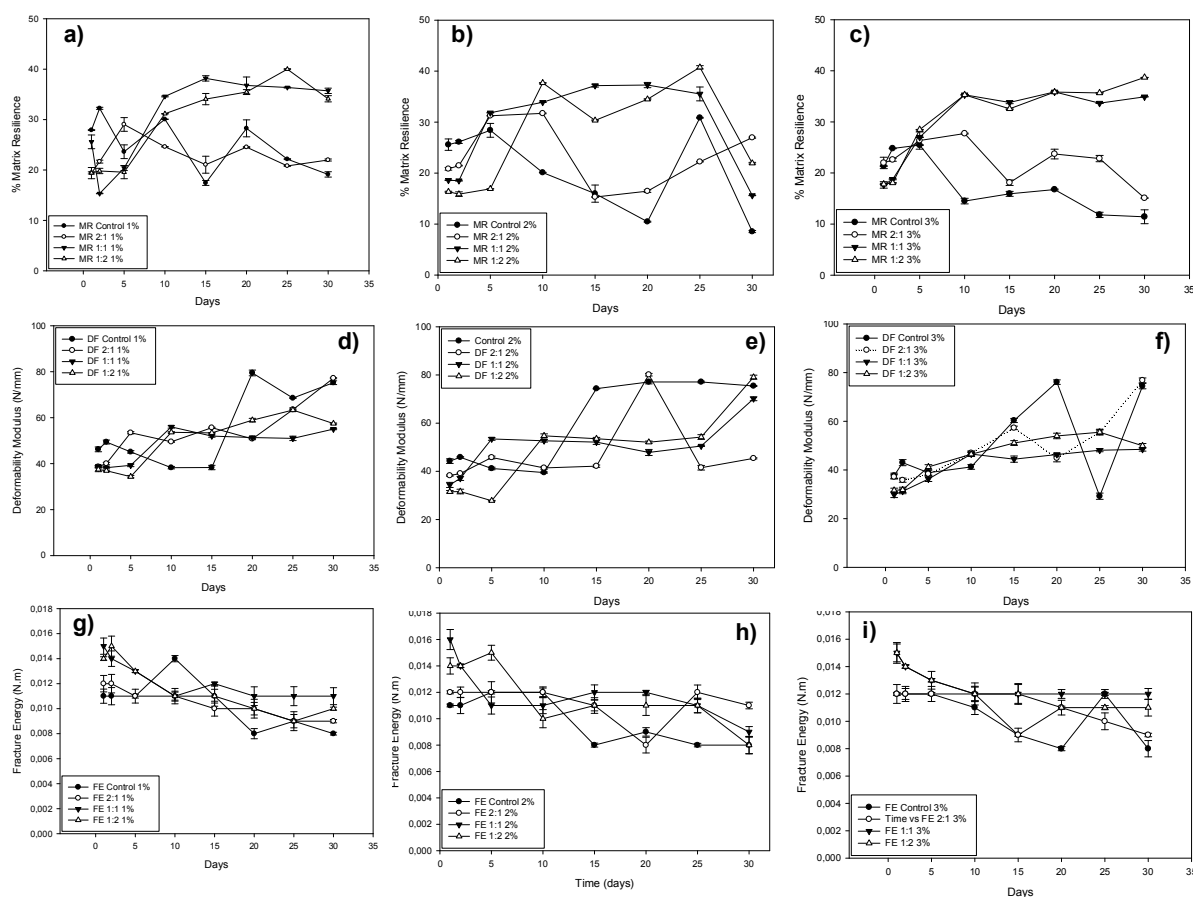


Figure 3.9: Textural analysis profiles featuring the %MR (a-c), DF (d-f) and FE (g-i) of the respective chitosphere formulations (n=3).

3.3.8. The influence of PMMA pristine particles on *in vitro* drug release studies

The different chitospheres formulations were able to provide complete and sustained release of indomethacin in a linear manner. From the release profiles depicted in Figure 3.10 it is evident that the addition of pristine PMMA particles to the chitospheres matrices were able to prolong the drug release rate where increase in pristine PMMA particles concentration lead to a reduction in the rate of drug release. The Control matrices, formulated from only chitosan, provided release up till only 72 hours whereas chitospheres containing pristine PMMA particles exhibited release profiles extending over 144 hours where the former was observed for chitospheres crosslinked in a 2%^{w/v} STPP solution. Although increase in the concentration of crosslinking solution used demonstrated a decrease in the release rates, the difference in this reduction was very little as the plotted drug release curves were often superimposable. Burst release has been defined as the immediate and large release of drug molecules from a delivery system within 24 hours of exposure to dissolution medium (Huang and Brazel, 2001). Significant burst release was not noted during the first sampling point however, after 24 hours, chitospheres released less drug compared to the Control

formulation which released between 55-64% of the incorporated drug within the first 24 hours. As the concentration of PMMA increased the total amount of drug released at 24 hours considerably decreased with the 2:1 chitospheres releasing 42-56%, the 1:1 chitospheres releasing 31-40%, and the 1:2 chitospheres releasing only 25-39% of the total encapsulated drug.

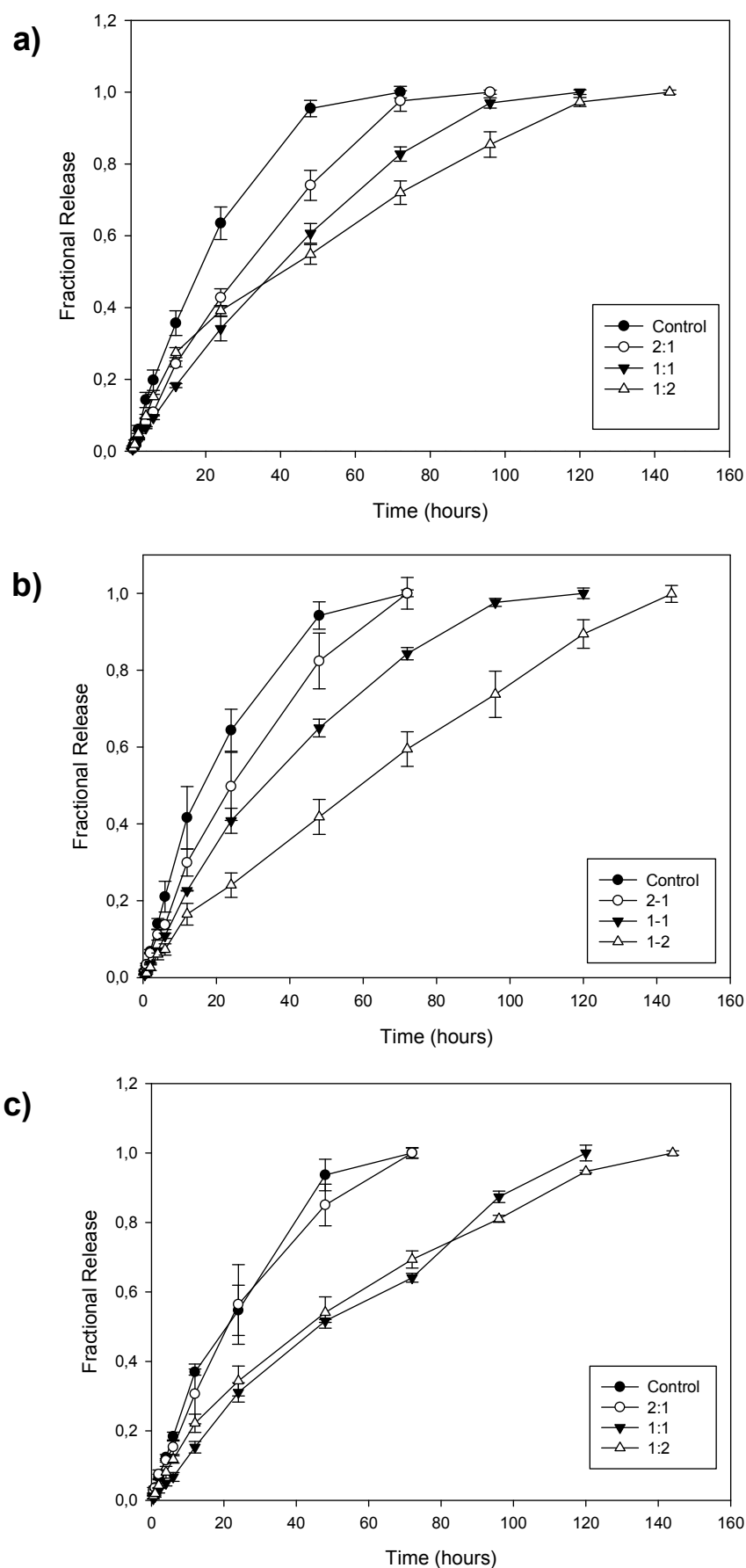


Figure 3.10: Drug-release profiles of the various chitospheres formulations indicating the effect of pristine PMMA particles concentration on the drug-release rate a) 1% w/v STPP b) 2% w/v STPP c) 3% w/v STPP (n=3).

Mathematical modelling of the release kinetics was performed using the zero-order, first-order, Higuchi, Korsmeyer-Peppas and Hixson-Crowell models as described by Shoaib and co-workers (2006). From Table 3.6, it is seen that the drug-release profiles of the chitospheres can be described by a combination of 2 models. The best-fit of 2 models were used since the R^2 values were of insignificant difference and hence could not be ignored. The Control followed the Hixson-Crowell and Korsmeyer-Peppas models, however, in the case of PMMA-loaded chitospheres the release profiles followed the Korsmeyer-Peppas and Higuchi models. Increase in the concentration of PMMA particles and the resultant increase of mesopore formation suggests the predominance of the Higuchi model in describing the release of drug. A review by Wang (2009) mentions that drug release from mesoporous materials is often described using the Higuchi relationship. The release exponent obtained from the n-value of the Korsmeyer-Peppas model showed that majority of the chitospheres exhibited values greater than 0.89 which is indicative of super case II transport whereas those with n-values of 0.45-0.89 demonstrate non-Fickian release or anomalous transport (Dash *et al.*, 2010; Singhvi and Singh, 2011). Both super case II and anomalous transport indicate that more than one mechanism is involved in the release of drug from the chitospheres which generally involves a combination of polymer chain erosion and diffusion of drug out of the matrix (Singh *et al.*, 2012; Maity and Sa, 2014). Furthermore, F1 and F2 values indicating the %difference and %similarity, respectively, between the Control formulations and the PMMA-containing chitospheres show that increasing the PMMA concentration produces a distinct variation in the dissolution profiles. The data in Table 3.7 shows that when the PMMA concentration increases the F1 values increase and the F2 values gradually decrease showing greater difference and less similarity between the two dissolution profiles. These values correspond to the deviation of the curves noted in the drug release profiles between the Controls and the chitosphere formulations with the 1:2 dissolution curve lying furthest away from that of the Control. The 1:2 formulation prepared with 2%^{w/v} STPP crosslinking showed the greatest %difference of 53.90% and the lowest %similarity of 57.86%.

Table 3.6: Kinetic modelling of indomethacin release from the Controls and PMMA-loaded chitospheres.

Formulation	Zero-Order		First-Order		Higuchi		Korsmeyer-Peppas			Hixson-Crowell		First best-fit model	Second best-fit model
	r^2	$k_0(h^{-1})$	r^2	$k_1(h^{-1})$	r^2	$k_H(h^{-1/2})$	r^2	n	$K_{KP}(h^{-n})$	r^2	$k_{HC}(h^{-1/3})$		
C 1%	0.9912	1.4884	0.9883	0.0652	0.9822	14.1440	0.9904	1.0497	2.7040	0.9979	-0.0643	Hixson-Crowell	Zero-Order
C 2%	0.9019	1.4645	0.9903	0.0636	0.9825	14.0190	0.9960	0.9671	3.4538	0.9971	-0.0632	Hixson-Crowell	Korsmeyer-Peppas
C 3%	0.9335	1.4692	0.9784	0.0631	0.9856	13.8450	0.9956	0.9149	3.4269	0.9902	-0.0629	Korsmeyer-Peppas	Hixson-Crowell
2-1 1%	0.9506	1.1458	0.9584	0.0486	0.9852	12.1470	0.9919	1.0740	1.6036	0.9851	-0.0471	Korsmeyer-Peppas	Higuchi
2-1 2%	0.9694	1.4266	0.9189	0.0573	0.9905	13.2250	0.9934	0.8927	3.0839	0.9527	-0.0590	Korsmeyer-Peppas	Higuchi
2-1 3%	0.9538	1.4328	0.9391	0.0583	0.9892	13.3820	0.9874	0.7860	4.1918	0.9664	-0.0597	Higuchi	Korsmeyer-Peppas
1-1 1%	0.9606	0.9145	0.9516	0.0362	0.9874	10.6690	0.9943	0.9988	1.4598	0.9760	-0.0358	Korsmeyer-Peppas	Higuchi
1-1 2%	0.9436	0.9095	0.9590	0.0371	0.9914	10.7280	0.9928	1.0127	1.5940	0.9835	-0.0362	Korsmeyer-Peppas	Higuchi
1-1 3%	0.9778	0.8329	0.8164	0.0295	0.9670	9.5269	0.9876	0.9794	1.1519	0.8693	-0.0309	Korsmeyer-Peppas	Zero-Order
1-2 1%	0.9455	0.7199	0.9352	0.0286	0.9971	9.2433	0.9708	0.9332	2.1548	0.9633	-0.0281	Higuchi	Korsmeyer-Peppas
1-2 2%	0.9755	0.6464	0.7910	0.0302	0.9862	8.7289	0.9908	0.8766	1.5150	0.9456	-0.0257	Korsmeyer-Peppas	Higuchi
1-2 3%	0.9608	0.7185	0.9125	0.0265	0.9977	9.1553	0.9931	0.8519	2.3211	0.9442	-0.0270	Higuchi	Korsmeyer-Peppas

Table 3.7: The percentage difference (F1) and similarity (F2) factors for the various chitosphere formulations in comparison to the Controls.

% Crosslinking	Formulation	F1 value (%)	F2 value (%)
1%	2:1	22.16	76.43
	1:1	35.86	66.94
	1:2	33.27	65.93
2%	2:1	14.14	84.19
	1:1	31.95	69.69
	1:2	53.90	57.86
3%	2:1	6.91	99.73
	1:1	45.71	62.71
	1:2	36.77	65.71

3.4. Discussion

The following section explains the mechanisms by which the pristine PMMA particles interact with chitosan leading to the observed effects on the swelling, erosion and drug release attributes of the chitosphere formulations.

The spherically shaped chitosan matrices are proposed to undergo water penetration and hydration in concentric layers as depicted in Figure 3.11. Upon splitting slightly swelled chitospheres in half, a hydrated slightly opaque outer layer and an unhydrated solid core can be seen as water moves from the outside of the chitospheres inwards. This mechanism of hydration may be further evidenced by the fluctuating peaks noted in the texture analysis profiles of MR and DF (Figure 3.9). Increases in MR may indicate the presence of the swollen peripheral layer of the chitospheres. As evidenced by SEM (Figure 3.2), after the outmost layer has reached its swelling capacity it ruptures and eventually sloughs off revealing the next solid drug-loaded layer to be hydrated via water-uptake hence, producing a decline in MR. Hydration of this fresh layer induces release of some of the drug molecules from the surface via diffusion (Maity and Sa, 2014). The sloughing of the previously hydrated layer suggests the mechanism by which erosion takes place. When the rate of penetration of water into the chitospheres matrix is less than the rate of mass loss of the chitospheres, surface erosion is considered to take place (Alexis, 2005; Yao *et al.*, 2011).

The surface erosion mechanism occurs particularly with chitospheres containing pristine PMMA particles whereas the chitosan-only multiparticulates may erode via bulk degradation since it displayed rapid loss of mass and increased swelling behaviour. The Control matrices, as visualised by SEM, had numerous cracks on its surface compared to chitospheres containing pristine PMMA particles. As indicated by the low MR and high DF

values these chitosan multiparticulates were brittle and depending on the depth of the cracks, water could easily penetrate the matrix hydrating the core and causing the sphere to rupture from within. Furthermore, cracks and chips on the sphere surface increase the surface area available for moisture hydration and as a result this allows the entry of large quantities of water and increases the escape of drug molecules via diffusion hence the rapid release of drug within 72 hours. Variation in drug release rates produced by changes in surface area is evidenced by the BET total surface areas where the Control with a surface area of $25.5898\text{m}^2/\text{g}$ released drug faster than the chitospheres which featured smaller BET surface areas of $7.5959\text{--}12.6663\text{m}^2/\text{g}$ and slower drug release rates. Although the 2:1 chitospheres swelled the most and underwent the greatest change in volume compared to the Control, it was able to release drug slower than the Control due to its mechanism of surface erosion and the subsequent peeling of hydrated layers as noted in the SEM images pertaining to Day 2 of degradation samples.

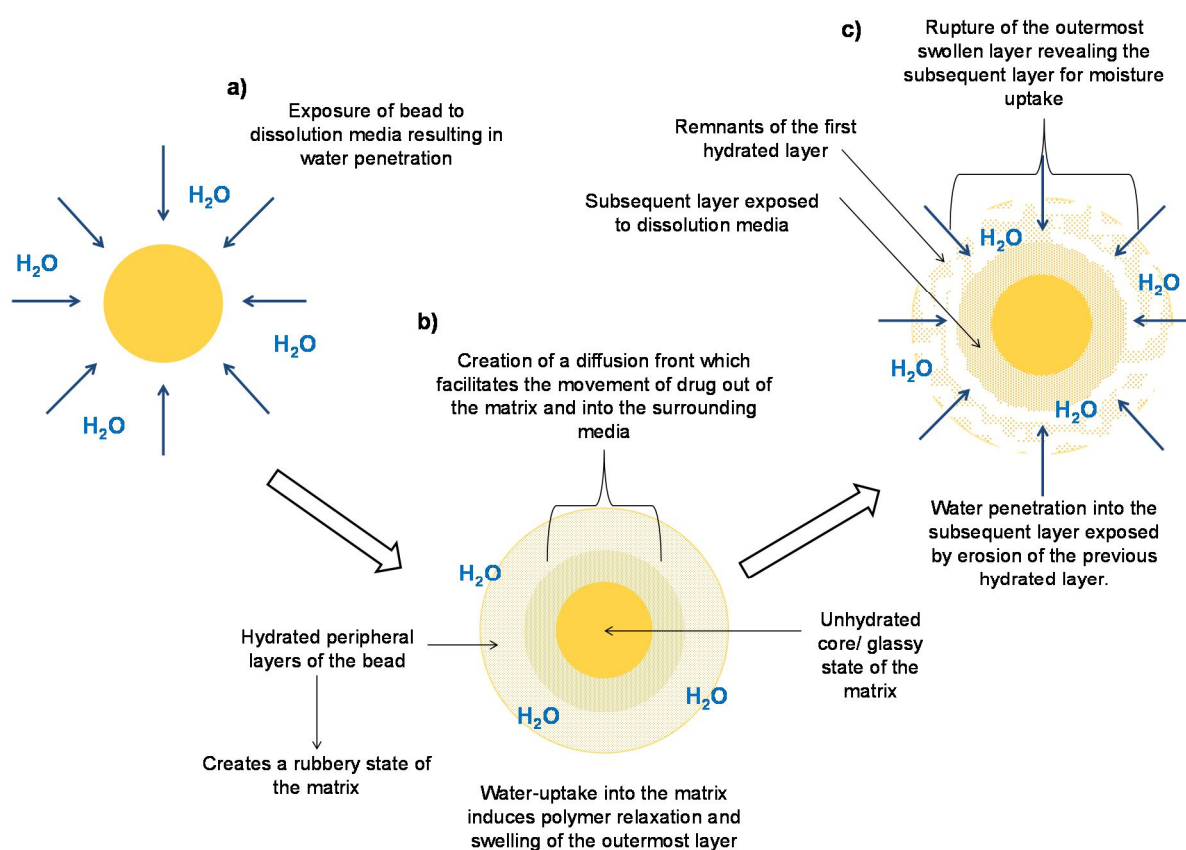


Figure 3.11: Schematic representing the hydration and erosion of chitospheres loaded with pristine PMMA particles occurring in concentric layers.

Drug release occurs by three primary mechanisms as described by Yao and associates (2011): 1) diffusion-controlled, 2) swelling-controlled and 3) chemically-controlled. The chemically-controlled drug release mechanism encompasses surface erosion, bulk

degradation and other interactions or processes taking place within the polymer matrix. It may be possible that one or more of these release mechanisms could be occurring simultaneously within a matrix system or one may be the dominant mechanism at certain stages of matrix hydration and erosion.

All the chitosphere formulations and the Control initially release drug via diffusion as the drug molecules located on the peripheral layers of the sphere hydrate and dissolve through the swelling gel layer into the surrounding media via a concentration gradient. A burst release of drug was not observed and was possibly due to washing of the spheres in 1M acetic acid to remove surface drug and unreacted polymer. As the chitospheres continue to hydrate from the outside towards the inner core, the polymer matrix undergoes a transition from a glassy to a rubbery state (Figure 3.11b) (Yao *et al.*, 2011). Drug molecules in the hydrated rubbery layer of the chitospheres are free to dissociate from the matrix and diffuse out whereas molecules located in the unhydrated glassy core remain static. A combination of erosion, swelling and chemically-based release may occur with chitospheres containing pristine PMMA particles. Chitospheres are proposed to release drug by an *in situ* leaching process where the pristine PMMA particles act as the porogen. PMMA, being a pH-responsive polymer, dissolves at pHs higher than 7.0. When the chitospheres are exposed to PBS of pH 7.4, the same pH-responsive dissolution is considered to take place leaving behind porous structures in its place.

Furthermore, since PMMA dissolves in the PBS, it may be possible that some of the dissolved matter remains in the chitosan matrix thereby initiating the formation of a PEC between the cationic chitosan and the dissolved molecules of the pristine PMMA particles (Figure 3.12). Since chitosan is a cationic polymer and PMMA an anionic polymer it is likely that some electrostatic interactions may have arisen resulting in the formation of a PEC with binding occurring between the positively charged amine and negatively charged carboxyl groups of chitosan and PMMA, respectively, leading to the synthesis of a carboxylate functional group indicated by the new peak formed at 1540cm^{-1} as depicted in the FTIR spectra in Figure 4 (Li and Huang, 2012). However, since the unreacted amine and carboxylic acid functional groups corresponding to the native polymers of chitosan and PMMA, respectively, are still present it suggests that only a certain ratio of these molecules reacted to form a new bond. It may be possible that parts of the PMMA particle may still be residing in a partially empty pore while the surface around this particle forms a PEC. Since the intensity of the new peak at 1540cm^{-1} is far less than the native peaks it may suggest that the PEC is present in small quantities. It is noted that unhydrated chitospheres also display this new peak although a very weak signal is produced; particularly when the crosslinker

concentration is increased, this peak becomes hardly evident. However, after hydration of the matrices in PBS pH 7.4, the intensity of the peak significantly increases. This presence of this peak in the unhydrated spheres is due to the alkaline pH of the crosslinking solution. In weak STPP solutions, diffusion of the crosslinking ions can easily occur from the outside to the centre of the sphere and its alkalinity causes a small degree of PMMA particle dissolution which readily forms a PEC with chitosan. In stronger STPP solutions, a hard crosslinked outer layer is rapidly formed which acts as a barrier and hinders the further entry of crosslinking ions towards the core, hence less diffusion of the crosslinker ions is able to take place, less solution enters the matrix and the dissolution of PMMA does not occur. This issue can simply be addressed by adjusting the pH of the crosslinking solution with dilute acid. However, the increase in the intensity of the 1540cm^{-1} peak from the unhydrated to the hydrated chitosphere matrices, as observed in FTIR, proves the spontaneous, largely *in situ* synthesis, of a PEC between these oppositely charge polymers. Furthermore, the weak thermal stability of unhydrated chitospheres suggests the presence of unreacted PMMA particles validating that PEC synthesis takes place only upon hydration. This formation of a PEC may explain the reduced swelling and the prolonged and slowed drug release rates. Moreover, the retention and entanglement of drug in the PEC regions further retard drug release.

The swelling behaviour of chitosan is dependent on the number of available amino groups for protonation however, with crosslinking interactions and PEC formation, the number of free amino groups for protonation is reduced due to complexation of STPP ions and PMMA anions with the amino groups of chitosan (Yao *et al.*, 1994; Berger *et al.*, 2004). This explains the low water-uptake and swelling ratios observed in the chitospheres loaded with PMMA pristine particles coupled with the resulting smaller surface areas which reduce the contact surface available for interaction with water molecules.

As visualized by SEM such chitospheres were able to maintain their structural integrity and also exhibited superior FE as indicated by textural profiling denoting that they possess improved mechanical strength requiring more energy to cause breakage of the chitospheres. Such PECs between chitosan and polymethacrylate copolymers are possible and have been previously confirmed where polymethacrylates were used as surface coating agents for pH-controlled release (Lorenzo-Lamosa *et al.*, 1998; Moustafine *et al.*, 2008; Hamman, 2010). To our knowledge, this is the first report of *in situ* polyelectrolyte synthesis in addition to *in situ* porogen leaching.

We postulate that the mechanism governing the release of drug from chitospheres containing pristine PMMA particles occurs primarily via an *in situ* PEC formation in addition to *in situ* leaching and surface porous microenvironment formation. The chitospheres first undergo hydration of its peripheral layers. When the matrix has absorbed sufficient water the pristine PMMA particles become hydrated. Adequate moisture-uptake is required to instigate the dissolution of the pristine PMMA particles resulting in its final discharge into the surrounding media. Hence, the complete dissolution of the pristine PMMA particles occurs only on the surface of the chitosphere for creation of pores and not throughout the entire matrix system. Chitospheres prepared in higher concentrations of STPP crosslinking solutions impeded the penetration of water therefore swelling minimally. In this case, the rate of water permeation into the polymer matrix becomes the rate-determining step in drug release as the rate of water penetration influences the rate of pristine PMMA particles dissolution and pore formation. The 1:2 chitospheres, containing the highest concentration of pristine PMMA particles, swelled the least suggesting only slight water-uptake therefore the pristine PMMA particles were not sufficiently hydrated to dissolve out of the matrix. Furthermore, the presence of more PMMA suggested the formation of more PEC regions. This, together with the slower rate of pore formation resulted in a slower release of the drug. From the SEM photographs, it is noted that 1:2 chitospheres display the least amount of pores on its surface compared to the 1:1 chitospheres which contain a greater number of porous structures on the outer surface. Furthermore, the formation of a PEC meant that drug molecules would be entrapped in the matrix for a longer time thereby prolonging the release of drug. This is confirmed by FTIR as the intensity of the peak at 1540cm^{-1} was greatest for chitospheres prepared with higher concentrations of PMMA suggesting that more regions of PEC was formed when more PMMA is available. Moreover, it was noted that 3% crosslinked 1:1 chitospheres featured the slowest release of indomethacin, however, it also exhibited the highest peak intensity corresponding to the polyelectrolyte formation and hence this may explain its prolonged release. The mechanism of surface erosion of the hydrated layer indicated that drug is released in concentric layers until the core is reached. This way, bulk fluid does not move into the centre of the chitosphere causing the premature release of drug molecules as with the Controls. The *in vitro* dissolution of the pristine PMMA particles and the potential formation of a PEC combined with processes of surface erosion can be considered as chemically-controlled drug mechanisms as interactions take place between the PMMA and chitosan molecules. Release of entrapped drug from the PEC regions occurs via erosion of the PEC when the amino groups of chitosan become deionized. Upon deionization of the amino groups, the binding affinity between the two electrostatically interacting molecules, $-\text{NH}_2$ of chitosan and $-\text{COOH}$ of PMMA, becomes

weaker resulting in increased water penetration into the polymer network and ultimate disintegration of the PEC (Berger *et al.*, 2004; Li *et al.*, 2012).

Mathematical modelling of the release kinetics reveals that the release exponent (n) decreases with increases in crosslinking and PMMA particles. This may suggest that increasing the quantity of crosslinker and PMMA particles, creates a shift in the release mechanism from a predominantly erosion-based (where $n > 1$) release towards a coupled polymer-relaxation, erosion and diffusion-oriented release termed as super case II transport (where $0.45 < n < 0.89$) (Maity and Sa, 2014; Nayak *et al.*, 2014). Since indomethacin is a poorly water soluble drug (Inada *et al.*, 2013), its release may occur mainly by surface erosion (Contreras *et al.*, 2012) of the chitospheres as depicted in the SEM images in Figure 3.2 in addition to diffusion occurring from the peripherally hydrated layers of the chitospheres and via the porous surface left by the dissolution of surface pristine PMMA particles and erosion of the PEC. The shift in the release mechanism of the chitospheres imparted by the addition of pristine PMMA particles corresponds to the F1 and F2 values showing dissimilarity of the drug release profiles as the concentration of PMMA particles is increased. Although F2 values exceeding 50 indicate similarity of the dissolution profiles (Shah *et al.*, 1998), the drug release profiles of the Controls and the chitosphere formulations may be deemed similar by the fact that they both release drug in a linear first-order manner. The large F1 values > 15 may be a result of the prolonged mechanism of drug release via PEC and pore formation which were absent in the Controls. Thus, the key factor differentiating the dissolution profiles is attributed to the spontaneous in situ PEC and pore formation.

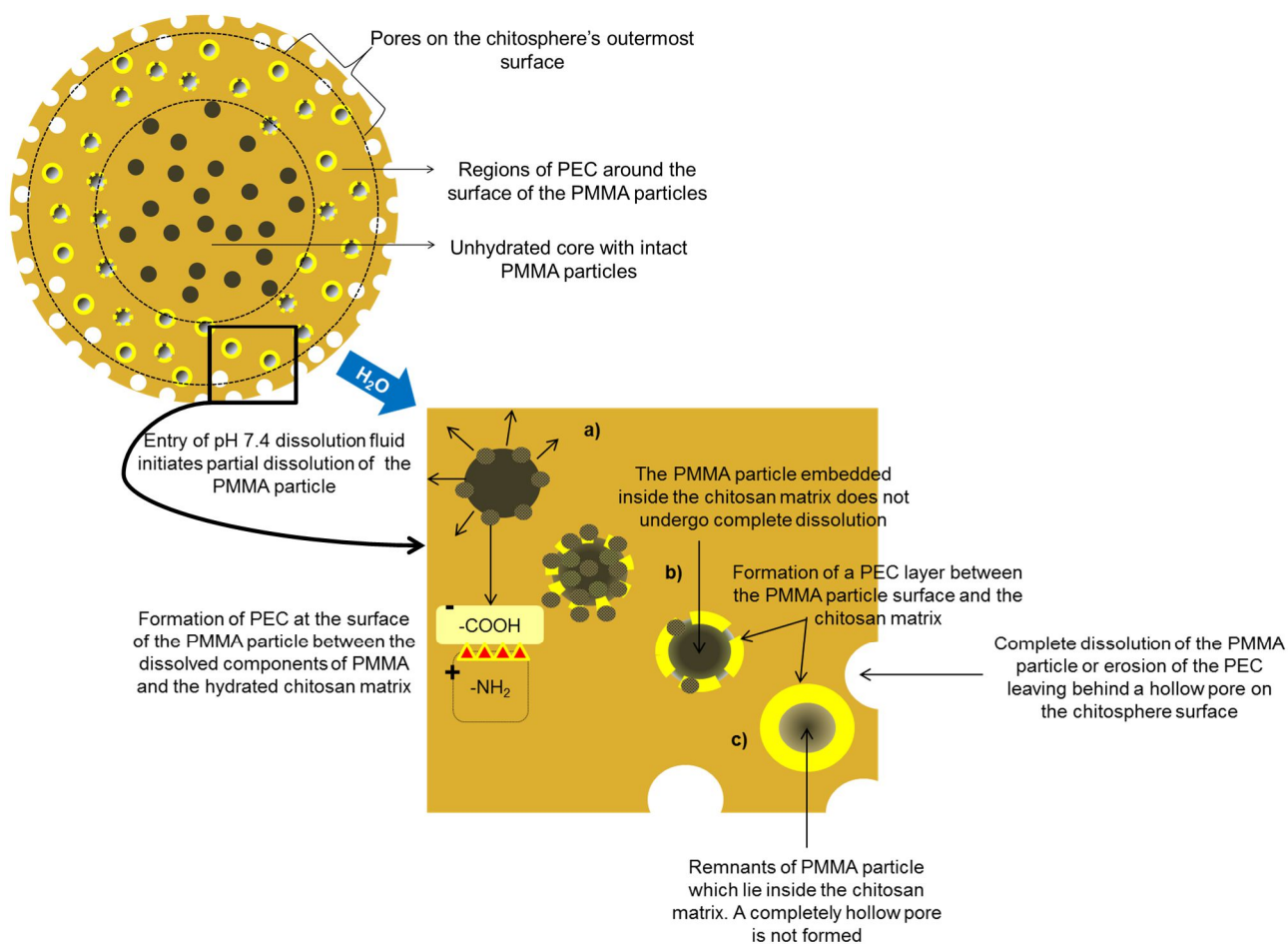


Figure 3.12: Depiction of the formation of surface pores and a PEC between pristine PMMA particle surfaces and the chitosan matrix.

3.5. Concluding Remarks

Chitospheres were prepared to use as a model matrix system for the investigation of the 1) *in situ* PEC synthesis and 2) *in situ* leaching and surface pore-formation effects of pristine PMMA particles validated by SEM imaging and porositometric characterization. Comparison of the chitosan-only multiparticulates and chitospheres containing pristine PMMA particles showed that the dispersion of pristine PMMA particles throughout a polymer matrix is capable of prolonging drug release in addition to enhancing mechanical strength of the matrix and shape of the chitospheres. Multiparticulates without pristine PMMA particles could control the release of indomethacin over 72 hours only whereas those containing pristine PMMA particles offered controlled release extending over 144 hours via super case II transport. The dissolution of pristine PMMA particles into the surrounding chitosan matrix resulting in the spontaneous formation of a PEC, confirmed by FTIR, indicates the variations in the outcomes of drug release between the different pristine PMMA particle ratios, swelling and erosion based on polymer selection. Although PMMA-loaded chitospheres exhibited superior textural properties and mechanical strength, they lacked ideal properties of thermal

stability in their unhydrated form compared to multiparticulates prepared from chitosan alone. The density of crosslinking of chitosan determines the rate of moisture permeation into the chitospheres thereby influencing the degree of hydration and consequential escape of the pristine PMMA particles out of the matrix leaving behind an impression of the space it occupied (artefact). However, the STPP crosslinking concentrations utilised in this study produced insignificant differences in the various characterisation studies between formulations of the same polymeric constituents, thus the pertinent alterations in characteristics and observed release profiles is largely attributed to the formation of a PEC due to the presence of PMMA particles. Such a matrix, modified by the inclusion of pristine polymer particles, may be of interest in the development of long-term controlled-release systems for the administration of potent small molecular weight therapeutic drugs. The enhanced matrix resilience, strength, porosity and minimal swelling may be attractive for the design of drug-releasing implantable tissue-engineering scaffolds such as those required for drug-delivery to the brain and other parts of the nervous system where tissue healing may be slow necessitating the need for an adequate scaffold.

The proof-of-concept study described in this chapter was essential to confirm the proposed action of pristine polymer particle intercalation on extending drug release rates and inducing porosity for microenvironment formation and modulation of matrix resilience properties before further progression. Following the validation of the particle-intercalation strategy, this technique can then be applied to the design of a novel hydrophilic polymeric matrix that is better suited for nerve repair, in terms of fabrication and processing and tissue biocompatibility. Such a design is discussed in Chapter 4 where a Box-Behnken experimental design is employed for the intent of further formulation optimization.

3.6. References

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CHAPTER 4

DESIGN, FORMULATION AND STATISTICAL OPTIMIZATION OF AN INTERCALATED INTERPENETRATING POLYSACCHARIDE HYDROGEL CONDUIT FOR THE DUAL DELIVERY OF PROTEIN AND ANTI-INFLAMMATORY DRUG

Abstract

The purpose of this study was to develop novel, porous neurodurable interpenetrating networks of gellan-xanthan hydrogel conduits intercalated with pristine polymethyl methacrylate (PMMA) particles for the sustained and concurrent release of two model compounds: bovine serum albumin (BSA) and diclofenac sodium. Hydrogel conduits were synthesised using a thermal-ionic crosslinking mechanism with direct incorporation of PMMA. The 15 formulations, generated using a Box-Behnken experimental design, were analysed for drug release, swelling, erosion, and textural properties. The 15 formulations provided a near zero-order release of BSA (37-75% fractional release) and diclofenac sodium (14-22% fractional release) over 20 and 30 days, respectively, modulated via a combination of pH-responsive (pH 7.4) dissolution of the intercalated pristine polymer particles and the unique gelling and erosion properties imparted by the graded addition of xanthan gum to the hydrogel blend. The concentration-dependent intercalation of PMMA extended drug release rates and enhanced matrix resilience from 31% to 56%. The gellan-xanthan ratio variability and the pore-inducing effects of intercalated PMMA yielded a means for fine tuning the mechanical attributes of the hydrogel matrices, particularly matrix rigidity and flexibility, offering an appealing strategy for the design and development of synthetic bioactive-releasing peripheral nerve repair conduits.

4.1. Introduction

Although the gold standard of peripheral nerve repair remains the autologous nerve graft and direct end-to-end tensionless suturing in the case of short gap nerve injuries, the drawback of donor tissue harvesting and resulting deformities associated with the nerve autograft potentiates the need for alternative treatment strategies (Strauch and Strauch, 2013). A number of artificial polymeric-constructed nerve guides are commercially available for clinical use and have proven to be beneficial in the regeneration of transected peripheral nerves, however, their efficacy in attaining satisfactory levels of functional recovery are considered substandard compared to the autograft (Kehoe *et al.*, 2012; Dinis *et al.*, 2015). This particularly pertains to the drug release mechanisms and resulting release kinetics as outlined in our review (Ramburrun *et al.*, 2014).

The inclusion of neurotrophic factors (NTFs) within nerve conduits has produced remarkable effects on peripheral nerve regeneration and functional recovery. NTFs enhance peripheral nerve regeneration by promotion of neuronal survival, Schwann cell migration and axonal regeneration (Thoenen and Sendtner, 2002; Catrina *et al.*, 2013). This research study utilized Bovine serum albumin (BSA) and diclofenac sodium (anti-inflammatory and analgesic) as the model protein and small molecular weight drug, respectively.

In order to obtain optimum levels of functional recovery in the treatment of peripheral nerve injuries, comparable to the nerve autograft, the following aspects of the tissue scaffold require particular focus: 1) the release kinetics relating to suboptimal and overdosing of bioactives, particularly during the initial stages of nerve regeneration; and 2) the mechanical properties in terms of the maintenance of structural integrity post-implantation, the ability to withstand compressive forces from surrounding tissues, flexibility and permeability for uninterrupted gas and nutrient exchange to regenerating tissues (Dalton *et al.*, 2008; Bian *et al.*, 2009; Cunha *et al.*, 2011; Kehoe *et al.*, 2012; Madduri and Gander, 2012).

Naturally-derived polymers are gaining attractiveness for the development of tissue repair scaffolds and ingestible drug delivery systems, particularly the large molecular weight polysaccharide-based polymers such as chitosan, starch, alginates, hyaluronan, dextran and gellan gum. The non-toxic, biocompatible, hemocompatible and biodegradable properties of this polymer class have popularised its use in scaffold-based materials for tissue engineering applications and drug delivery systems (Coviello *et al.*, 2006; Malafaya *et al.*, 2007).

Gellan gum, an anionic bacterial exopolysaccharide, consisting of repeating tetrasaccharide units of two glucose, one glucuronic acid and one rhamnose molecule, readily undergoes

ionotropic gelation to form physically crosslinked gels, hence its wide applications relating to the manufacture of *in situ* gelling ophthalmic preparations for enhanced drug delivery and bioavailability (Coutinho *et al.*, 2010). Similarly, xanthan gum is a high molecular weight anionic bacterial heteropolysaccharide. Due to its hydrophilic, inert and biocompatible nature, it has further been proved to retard drug release thereby providing time dependent release kinetics (Pongjanyakul and Puttipipatkachorn, 2007; Singh *et al.*, 2011). However, its weak gelling properties following physical crosslinking is unsuitable for use in the development of long-term tissue implants and scaffolds.

Polymethyl methacrylates (PMMA) are anionic pH-responsive synthetic acrylate copolymers comprising varying ratios of methacrylic acid and methyl methacrylate. Eudragit S100 consisting of poly(methacrylic acid-co-methyl methacrylate) in a 1:2 ratio undergoes dissolution at a pH > 7.0. Therefore, intercalation of such particles into a gel matrix results in the *in situ* formation of pores, via particulate leaching, for controlled delivery of bioactives and improvement of scaffold resilience properties as established in the previously conducted proof-of-concept study described in Chapter 3.

The present study uses a dual heat and calcium crosslinked gellan and xanthan gum combination for the fabrication of hydrogel nerve conduits. Further modification of the hydrogel matrix was achieved via the intercalation of pristine polymethylmethacrylate (PMMA) particles to modulate drug release and attune scaffold resilience properties via particulate leaching for the *in situ* formation of pores, as established in our previously conducted proof-of-concept study (Ramburrun *et al.*, 2015). The resulting hydrogels were polymethylmethacrylate pristine polymer particle intercalated gellan-xanthan interpenetrating networks P⁴[Gel-Xan]IPNs. The conduits were analysed for drug release, swelling, erosion, thermal behaviour, chemical transitions and mechanical properties upon utilization of varying polymer, crosslinker and pristine polymer particles concentrations.

4.2. Materials and Methods

4.2.1. Materials

Gellan gum (low acyl GelzanTM CM), Xanthan gum, Diclofenac sodium and Bovine serum albumin (BSA) were purchased from Sigma Aldrich, Steinham, Germany. Polymethyl methacrylate (PMMA) (Eudragit S100) was purchased from Evonik, Midrand, Johannesburg, South Africa and was used without further modifications. Propylene glycol was purchased from Merck, Darmstadt, Germany and calcium chloride anhydrous was purchased from Rochelle Chemicals, Johannesburg, South Africa.

4.2.2. Determination of the upper and lower variable limits for formulation synthesis and Box-Behnken design generation

A 3-factor Box-Behnken experimental design was utilized for the establishment of an optimized formulation. For the construction of the Box-Behnken design, a set of independent variable and their upper and lower limits had to be identified. Following the selection of appropriate polymers, preliminary investigations assisted in determining the formulation variables and the required limits. The gellan and xanthan gum ratios, CaCl_2 crosslinker concentration and pristine polymer particle concentration were identified as the variables for input into the Box-Behnken design. From the proof-of-concept study, detailed in Chapter 3, it is evident that the degree of crosslinking and the concentration of pristine polymer particle intercalation influences important matrix characteristics of swelling, erosion, bioactive release and mechanical properties. The upper and lower limits of the identified variables were determined, predominantly, based on the prospect of reproducible and effortless conduit fabrication via a mould casting technique. The following criteria, pertaining to formulation synthesis, were considered during establishment of the variable limits:

- The blended gel solution should facilitate easy pouring and moulding for the fabrication of seamless hollow conduits.
- Upon mould casting, the gel solution should snap set in minimal time to avoid settling of the suspended pristine polymer particles.
- The gelation temperature should be appropriate (37°C or less) to allow magnetic agitation for the homogenous incorporation of thermo-labile protein and peptide-based bioactives.

Table 4.1 lists the preliminary trial formulations prepared and the effects observed on conduit formation for the identification of the independent variables and their limits. Pristine polymer particle intercalation was obtained by the addition of PMMA in grams per gram of dry gum powders. Formulations were mould-cast and film-cast to assess conduit formation. Certain formulations were subjected to 14-day dissolution trials in PBS pH 7.4 to observe swelling characteristics and maintenance of a solid gel structure upon hydration.

Table 4.1: Composition and observations of preliminary trial formulations

Trial	Formulation Composition	Observation
1	1% ^{w/v} XG, 20% ^{w/v} PG	Xanthan gum alone was unable to maintain a solid architecture and dissolved into a liquid gel state after 12 days dissolution in PBS pH 7.4. Excessive PG would leach out of matrices after drying of scaffolds. Increase in crosslinker concentration
	1% ^{w/v} XG, 10% ^{w/v} PG	
	1% ^{w/v} XG, 5% ^{w/v} PG	
	1% ^{w/v} XG, 2% ^{w/v} PG	
	1% ^{w/v} XG, 5% ^{w/v} PG, 0.125% ^{w/v} CaCl_2	
	1% ^{w/v} XG, 5% ^{w/v} PG, 0.1% ^{w/v} CaCl_2	

		resulted only in increased viscosity of the gel but no formation of solid gel structure.
2	1% ^{w/v} GG, 5% ^{w/v} PG 1% ^{w/v} GG, 5% ^{w/v} PG, 0.05% ^{w/v} CaCl ₂ 1% ^{w/v} GG, 5% ^{w/v} PG, 0.1% ^{w/v} CaCl ₂ 1% ^{w/v} GG, 5% ^{w/v} PG, 0.3% ^{w/v} CaCl ₂	Gellan gum alone produced well-formed solid gels with rapid swelling propensity in PBS pH 7.4 where increased crosslinker resulted in the formation of harder and brittle gels undergoing immediate snap-setting upon decrease in temperature. Matrices maintained rigid form and shape upon swelling.
3	1% ^{w/v} GG:XG 50:50, 5% ^{w/v} PG, 0.05% ^{w/v} CaCl ₂ 1% ^{w/v} GG:XG 70:30, 5% ^{w/v} PG, 0.05% ^{w/v} CaCl ₂ , PMMA 1g/g 1% ^{w/v} GG:XG 80:20, 5% ^{w/v} PG, 0.05% ^{w/v} CaCl ₂	Combinations of gellan and xanthan provided a means to adjust matrix rigidity and swelling characteristics. Increased gellan produced brittle gels that were difficult to mould whereas increased xanthan produced weak gels that would collapse after removal from moulds.
4	1% ^{w/v} GG:XG 60:40, 5% ^{w/v} PG, 0.15% ^{w/v} CaCl ₂ , PMMA 1g/g 1% ^{w/v} GG:XG 60:40, 5% ^{w/v} PG, 0.05% ^{w/v} CaCl ₂ , PMMA 0.25g/g 1% ^{w/v} GG:XG 80:20, 5% ^{w/v} PG, 0.15% ^{w/v} CaCl ₂ , PMMA 1g/g 1% ^{w/v} GG:XG 80:20, 5% ^{w/v} PG, 0.1% ^{w/v} CaCl ₂ , PMMA 1g/g 1% ^{w/v} GG:XG 80:20, 5% ^{w/v} PG, 0.05% ^{w/v} CaCl ₂ , PMMA 0.25g/g	Produced solidified gel scaffolds of sufficient strength upon removal from moulds. Gel solutions solidified rapidly forming cracks and were unable to form conduits. Gelation temperature was 42°C – cause thermal degradation of protein bioactives. Gelation temperature was 38°C upon cooling of solution. PMMA concentration produced clumping of particles. Gelation temperature was 35°C upon cooling of solution.

The preliminary trial formulations mentioned above, allowed the identification of the independent formulation variables and the upper and lower limits for successful fabrication of conduits and loading of thermo-labile protein-based bioactives. Table 4.2 lists the identified variable upper and lower limits and the effects on conduit formulation.

Table 4.2: The identified variables and the upper and lower limits for generation of a Box-Behnken experimental design

Variable		Effect
Gellan:Xanthan	Lower limit: 60:40	Formulations composed of an equal or greater concentration of xanthan gum resulted in the formation of high-swelling, soft and rapid-dissolving matrices in PBS pH 7.4. Due to formation of a weak and insufficiently rigid gel, removal from the moulds upon setting resulted in frequent collapse of the scaffold.
	Upper limit 80:20	Formulations composed of greater gellan gum concentrations result in rigid and brittle gels with increased propensity of cracking during removal from moulds.
PMMA particles	Lower limit: 0.25g/g	Concentrations <0.25g/g produced insufficient particle intercalation as per microscope observations. Low particle intercalation would produce negligible changes in mechanical properties and bioactive release rates.
	Upper limit: 0.75g/g	Concentrations exceeding 0.75g/g and of equal gum concentration produced scaffolds of deformed surface morphology as per microscope imaging and increased potential of scaffold breakage during removal from moulds. Higher concentrations produced clumping of particles.
CaCl₂	Lower limit: 0.05% ^{w/w}	Concentrations <0.05% ^{w/w} provided insufficient crosslinking producing weak liquid-like gels resulting in collapse of the scaffold.
	Upper limit: 0.1% ^{w/w}	Concentrations >0.1% ^{w/w} resulted in the formation of rigid and brittle gels exhibiting immediate snap-setting and hardening at temperatures >38°C with reduced pourability for moulding purposes.
Propylene glycol	Fixed: 5% ^{w/v}	Concentrations >0.5% ^{w/v} resulted in inadequate drying of conduits and leaching out of excessive plasticiser forming oily matrices.

4.2.3. Synthesis of hydrogel nerve conduits

4.2.3.1. Preparation of the gel blend and pristine polymer particle suspension

Gellan gum and xanthan gum powders of varying ratios were combined and added to deionised water to form a 1%^{w/v} gel solution. After solubilisation of the gum powders, propylene glycol and calcium chloride were added as the plasticiser and crosslinker in

concentrations of 5%^{w/v} and 0.05%^{w/v}-0.1%^{w/v}, respectively. The gel blend was then heated to 100°C and maintained at that temperature for 10 minutes. Addition of polymethyl methacrylate (PMMA) nano-sized particles was introduced into the gel blend, in concentrations of 0.25g (low), 0.5g (intermediate) and 0.75g (high) per gram of dry polysaccharide powder blend, after cooling the solution to 60°C. The gel-polymer particle suspension was slowly cooled to temperatures between 35-37°C to accommodate the incorporation of drug and model protein (diclofenac sodium and bovine serum albumin, respectively). Cylindrical moulds (Figure 4.1), measuring 50mm in length and 12mm in diameter, were constructed. A removable glass rod of 1.88mm diameter was inserted into the centre of the mould to form the lumen of the conduit. The polymer gel suspension was carefully syringed into pre-lubricated cylindrical moulds and allowed to set at room temperature for 20 minutes before being removed and left to air-dry, while on the glass rods, in a fumehood for 24 hours. A series of 15 experimental gellan-xanthan gum-pristine polymer particle intercalated formulations were synthesised employing a 3-factor Box-Behnken design utilizing Minitab[®] statistical software (V15, Minitab Inc., PA, USA) as listed in Table 4.3.

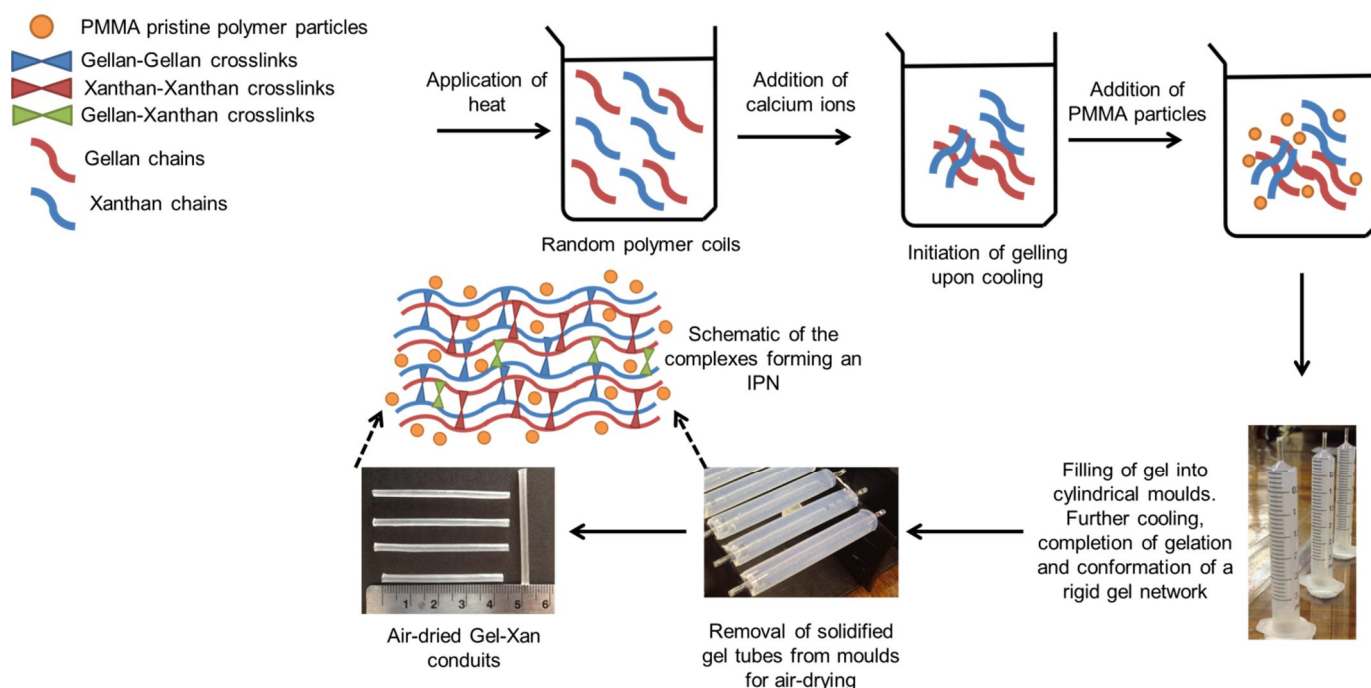


Figure 4.1: Schematic depicting the synthesis of the Gel-Xan conduits and formation of an IPN hydrogel.

Table 4.3: Formulations obtained via the Box-Behnken design

Formulation	Gellan:Xanthan	CaCl ₂ (% ^w / _v)	PMMA (g) [†]
1	60:40	0.1	0.5
2	70:30	0.075	0.5
3	80:20	0.1	0.5
4	70:30	0.05	0.75
5	70:30	0.1	0.75
6	70:30	0.075	0.5
7	80:20	0.05	0.5
8	80:20	0.075	0.25
9	60:40	0.075	0.75
10	70:30	0.05	0.25
11	60:40	0.075	0.25
12	60:40	0.05	0.5
13	70:30	0.075	0.5
14	80:20	0.075	0.75
15	70:30	0.1	0.25

[†] Amount of PMMA (g) per 1 gram of dry polysaccharide powder blend.

4.2.4. Response surface analysis as per the Box-Behnken design

Response surface analysis of the 15 design formulations were carried out employing Minitab® statistical software (V15, Minitab Inc., PA, USA). The *in vitro* performance and design optimization of the experimental formulations were evaluated in terms of drug release, swelling, erosion and textural properties (matrix resilience, deformation and fracture) to elucidate the influence of polymer ratios, crosslinker and pristine particle concentration on the studied response outcomes.

4.2.5. Characterization of the pristine polymer particle intercalated gel conduits

4.2.5.1. Detection of vibrational molecular changes of the nerve conduits

To confirm the chemical structures and detect molecular transitions thereof, samples of the conduits (listed in Table 1) were analysed for the presence of crosslinking and interactions between the native polymers (gellan, xanthan and PMMA). Samples were analysed over an FTIR spectra of wavelengths between 4000-600cm⁻¹ using a PerkinElmer Spectrum 2000 ATR-FTIR (PerkinElmer 100, Llantrisant, Wales, UK) spectrometer fitted with a single-reflection diamond MIRTGS detector.

4.2.5.2. Thermophysical characterization of nerve conduits

The thermodynamic properties of the conduits were determined using a Temperature Modulated Differential Scanning Calorimeter (TMDSC) (Mettler Toledo, DSC, STARe System, Swchwerzenback, ZH, Switzerland). Samples of 15mg were sealed in aluminium

crucibles and heated over a temperature range between 0-300°C at a heating rate of 10°C/min. DSC curves were obtained by plots of sample weight over sample temperature. Differences in the heat flow and phase transitions of the formulations provided insight into the effects of polymer blending and crosslinking. The determination of change in sample weight as a function of temperature was performed using a Thermogravimetric analyser (TGA) (PerkinElmer, TGA 4000, Llantrisant, Wales, UK). Samples were heated at a rate of 10°C/min from 30-900°C under continuous nitrogen purging. Thermograms were generated as percentage weight vs. temperature and analysed using the Pyris™ software. Correlation of DSC and TGA data assists in determining the effect of crosslinking in relation to polymer thermal degradation properties.

4.2.5.3. Porositometric analysis

The surface area and pore size of 24-hour hydrated hydrogel conduits in PBS pH 7.4 were evaluated using a Porositometric Analyzer (Micromeritics ASAP 2020, Norcross, GA, USA). Samples were frozen at -85°C and lyophilized (Freezone 12, Labcono, Kansas City, USA) before degassing samples for elimination of residual moisture and contaminants. A glass filler rod inserted into the sample tube reduced the total free space volume in the tube thus hastening the degassing stage involving an evacuation and heating phase. Thereafter, the sample tube was transferred to the analysis port for determination of surface area, pore size and pore volume in accordance with the BET and BJH models. An evacuation rate of 50.0mmHg/s with a temperature ramp rate of 10°C/min, target temperature of 40°C and a hold temperature of 30°C for 900min was used. The BET linear equation (Equation 4.1) was used to determine the monolayer capacity.

$$p/n^a(p^0-p) = 1/n_m^a \times C + (C-1)p/n_m^a \times Cp^0 \quad \text{Equation 4.1}$$

Where, n^a refers to the quantity of N₂ adsorbed at the relative pressure p/p^0 , n_m^a was the monolayer capacity and C was exponentially related to the enthalpy of adsorption in the first adsorbed layer.

Using the molecular cross-sectional value a^m , the surface area was determined from the monolayer capacity employing Equations 4.2 and 4.3.

$$A_s(BET) = n_m^a \times L \times a_m \quad \text{Equation 4.2}$$

$$a_s(BET) = A_s(BET)/m \quad \text{Equation 4.3}$$

Where $A_s(\text{BET})$ and $a_s(\text{BET})$ are the total and specific surface areas, respectively, of the adsorbent of mass m and L is the Avogadro constant.

4.2.5.4. Scanning Electron Microscopy for visualisation of surface morphology

For evaluation of the surface morphology, lyophilized hydrogel samples, post 24 hours dissolution in PBS pH 7.4, were fixed to aluminium stubs using double-sided adhesive carbon tape and then sputter coated with carbon and gold-palladium for 120 seconds (SPI Module™ Sputter-Coater and Control Unit, West Chester, PA, USA). Samples were viewed using an FEI Nova NanoLab 600™ Scanning Electron Microscope (SEM) (FEI Company, Hillsboro, OR, USA) under different magnifications and images were taken of the matrix surface architecture.

4.2.5.5. Swelling and erosion studies

Swelling and erosion studies were performed in triplicate by immersing 10mm conduit samples in 2mL of PBS pH 7.4 in an orbital shaking incubator (LM-530-2, MRC Laboratory Instruments Ltd., Hahistadrut, Holon, Israel) maintained at 37°C and set at 50rpm for sample agitation. Samples were removed at predetermined time intervals and the change in mass were measured for the determination of swelling ratio after carefully blotting the hydrated samples with tissue paper for the removal of excess water. The mean wall thickness of the hydrated conduits at each sampling point was measured at four different angles using a digital Vernier calliper (Krafft, DV150GW, Schoellerstr Düren, Germany). The conduit samples were then dried at 40°C for 72 hours to ensure complete removal of residual moisture before being weighed for the determination of the eroded mass. Equations 4.4 and 4.5 were employed for the determination of swelling ratio and erosion, respectively.

$$\% \text{ Swelling ratio} = \frac{W_s - W_i}{W_i} \times 100 \quad \text{Equation 4.4}$$

$$\% \text{ Erosion} = \frac{W_f - W_i}{W_i} \times 100 \quad \text{Equation 4.5}$$

Where W_i is the initial weight of the conduits, W_s is the weight of conduits after hydration and W_f is the weight of eroded conduits.

4.2.5.6. Textural profiling of hydrated conduits

Textural analysis characterization using a Texture Analyser (TAXTplus Stable Microsystems, Surrey, UK) fitted with a 10mm diameter delrin probe was undertaken on hydrated conduit samples at pre-determined sampling points. The Matrix Resilience (MR), Deformability Modulus (DF) and Fracture Energy (FE) were determined. MR (N.sec) was determined by

the ratio of the areas under the compression and decompression curves generated by Force-Time textural profiles. This represented the ability of the hydrogel conduit to recover to its original form after application of an external deforming stress. The Area under the Curve (AUC) from the Force-Distance textural profile allowed the determination of the FE (N.mm) which equates to the energy in joules required to cause rupture of or a break in the matrix of the hydrated swollen conduits. The DM (N/mm) is an indication of the hydrogel matrix flexibility and was computed from the gradient of the Force-Distance profile from zero to the point of the peak force. The textural analysis parameters utilized include a pre-test and post-test speed of 1.00mm/sec, a test speed of 0.5mm/sec, a trigger force of 0.5N and a load cell of 5kg with a target mode of 10% strain.

4.2.5.7. *In vitro* release studies and kinetic modelling

In vitro release studies were performed using 10mm samples of conduit each loaded with 3mg BSA and diclofenac sodium. Samples were placed in glass vials containing 1mL PBS pH 7.4 and stored in an orbital shaking incubator. At pre-determined time intervals, 0.3ml of dissolution media was removed for drug content analysis and replaced with an equal fresh volume of media to maintain sink conditions. Samples were analysed for the quantity of BSA and diclofenac sodium by UV-spectrophotometry set at 595nm and 276nm, respectively, using a Bradford protein assay and an absorbance multi-plate reader (BioTek, USA). Further insight into the drug-release mechanisms was obtained via kinetic modelling of the drug-release profiles using the models listed in Table 4.4.

Table 4.4: Models and parameters employed for kinetic modelling of drug release profiles

Model Name	Parameters	Reference
Zero-order: $C = K_0 t$	K_0 = zero-order rate constant t = time	Dash <i>et al.</i> , 2010
First-order: $\text{Log} C = \text{Log} C_0 - kt/2.303$	C_0 = initial concentration of drug K = first-order constant	Dash <i>et al.</i> , 2010
Higuchi: $Q = K t^{1/2}$	Q = % drug release K = design variable of the system t = time	Dash <i>et al.</i> , 2010 Siepmann and Peppas, 2012
Hixson-Crowell: $Q_0^{1/3} - Q_t^{1/3} = K_{HC} t$	Q_0 = initial amount of drug loaded Q_t = amount of drug released in time t K_{HC} = rate constant t = time	Dash <i>et al.</i> , 2010
Hopfenberg: $M_t/M_\infty = 1 - (1 - K_1 t)^n$	M_t / M_∞ = fractional drug release at time t K = rate constant	Dash <i>et al.</i> , 2010

	t = time $n = 1, 2$ or 3 for slab, cylinder or sphere geometries, respectively	
Korsmeyer-Peppas/Power Law: $M_t/M_\infty = Kt^n$	M_t / M_∞ = fractional drug release at time t K = rate constant t = time n = release exponent	Siepmann and Peppas, 2012 Maity and Sa, 2014
Peppas-Sahlin: $M_t/M_\infty = K_1t^m + K_2t^{2m}$	M_t / M_∞ = fractional drug release at time t K_1 = Fickian diffusional contribution (F) K_2 = Case-II relaxational contribution (R) t = time m = constant	Siepmann and Peppas, 2012 Mady, 2013

4.3. Results and Discussion

4.3.1. Vibrational molecular transitions and thermodynamic analyses validating crosslinking and formation of an IPN hydrogel network

FTIR spectroscopy was used to identify the similarities and differences in the chemical structures of the crosslinked gellan-xanthan hydrogel blends. Spectroscopy of the native polymers served as a comparison for the detection of polymer-polymer and polymer-crosslinker interactions thus providing validation of and the degree of crosslinking achieved. The FTIR spectra of both the pure forms of gellan and xanthan gum are very similar and correspond to the typical chemical structure of polysaccharides. Both polymers exhibited a broad band at 3316cm^{-1} and 3281cm^{-1} indicative of --OH stretching of gellan and xanthan gum, respectively. Stretching vibrations of the --CH_2 groups occurred at wavelengths of 2884cm^{-1} for gellan gum. Xanthan gum produced a peak at 2901cm^{-1} corresponding to the C-H stretching of the CH_3 alkyl groups (Faria *et al.*, 2011). Bands appearing at 1600cm^{-1} and 1400cm^{-1} are due to the asymmetric and symmetric stretching of COO^- groups in both polymers, respectively (Faria *et al.*, 2011). Defined bands occurring towards the end of the spectra at wavenumbers of $1018\text{--}1017\text{cm}^{-1}$ are characteristic of C–O stretching in both gellan and xanthan gum. The strong peak produced at 1722cm^{-1} distinguishes xanthan from gellan gum and is indicative of the C=O vibrations of the carboxylate anion whereas further peaks at 1370cm^{-1} and 1246cm^{-1} present the C-H bending of methyl groups and the presence of *o*-acetyl esters (Faria *et al.*, 2011). The presence of PMMA is identified by its esterified carboxylic acids peaking at 1722cm^{-1} , however, when combined within the gel formulations this peak overlaps with that corresponding to the carbonyls of xanthan gum resulting in increased intensity of this peak.

The PMMA intercalated gellan-xanthan gum formulations maintained the characteristic chemical compositions of the parent polymers as shown in Figure 4.2a. The broad band of –OH stretching shifts to higher wavenumbers ($3370\text{--}3390\text{cm}^{-1}$) becoming narrower and decreasing in intensity thus suggesting interaction of hydroxyls with Ca^{2+} during intramolecular and intermolecular hydrogen-bonding. The peak corresponding to stretching of methyl (CH_3) and methylene (CH_2) groups of gellan and xanthan, respectively, shifts to higher wavenumbers increasing in intensity and slightly broadening before splitting into two peaks at 2920cm^{-1} and 2870cm^{-1} . The change in stretching vibration of these groups can be attributed to the interaction and bonding of Ca^{2+} ions with the carboxylate groups on the glucuronate and pyruvate moieties present in the polysaccharide polymers thereby affecting the nearby CH_2 and CH_3 groups. The peak corresponding to the carboxylate ion of xanthan becomes narrower with a slight increase in intensity due to the addition and overlapping of the PMMA carboxylate peak appearing at the same wavenumber, however shifting of the peak to a higher wavenumber of 1730cm^{-1} is noted. Formulations where polymer composition and PMMA particle concentration was constant, differing only in crosslinker concentration, were selected for clarified comparison {60:40: F1 ($0.1\%\text{w/v}$) and F12 ($0.05\%\text{w/v}$); 70:30: F15 ($0.1\%\text{w/v}$) and F10 ($0.05\%\text{w/v}$); 80:20: F3 ($0.1\%\text{w/v}$) and F7 ($0.05\%\text{w/v}$)}. Formulations containing higher concentrations of calcium crosslinker exhibited carboxylate peaks of decreased intensity suggesting either the interaction of COO^- groups of Xanthan gum with Ca^{2+} or its participation in hydrogen-bonding (Pongjanyakul and Puttipipatkachorn, 2007) (Figure 4.2b). The peaks at 1600cm^{-1} and 1400cm^{-1} indicative of COO^- asymmetric and symmetric stretching, respectively, shifts to higher wavenumbers of 1615cm^{-1} and 1435cm^{-1} undergoing a significant decrease in intensity, particularly the asymmetric stretching peak. This indicates the disappearance of the carboxylate anions as it interacts with Ca^{2+} cations leading to the formation of a calcium carboxylate salt (Verma and Pandit, 2012; Yang *et al.*, 2013). As described in another study (Elmowafy *et al.*, 2008), the peaks occurring between $1017\text{--}1018\text{cm}^{-1}$ associated with the C-O stretching in both gellan and xanthan native polymers undergoes a substantial change in the respective formulations upon ionic interaction with calcium ions. This peak significantly increases in intensity towards higher wavenumbers forming a broad peak and then splitting into a 1065cm^{-1} and 1031cm^{-1} peak indicating Ca^{2+} bonding to the COO^- groups of gellan and xanthan, respectively, thereby changing the C-O stretching vibration via steric hindrance on the carbonyl functional group. This interaction may further explain the formation of the new peak at 946cm^{-1} shouldering off the broad $1065\text{--}1031\text{cm}^{-1}$ peak.

In addition, the FTIR spectra show that PMMA particles are merely present in its pristine form in the hydrogel formulations and do not partake in the crosslinking mechanism between

the divalent salt and the polysaccharides. This is plausible considering that during the hydrogel synthesis the PMMA particles were incorporated into the aqueous gel solution in its non-dissolved solid state after the addition of CaCl_2 and initiation of gelation.

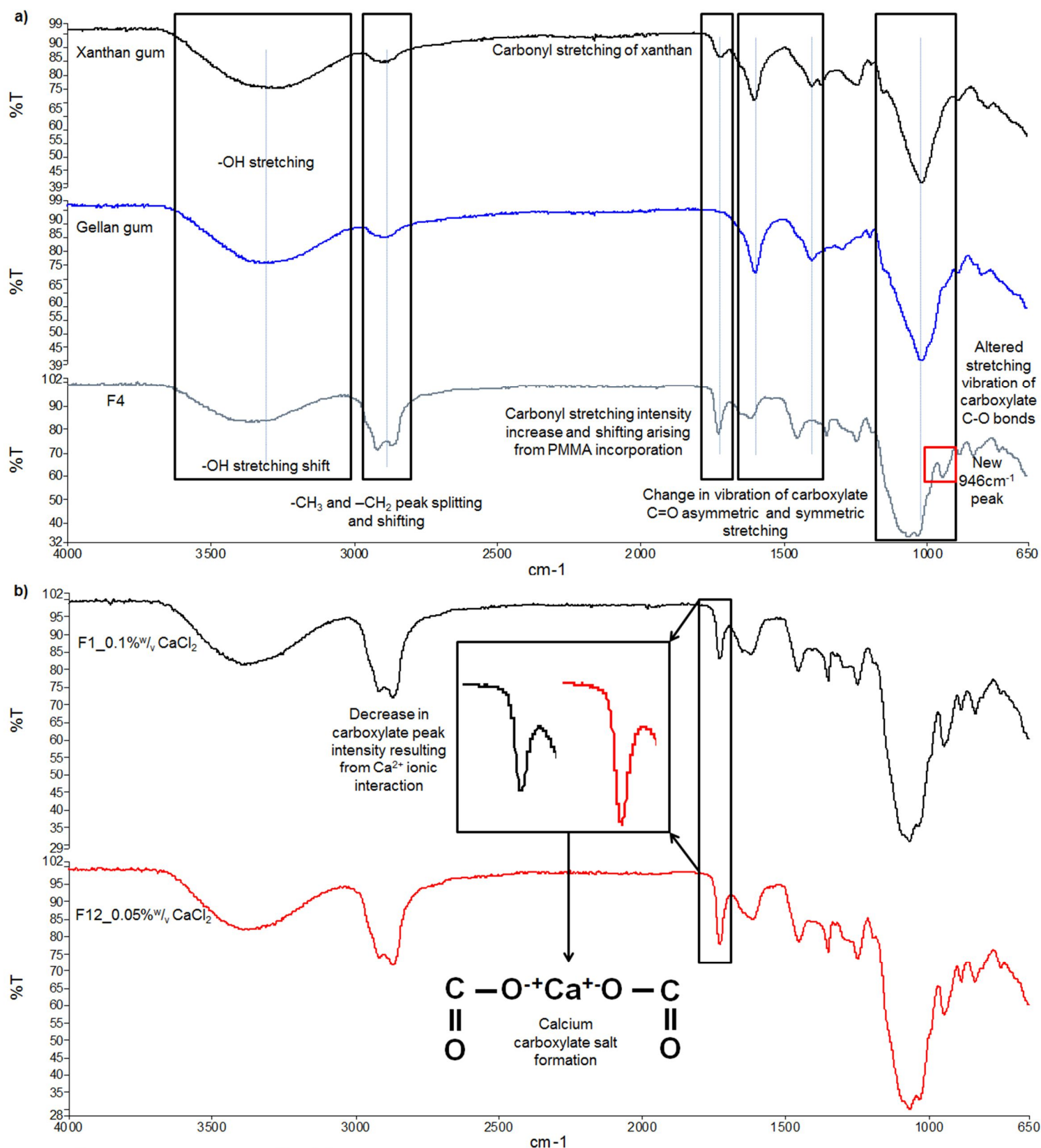


Figure 4.2: FTIR Spectra a) showing the vibrational changes between constituent polymers and F4 upon crosslinking with CaCl₂ b) validation and comparison of crosslinking degree via carboxylate-calcium interactions between F1 and F12 (60:40 with intermediate PMMA intercalation).

DSC and TGA were employed in addition to FTIR spectroscopy to further validate the formation of crosslinks resulting in a gellan-xanthan hydrogel IPN by measuring the changes in energy transfers and mass polymer decomposition. The first endothermic peak in the DSC thermograms appearing around 100°C is probably the presence of crystalline water entrapped between the polymer chains. No defined melting peaks were observed in both the native polysaccharides and the formulations. Gellan gum displays its exothermic peak at 253°C whereas Xanthan gum exhibits an exothermic peak at 282°C indicating its superior thermal stability compared to gellan. The DSC thermogram of PMMA shows two endothermic peaks: a weak peak at 93°C which may be attributed to the evaporation of moisture residues and a prominent peak at 220°C where melting is initiated. The broad exothermic peak occurring towards the end of the experimental run around 270°C indicates polymer decomposition; however this peak exhibited significant changes in intensity with varying concentrations of crosslinker and thus provides validation and determination of the degree of crosslinking. It was noted that increase in CaCl_2 concentration generally resulted in decreased height and area of the exothermic peak suggesting calcium-polymer bond formation resulting from the Ca^{2+} cation chelation of anionic carboxylates thus improving thermal stability of the formulations via formation of a more rigid matrix network. The energy associated with this phase of degradation provides an indication of the ease of bond-breaking and hence the crosslinking degree. Highly crosslinked matrices require greater temperatures for the initiation of thermal decomposition thereby giving off less energy due to a smaller number of bond-breakage at that specific temperature. Increase in CaCl_2 concentration from 0.05% $_{\text{w/v}}$ to 0.1% $_{\text{w/v}}$ across the three different gum ratios increased the temperature at which the exothermic peak appeared hence increasing the temperature of the onset of degradation (Figure 4.3). Moreover, this decomposition stage resulted in the release of less energy, at that particular temperature range, which equates to less bond-breakage ensuing in the polymer matrix during thermal degradation. The improved thermal stability arises from ionic bonds between calcium cations and carboxylate anions present in gellan and xanthan gum. Comparing the parent polymers, although the temperature corresponding to the onset of degradation remained similar, the enthalpy change during polymer disintegration decreased from 1684mJ and 1240mJ for gellan and xanthan gum, respectively, to values in the range of 91-192mJ in the various hydrogel formulations where increased concentration of crosslinker decreased the change in energy transferred. This indicates that the unmodified polymer powders underwent extensive thermal degradation due to absence of crosslinks compared to the crosslinked hydrogel IPNs.

In addition, the glass transition temperature (T_g) may be used to provide an indication of crosslinking, crystallinity and homogeneity of the constituent polymer components. The

presence of only one T_g including a single exothermic peak indicates the formation of a homogenous crosslinked IPN matrix whereas basic polymer blends would result in the emergence of multiple distinct glass transition temperatures each corresponding to the individual constituent polymers (Elmowafy *et al.*, 2008). Increase in crystallinity is reflected by a larger and broader glass transition whereas increase in crosslinking shifts the T_g to higher temperatures. The T_g of gellan and xanthan gum were not evident in the experimental run and may possibly occur at temperatures lower than zero. However, well-defined glass transitions, peaking at around 20°C (Figure 4.3), of the crosslinked formulations were evident on the thermograms indicating the presence of crosslinks and the formation of a partly rigid matrix structure. Furthermore, a slight endothermic dip noted in the thermograms between 220-230°C prior to the onset of polymer decomposition may be indicative of PMMA melting as formulations containing higher PMMA concentrations ranging from 0.5g-0.75g/g present with a slightly pronounced endothermic slope.

The TGA thermograms in conjunction with the derivatisation curves were correlated to the results obtained from DSC. As noted in DSC, all TGA thermograms presented with an initial minimal decomposition step which corresponded to the dehydration process of entrapped crystalline water molecules. Pure gellan and xanthan gum underwent the greatest loss in weight at temperatures of 270°C and 295°C, respectively, corresponding to the polymer decomposition indicated by the exothermic peak in DSC with complete decomposition occurring at 615°C for gellan whereas an 8% mass of xanthan remained at the end of the 900°C run. According to the derivatisation curves, a further decomposition step occurred peaking at 606°C and 634°C for gellan and xanthan, respectively. PMMA exhibited two decomposition steps: at 237°C where less than 5% weight loss occurred indicative of polymer melting and at 395°C which was indicative of polymer decomposition with complete sample degradation occurring at 434°C. CaCl_2 exhibited the first decomposition peak at 190°C pertaining to the decomposition of chloride with a steady 39% remaining mass towards the end temperature of 900°C. According to literature, calcium only melts at 840°C (Kaye and Laby, 1995) explaining the flat linear portion of the thermogram starting from 280°C onwards indicating only slight changes in sample weight.

TGA thermograms of the experimental formulations exhibited decomposition in 4 steps: Step 1 occurring between 80-100°C corresponds to the loss of residual moisture from the samples; Step 2 occurring between 250-260°C and ending at 350°C was attributed to polysaccharide degradation where the major loss of sample weight was observed; Step 3 initiating at approximately 395-399°C and ending at 436°C was due to the decomposition of the pristine polymer particles; Step 4 was assigned to the final stage of polymer degradation

and occurred in the range of 570-580°C. Analysis of the derivatisation curves revealed that the most significant change in weight of the samples occurred at Step 2 and Step 4. The peak corresponding to Step 3 fluctuated in intensity in response to increases and decreases in the concentration of PMMA particles. From the derivative curve, it was further noted that highly crosslinked formulations, F1, F3, and F15 containing 0.1%^{w/v} CaCl₂ and F8 and F11 containing 0.075%^{w/v} CaCl₂ presented with a new peak at 319°C shouldering off the Step 2 decomposition peak. This may indicate the decomposition of calcium carboxylate salt in the form of carboxylate-Ca-carboxylate crosslinks.

The results indicate that an IPN is indeed formed where the two polymer components are simultaneously crosslinked via two crosslinking mechanisms. FTIR indicates the types of bonding that may have occurred to form crosslinks between polymers whereas thermal analysis indicates that the constituent polysaccharides are in fact crosslinked and not merely present as a blend.

Crosslinking in the hydrogel matrices could occur via two mechanisms: thermo-induced physical crosslinking and ionotropic crosslinking with bivalent calcium ions. Upon heating above 80°C and subsequent cooling of the gel solutions, gellan and xanthan gum are known to undergo a change in structural conformation of the polymer chains from random coils to ordered helical structures (Tang *et al.*, 2001; Bergmann *et al.*, 2008). The double helix structures in gellan gum creating junction zones are further reinforced and stabilised via Ca²⁺ chelation, specifically between two carboxyl anions on opposite chains. This leads to the formation of 3D physical solid gels. Similarly, xanthan gum undergoes conformational change forming either double or single helices via calcium chelation where the presence of cations result in the reduction of electrostatic repulsion (Baumgartner *et al.*, 2008) between the negative carboxyls leading to wrapping of the side chains around the polymer backbone via hydrogen bonding thereby forming a helical rod. Ionotropic crosslinking and gelation may occur via a combination of intramolecular and intermolecular crosslinks.

Intramolecular interactions may occur via hydrogen-bonding amongst single chains and the polymer backbone and additionally between hydrogens and carboxylates on the terminal ends of a single chain resulting in chain contraction (Dário *et al.*, 2011). Likewise, an intramolecular crosslink may occur between a single calcium cation and the terminal ends of two carboxylate side chains, present on the pyruvate and glucuronate moieties, along a single main polymer chain (Bergmann *et al.*, 2008; Dário *et al.*, 2011; Bueno *et al.*, 2013)

Intermolecular crosslinking may occur via hydrogen-bonding between formed helical structures and between hydroxyls and carboxylates of xanthan and gellan, respectively and vice versa (Chandrasekaran and Radha, 1997; Pongjanyakul and Puttipipatkachorn, 2007).

Intermolecular cation chelation arises from electrostatic interaction between a central Ca^{2+} ion and two carboxyl anions on separate chains of gellan-gellan, xanthan-xanthan or gellan-xanthan resulting in the formation of a rigid structure attributed to the stabilization of junction zones by Ca^{2+} and the resulting reduction in electrostatic repulsion between negatively charged side chains, specifically the pyruvate and glucuronate (Dário *et al.*, 2011) functional moieties of xanthan gum. The above intra- and intermolecular interactions and the resulting structural conformations may occur randomly in any ratio as the reaction during thermal treatment proceeds.

As depicted in Figure 4.3, this method of non-covalent crosslinking and the potential existence of various crosslink-types within a single matrix may yield the formation of an interpenetrating hydrogel network where two crosslinked polymer networks are partially interlaced on a molecular scale but not covalently bonded to each other and where the constituent polymers can only be separated upon breaking of chemical bonds (Matricardi *et al.*, 2013; Aminabhavi *et al.*, 2015).

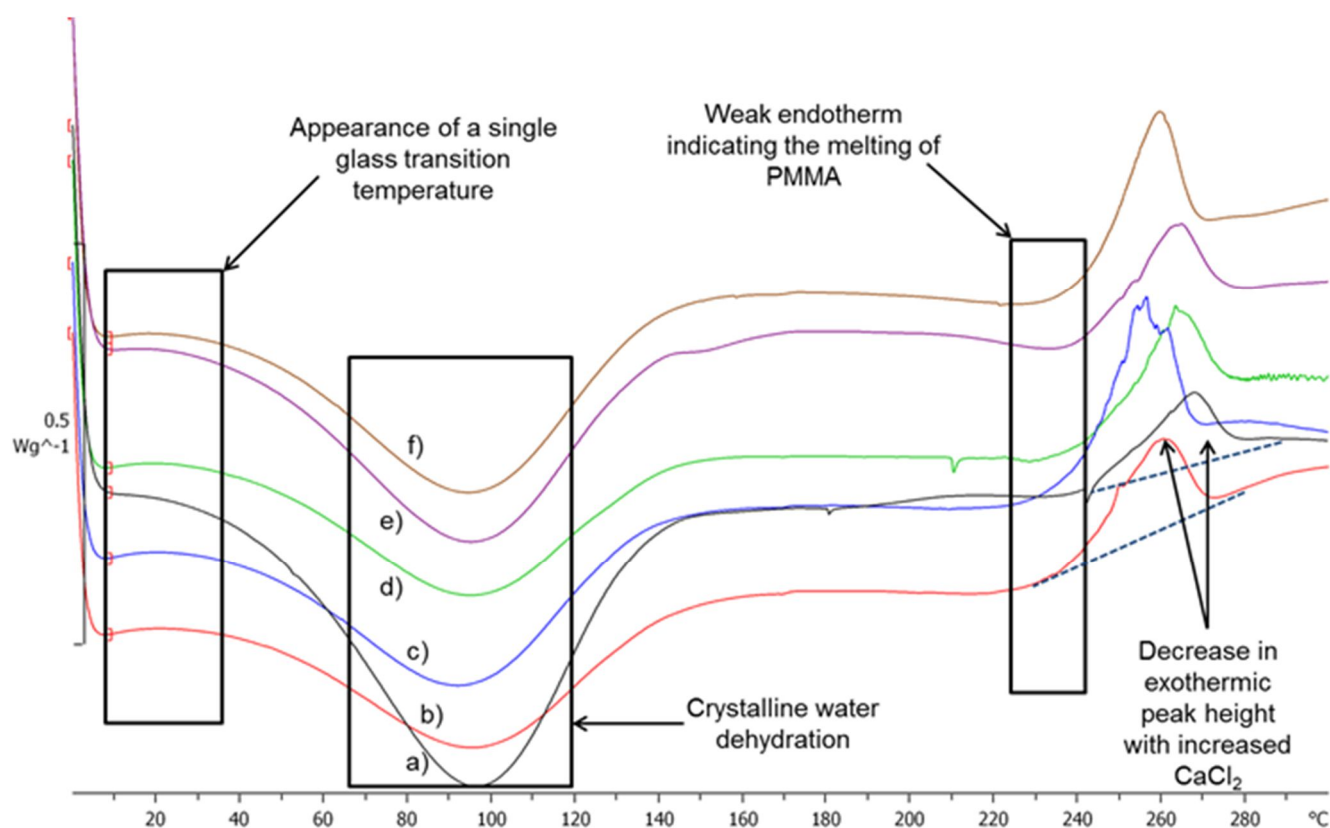


Figure 4.3: DSC thermograms of formulations varying in gellan-xanthan ratios with the following CaCl_2 crosslinker concentrations 60:40 a) F1-0.1% $^{w/v}$ and b) F12-0.05% $^{w/v}$; 70:30 c) F10-0.05% $^{w/v}$ and d) F15-0.1% $^{w/v}$; 80:20 e) F3-0.1% $^{w/v}$ and f) F7-0.05% $^{w/v}$.

4.3.2. Analysis of pH-responsive in situ particulate leaching of intercalated PMMA particles for formation of porous microenvironment structures

We have previously reported elsewhere the mechanism of pH-responsive particulate leaching of the intercalated pristine PMMA particles via dissolution at pH 7.4 leading to the formation of a pore-like microenvironment network (Ramburrun *et al.*, 2015). We postulated that the intercalation of pristine polymer particles of nano-size distribution within a hydrophilic polymer matrix would result in pore formation in the form of a core microenvironment array. Increase in PMMA particle concentration would lead to an increased number of core microenvironment structures. Similar to the previous proof-of-concept study in chitospheres, the gellan-xanthan IPN hydrogels exhibited an increase in the quantity of pores formed with increasing PMMA particle concentration where such particles elicited the induction of pores by serving as a pH-responsive porogen *in situ*. Matrix resilience and porosimetric analyses validated the formation of such microporous environments within the hydrogel matrix post-*in vitro* dissolution.

Matrix resilience may be used to indicate the presence of porous structures within a polymer matrix. Loose, empty voids within a polymer matrix allows the matrix to undergo a transient change in shape upon compression to a certain extent before reverting to its original form and shape after removal of the compressive force. The matrix resilience profiles in Figure 4.4 show marked increased resilience in formulations containing larger quantities of pristine PMMA particles. This is particularly observed with F5 and F15, F9 and F11 and F14 and F8 where polymer and crosslinker compositions are identical but differ only in PMMA particle concentration. As the dissolution media penetrates the matrix initiating the dissolution of the PMMA particles, porous microenvironment structures are continuously formed until complete leaching of the particles hence explaining the progressive increase in %matrix resilience.

It was further noted that formulations containing higher concentrations of particles displayed superior matrix resilience, particularly F4, F5 and F14 containing 0.75g/g of pristine particles and F7 and F12 synthesised with 0.5g/g of PMMA particles. At the end of the study period, these formulations exhibited resilience values between 45-56% compared to formulations containing 0.25g/g of PMMA particles displaying resilience values between 31-40%. The observed increase in matrix resilience conferred by the pH-responsive particulate leaching of the PMMA particles for the formation of pores corresponds to the loss in weight of the conduits.

Porositometric analysis performed in accordance to the BET and BJH models, using nitrogen as the adsorptive gas, was used to ascertain the pore size, pore surface area and shape by assessing the shape of the isotherm and resulting hysteresis loop. Presence of hysteresis indicates the presence of pores whereas its shape may assist in determining pore shape. Formulations, after 24 hours of dissolution, containing higher concentrations of PMMA particles presented with greater BET surface and micropore areas as a result of multiple empty impressions left behind after the *in situ* porogen leaching effect of the intercalated particles (Table 4.5). The pores formed by the dissolution of the PMMA particles measure between 2-3.6nm indicating a pore size distribution within the mesopore range (Sing, 1984; Groen *et al.*, 2003). In addition, such formulations exhibited slightly larger pore volumes and pore diameters compared to formulations containing less PMMA particles. Type II and IV isotherms were noted for formulations comprising 70:30 and 60:40 gum ratios, respectively, whereas F14 composed of an 80:20 polymer ratio and 0.75g/g PMMA particles featured a Type II isotherm while 0.25g/g PMMA particle intercalation presented a Type IV isotherm. The presentation of H3 and H4 hysteresis may suggest a varying slit-like ranging to a conical-like shape of the pores.

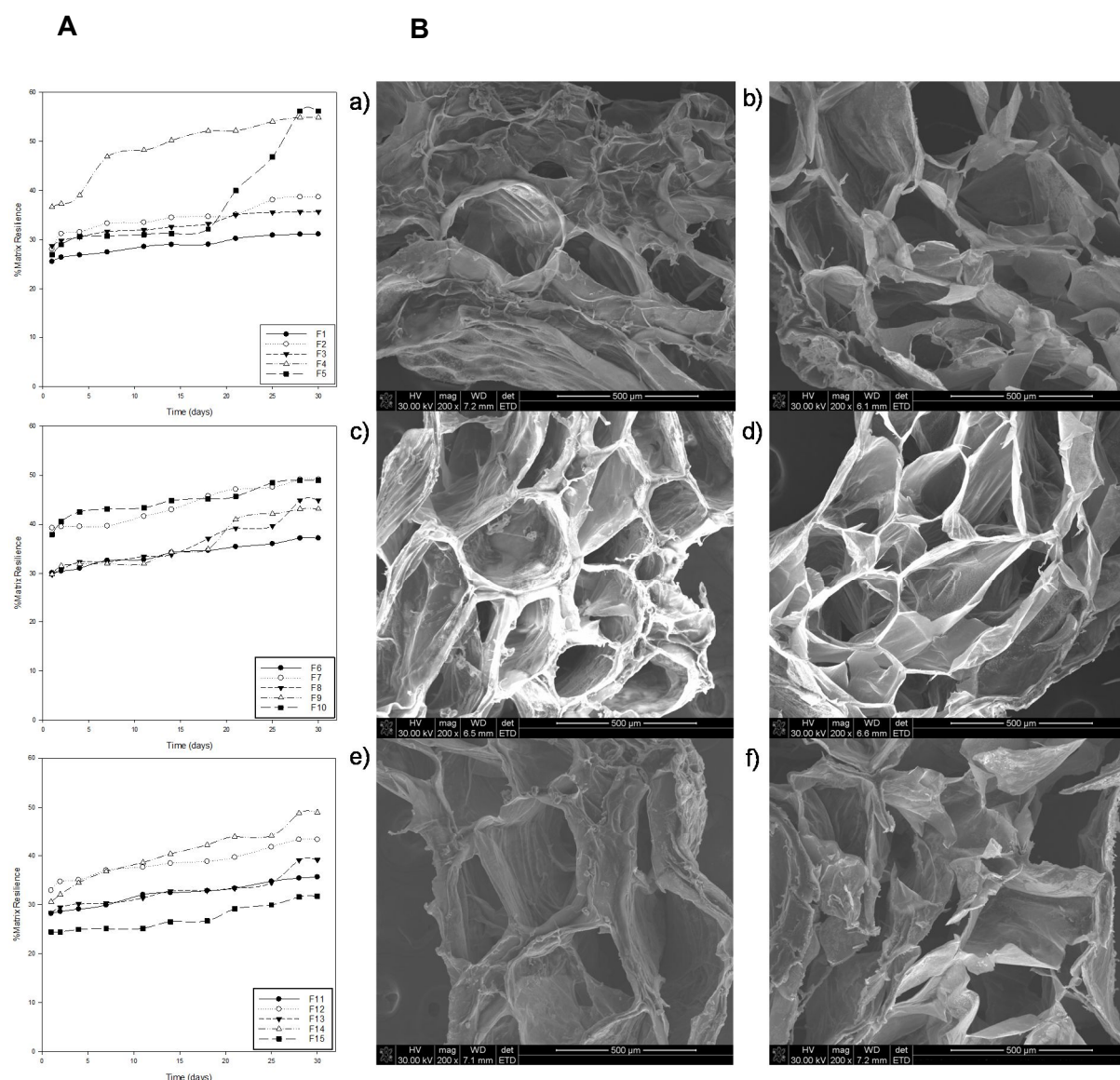


Figure 4.4: (A) Textural profiling matrix resilience analysis of the experimental formulations conducted over 30 days of dissolution in PBS pH 7.4 and (B) SEM cross-section imaging, at 200x magnification, of selected P4[Gel-Xan]IPN formulations for visualisation of morphological properties: 60:40 a) F9 and b) F11; 70:30 c) F5 and d) F15; 80:20 e) F14 and f) F8.

SEM cross-section morphology, shown in Figure 4.4, reveals the appearance of large honeycomb-like macroporous structures post-dissolution and lyophilisation of the hydrated conduits. The presence of this large porous network further enhances the resilience of the hydrogels and improves its water holding capacity.

The induction of porous structures is not only critical for optimal mechanical properties but moreover functions as reservoirs for the temporary accumulation of bioactive molecules in achieving controlled drug release. A porous matrix further allows the uninterrupted

physiological exchange of nutrient and waste products between the regenerating tissues confined within the interior environment of the implant (Garg and Goyal, 2014). Porosity enhances resilience which allows the scaffold to maintain its former architectural integrity when subjected to alternating compressive and decompressive forces acting on the scaffold from the movement of surrounding tissues at the implantation site, thereby making highly porous hydrogel scaffolds a favourable characteristic for tissue regeneration applications (Mastropietro *et al.*, 2012).

Table 4.5: Porosity analysis of 60:40 (F9 and F11); 70:30 (F5 and F15) and 80:20 (F14 and F8) P⁴[Gel-Xan]IPNs after 24 hours dissolution in PBS pH 7.4

Porositometric Parameters	0.75g/g PMMA Particles			0.25g/g PMMA Particles		
	F9	F5	F14	F11	F15	F8
BET Surface Area (m²/g)	4137.046	41.233	30.474	92.086	-	44.677
Micropore Area (m²/g)	4073.272	2.127	-	32.987	-	-
BET adsorption average pore width (4V/A) (nm)	3.600	2.722	3.554	1.542	-	2.998
BJH adsorption average pore diameter (4V/A) (nm)	4.690	5.340	5.230	4.670	4.478	5.016
BJH desorption average pore diameter (4V/A) (nm)	3.627	3.840	4.168	4.016	3.932	3.676

4.3.3. Response surface and statistical analysis of the Box-Behnken experimental design

Response analysis was performed of the following selected response outcomes after a 30-day period of *in vitro* studies: %swelling, %erosion, textural properties comprising %matrix resilience, deformation and fracture and drug release of BSA and diclofenac sodium based on mean dissolution time (MDT).

Increases in xanthan gum and CaCl₂ concentrations results in potentiated swelling via increased water-uptake whereas higher concentrations of gellan gum and PMMA particles results in an opposing effect on swelling ability of the conduits (Figure 4.5). Lower CaCl₂ concentrations in the range of 0.05-0.075%_{w/v} may assist in producing ideal swelling capacities with the consequential increase in mass being maintained between 500-1000%, after water absorption into the matrix. As shown in Figure 4.6, the investigated CaCl₂ concentrations between 0.05-0.1%_{w/v} result in steady erosion rates of the scaffolds with 67-70% of the conduit eroding at the end of 30 days. Similarly, the three polymer blend ratios produce steady erosion rates; however, gellan has a slight hindering effect on erosion with less than 65% matrix loss occurring after a 30-day period. The pH-responsive particulate leaching of the intercalated PMMA pristine particles leads to substantial increase in erosion.

The main affecters of matrix resilience, shown in Figure 4.7, were increasing PMMA particle and decreasing CaCl_2 concentrations. PMMA and CaCl_2 concentrations of 0.4g/g and 0.05%^{w/v}, respectively, are likely to produce scaffolds of at least 50% in resilience behaviour. Increase in CaCl_2 concentration from 0.05%^{w/v} to 0.1%^{w/v} results in a linear decline in resilience from 50% to 30%, attributed to formation of rigid polymer chains upon crosslinking. The maximum gellan gum concentration in the polymer blend, 80%^{w/w}, produces a slight enhancement in resilience by only 5%. Altering the ratios of gellan xanthan gum allows adjustment of the matrix to produce ideal deformation characteristics in the range of 1.2-1.5N/mm. Gellan and CaCl_2 concentrations between 70-80%^{w/w} and 0.05%^{w/v}, respectively, result in stiffer matrices, ascribed to decreased water-uptake and swelling, with deformation moduli exceeding 1.4N/mm after 30 days of dissolution (Figure 4.8). Increased CaCl_2 and decreased PMMA particle concentrations results in enhanced fracture energies (Figure 4.9) of the conduits signifying the requirement of higher amounts of energy for rupturing of the hydrated matrices.

The effects of the experimental variables on the release of BSA and diclofenac sodium varied in comparison to each other. Response analysis in Figure 4.10 showed that CaCl_2 concentrations hasten the release of BSA and diclofenac sodium whereas increases in xanthan gum and PMMA particle concentration promote slowed release of the model protein and drug. Although higher xanthan ratios were indicative of slowed diclofenac sodium release, analyses depict that combined lower CaCl_2 concentrations (0.05%^{w/v}) and an optimal PMMA concentration of 0.4g/g suggest a further enhanced reduction in the release rates, specifically of diclofenac sodium (Figure 4.11).

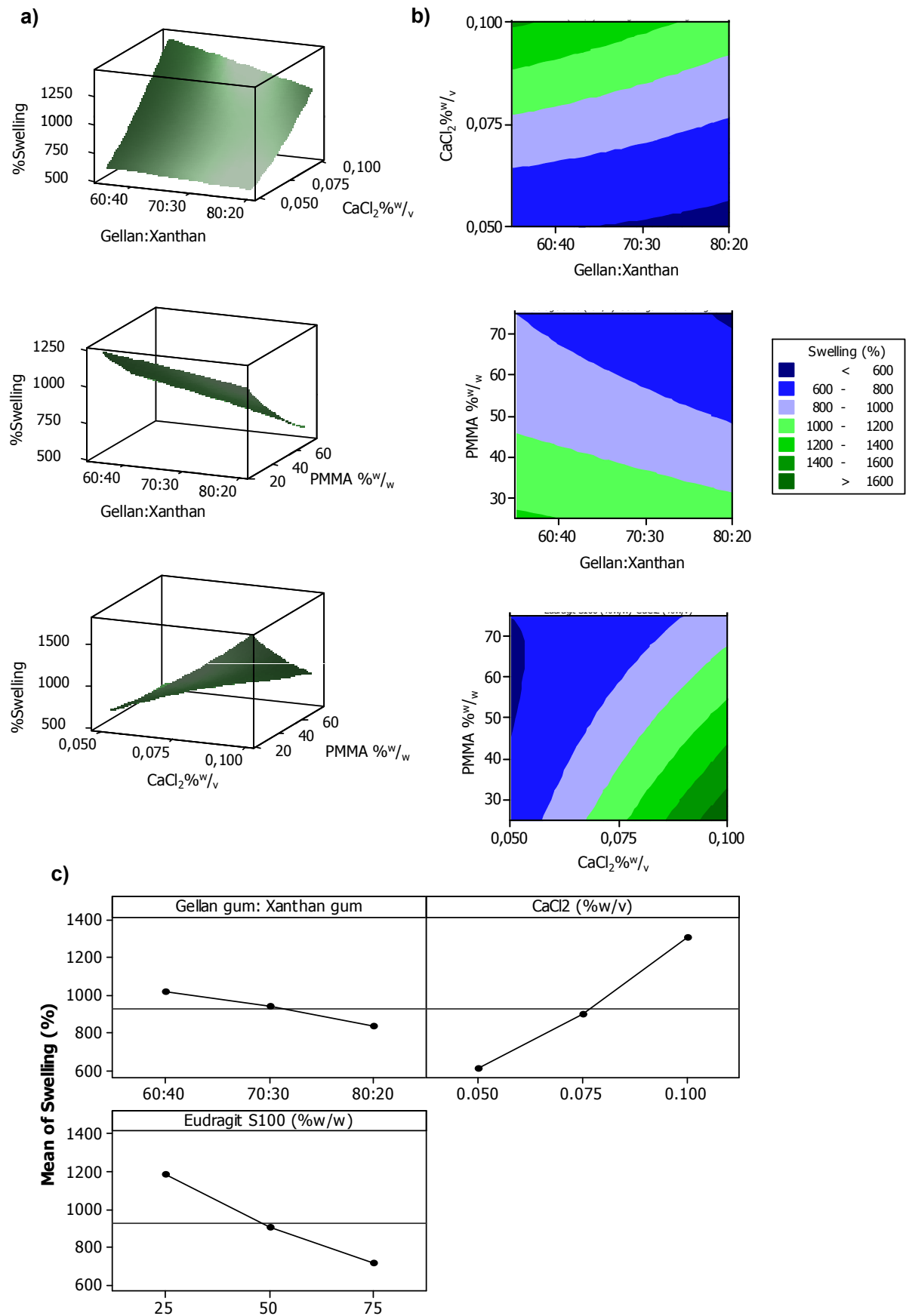


Figure 4.5: (a) Response surface, (b) contour and (c) main effects plots depicting the effects of gellan-xanthan ratios, CaCl_2 and PMMA concentrations on %swelling.

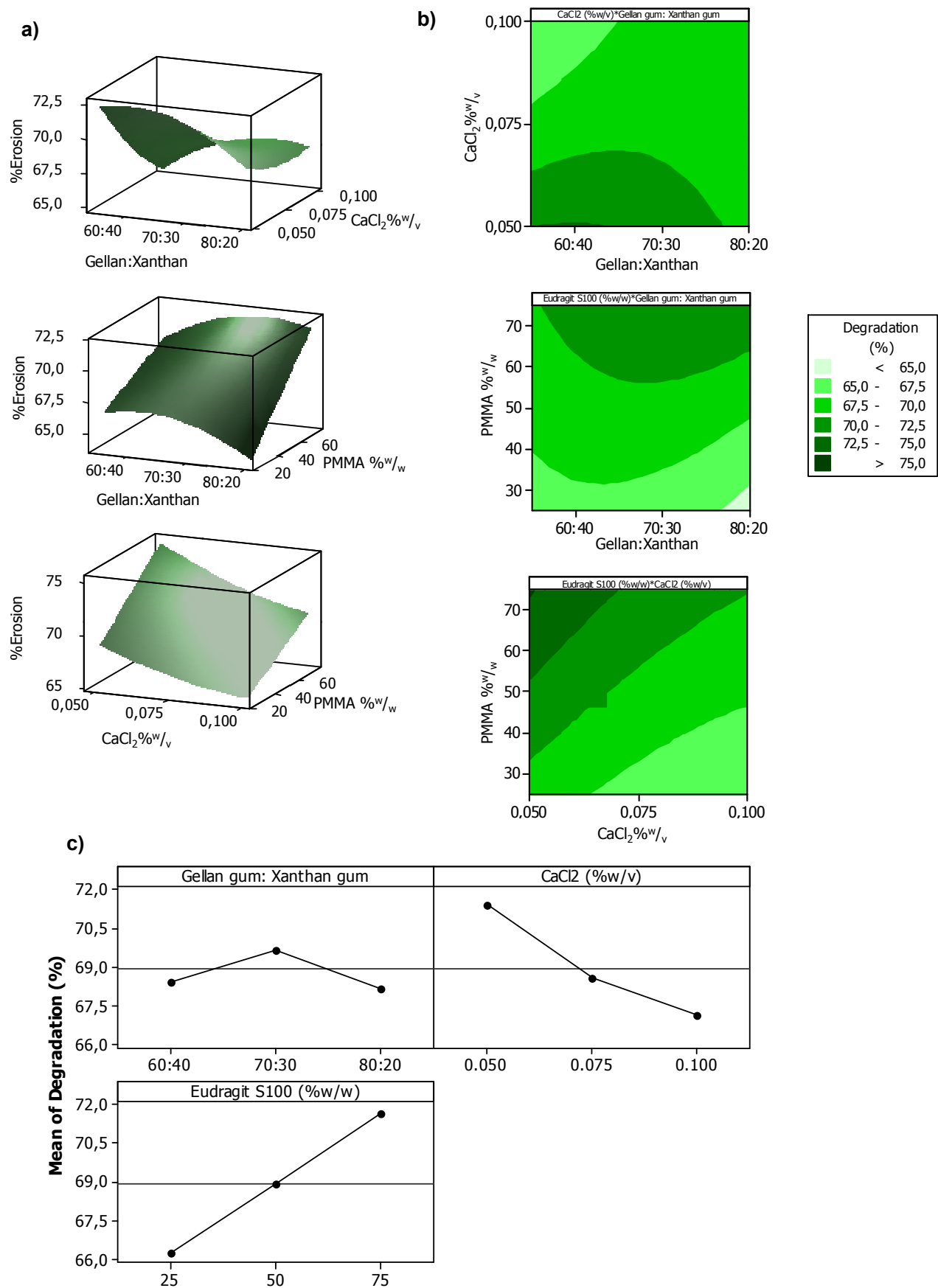


Figure 4.6: a) Response surface, b) contour and c) main effects plots depicting the effects of gellan-xanthan ratios, CaCl_2 and PMMA concentrations on %erosion.

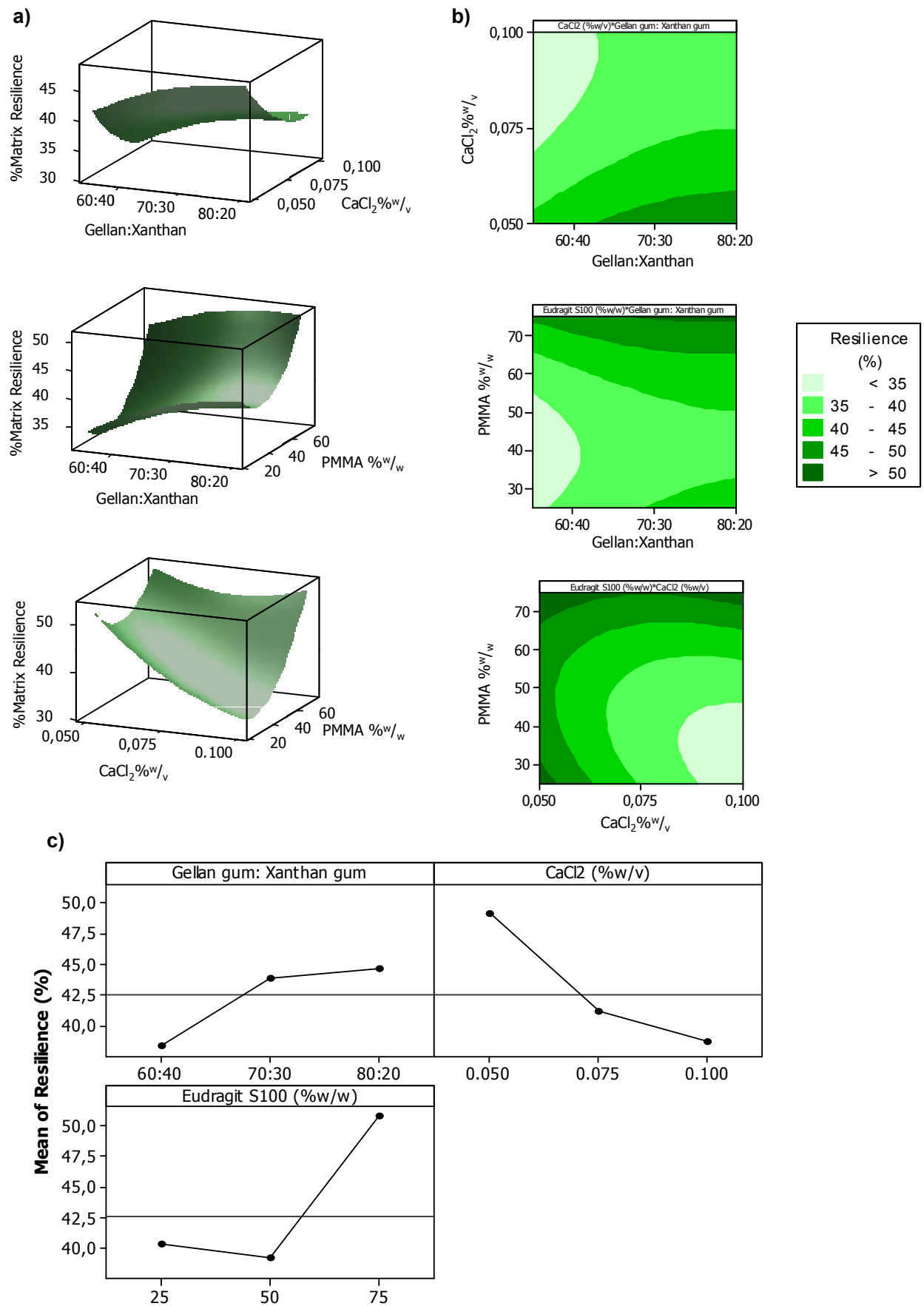


Figure 4.7: a) Response surface, b) contour and c) main effects plots depicting the effects of gellan-xanthan ratios, CaCl_2 and PMMA concentrations on %matrix resilience.

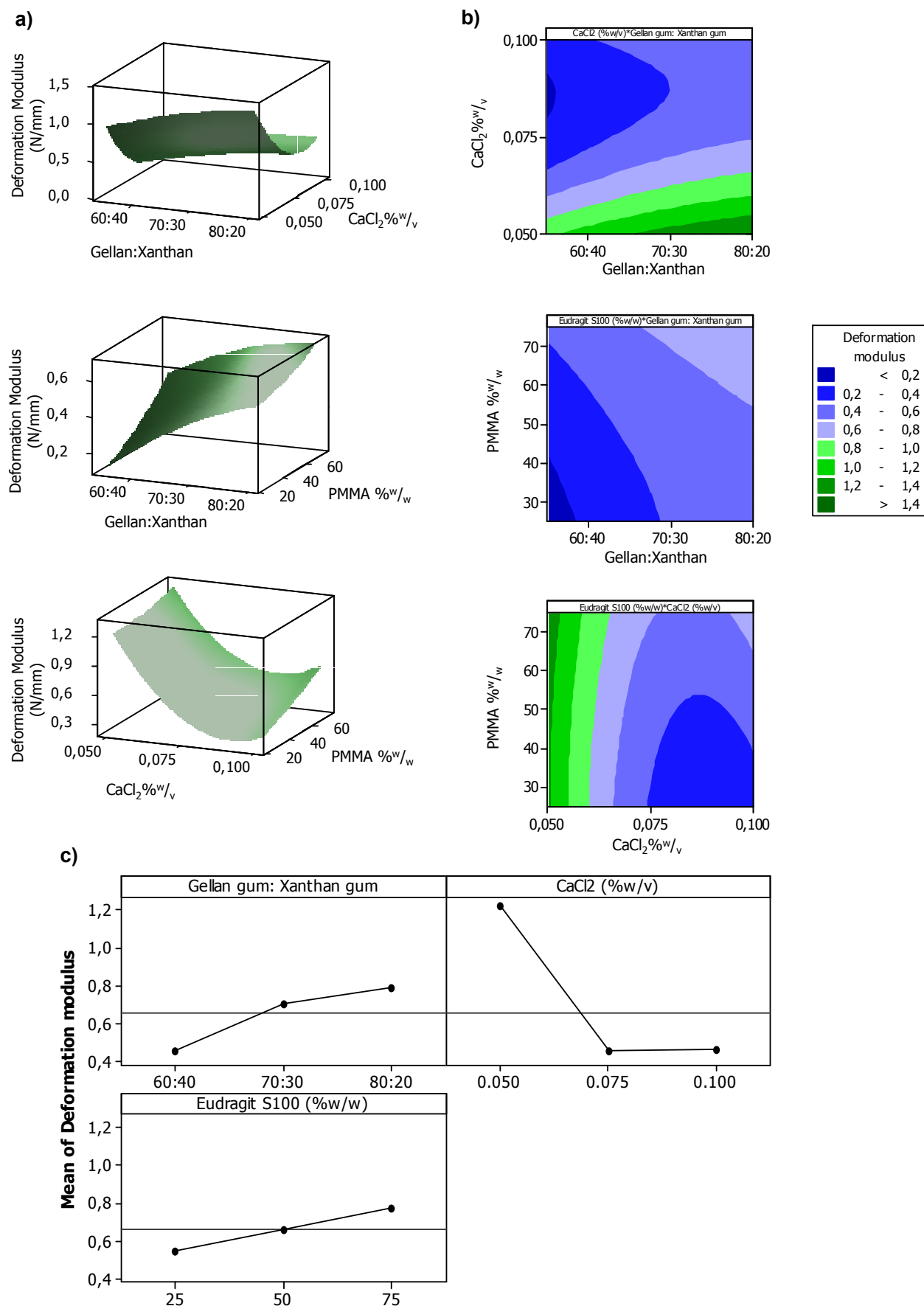


Figure 4.8: a) Response surface, b) contour and c) main effects plots depicting the effects of gellan-xanthan ratios, CaCl_2 and PMMA concentrations on the deformation modulus.

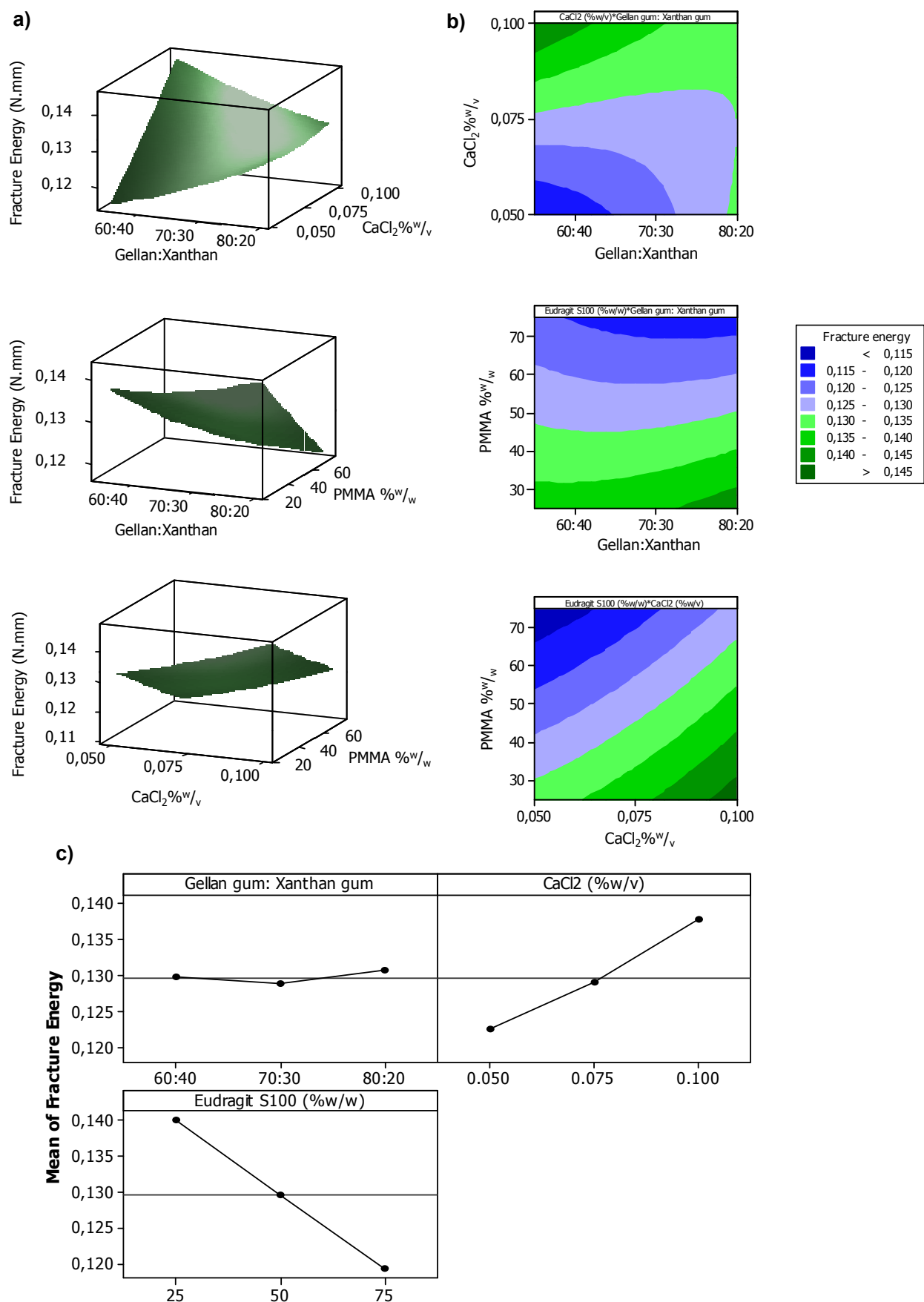


Figure 4.9: a) Response surface, b) contour and c) main effects plots depicting the effects of gellan-xanthan ratios, CaCl₂ and PMMA concentrations on the fracture energy.

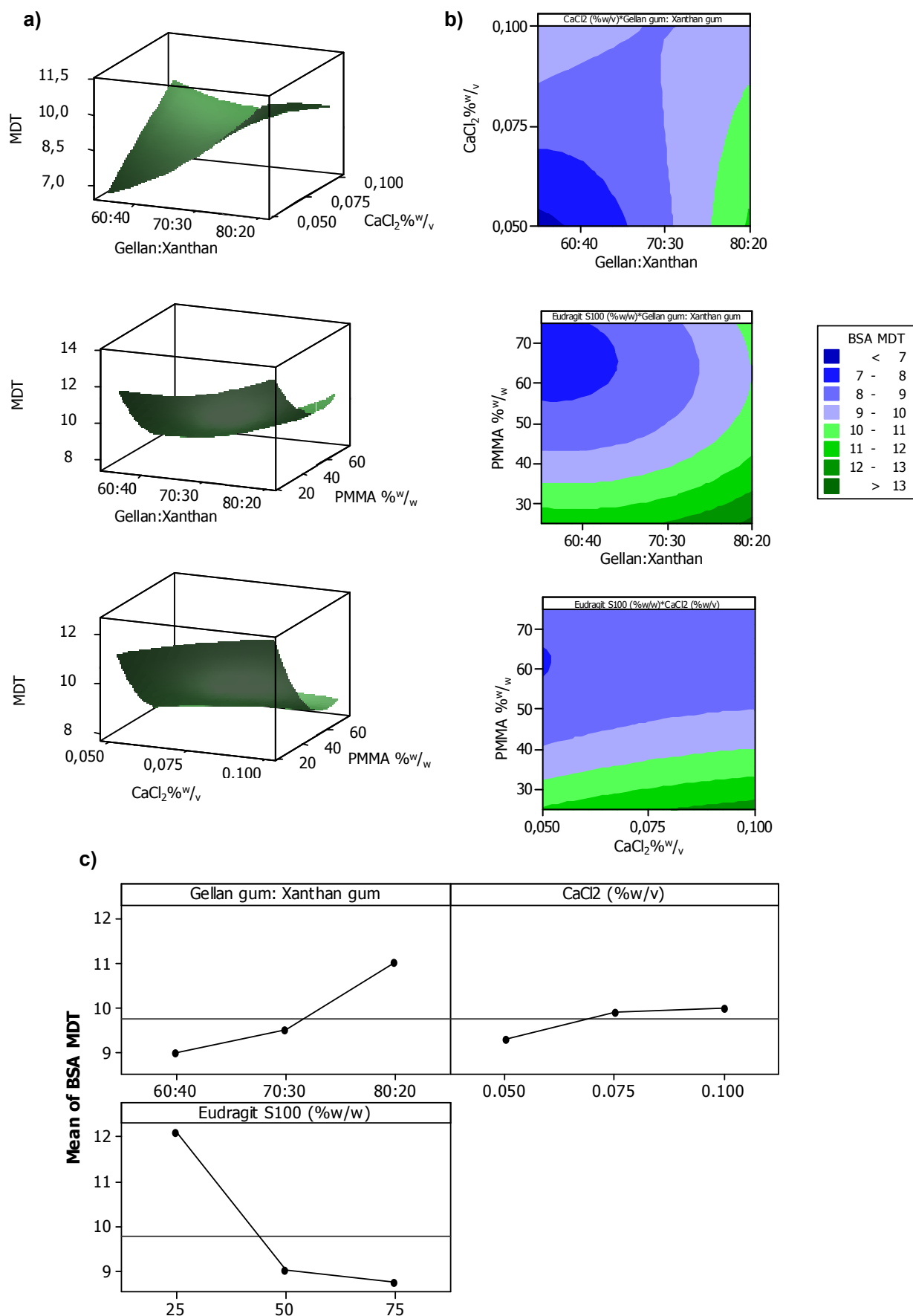


Figure 4.10: a) Response surface, b) contour and c) main effects plots depicting the effects of gellan-xanthan ratios, CaCl₂ and PMMA concentrations on BSA MDT.

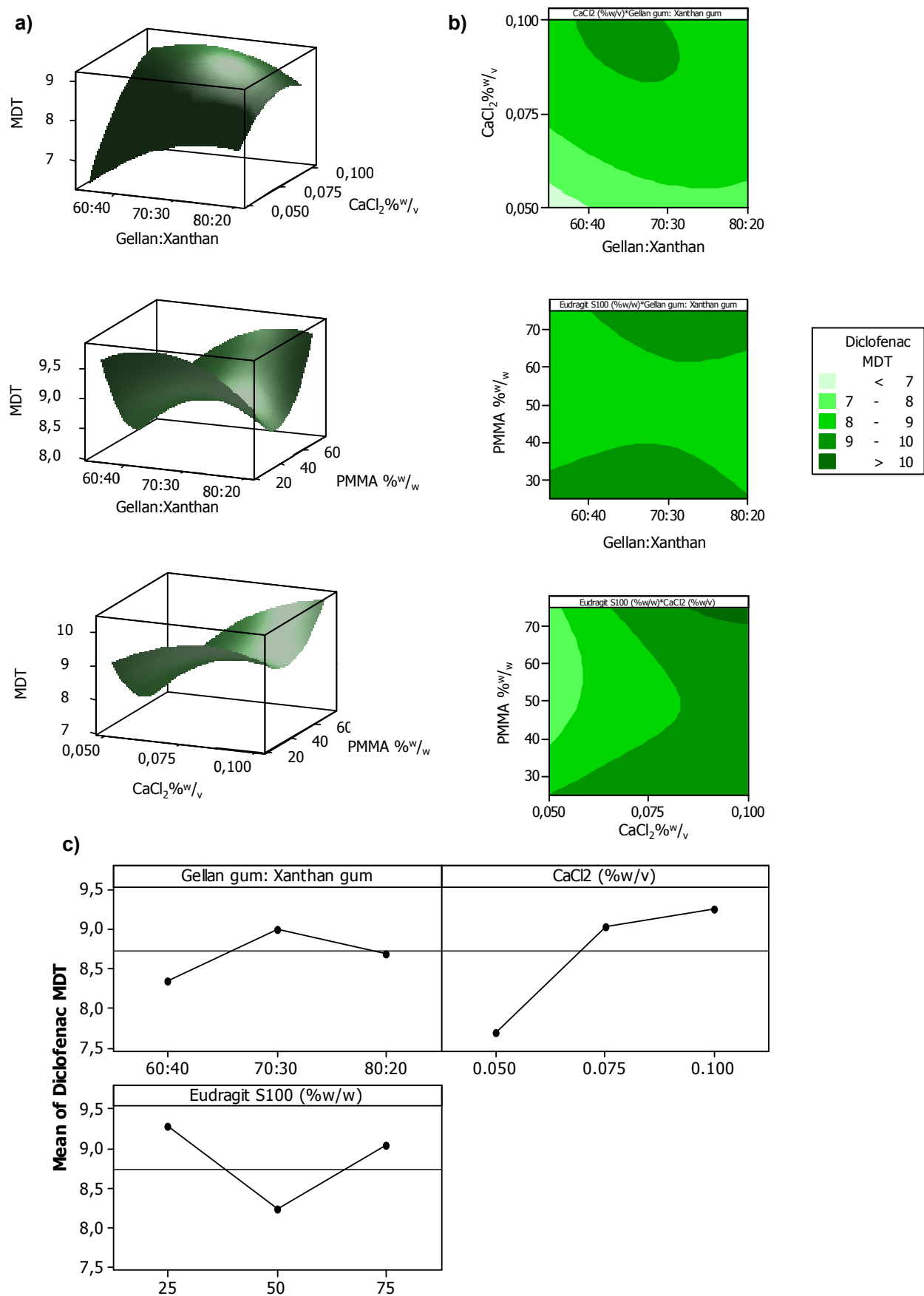


Figure 4.11: a) Response surface, b) contour and c) main effects plots depicting the effects of gellan-xanthan ratios, CaCl₂ and PMMA concentrations on diclofenac sodium MDT.

A complete ANOVA analysis (presented in Table 4.6) was further conducted on the selected experimental response outcomes of %swelling, %erosion, %matrix resilience, deformation moduli, fracture energies and bioactive release represented as BSA and diclofenac MDTs to determine the correlation between the response variables and the predictor variables. A p-value ≤ 0.05 indicates a significant effect between a response factor and the corresponding variable. The p-values highlighted in Table 4.6 indicate a significant effect of CaCl_2 and PMMA concentrations on %swelling ($\text{CaCl}_2 \times \text{PMMA}$, $p = 0.014$) and %matrix resilience (CaCl_2 , $p = 0.043$; PMMA, $p = 0.021$; PMMA $\times$ PMMA, $p = 0.016$; $\text{CaCl}_2 \times \text{PMMA}$, $p = 0.044$) whereas CaCl_2 , solely, had a significant effect on deformation moduli (CaCl_2 , $p = 0.013$; $\text{CaCl}_2 \times \text{CaCl}_2$, $p = 0.015$) of the hydrogel formulations. The collective concentrations of gellan, xanthan and CaCl_2 exhibited notable effects on the fracture energies (Gellan $\times$ Xanthan $\times$ CaCl_2 , $p = 0.028$) of the formulations. The response factors of %erosion, BSA MDT and diclofenac sodium MDT presented no significant effects with regards to the selected variable factors.

Additional statistical evaluation of the experimental model was acquired via the analysis of residual plots (Figure 4.12) where uniformity and distribution of the obtained data points were assessed. The normal probability plots of the residuals concerning the %swelling, %erosion, %matrix resilience, deformation modulus, BSA MDT and diclofenac sodium MDT present strong linear arrangement of the points indicating normal distribution whereas that of fracture energy deviated from the line with clustering of the points towards a linear arrangement thus indicating the potential influence of an external unaccounted variable. The residuals versus fitted plots of the 7 aforementioned response variables present random scattering of the lack of clustering of the points on either side of the zero line therefore suggesting the absence of trends. The histograms pertaining to the effects of BSA MDT, deformation modulus and %matrix resilience depict bimodal and symmetrical distribution of the residuals thus indicating probability of a uniform distribution of the acquired dataset with non-random error as noted from the evenly distributed points in the plots representative of the relationship between the residual and observation order. The histograms relating to %swelling, %erosion, fracture energy and diclofenac sodium MDT lacked symmetrical distribution denoting the occurrence of a random error of the dataset as further indicated by the declined even distribution of the data points along the zero line of the observation order plots.

Table 4.6: ANOVA analysis for the measured responses.

Response Variables	p-values						
	%Swelling	%Erosion	%Matrix Resilience	Deformation Modulus	Fracture Energy	BSA MDT	Diclofenac Sodium MDT
Constant	0.579	0.000	0.011	0.044	0.061	0.433	0.507
Gellan:Xanthan	0.627	0.767	0.253	0.334	0.206	0.675	0.383
CaCl ₂	0.215	0.112	0.043	0.013	0.373	0.338	0.284
PMMA	0.511	0.680	0.021	0.865	0.650	0.088	0.065
Gellan:Xanthan*	0.915	0.100	0.405	0.649	0.569	0.375	0.387
Gellan:Xanthan CaCl ₂ *CaCl ₂	0.334	0.384	0.144	0.015	0.633	0.855	0.322
PMMA*PMMA	0.424	0.967	0.016	0.810	0.923	0.068	0.134
Gellan:Xanthan*CaCl ₂	0.298	0.060	0.854	0.610	0.028	0.095	0.388
Gellan:Xanthan*PMMA	0.713	0.135	0.638	0.708	0.385	0.699	0.379
CaCl ₂ *PMMA	0.014	0.547	0.044	0.544	1.000	0.626	0.306

The complete regression equations regenerated for the responses of %swelling, %erosion, %matrix resilience, deformation modulus, fracture energy, BSA MDT and diclofenac sodium MDT are presented as follows:

$$\%Swelling = 559 - 73.1[Gellan:Xanthan] + 13836[CaCl_2] - 9.32 [PMMA]$$

Equation 4.6

$$\% Erosion = 70.3 - 0.112[Gellan:Xanthan] - 85.7[CaCl_2] + 0.108[PMMA]$$

Equation 4.7

$$\%Matrix Resilience = 40.7 + 2.55[Gellan:Xanthan] - 207[CaCl_2] + 0.209[PMMA]$$

Equation 4.8

$$Deformation \ Modulus \ (N/mm) = 1.20 + 0.135[Gellan:Xanthan] - 15.2[CaCl_2] + 0.00466[PMMA]$$

Equation 4.9

$$Fracture \ Energy \ (N.mm) = 0.126 + 0.00040[Gellan:Xanthan] + 0.305[CaCl_2] - 0.000415[PMMA]$$

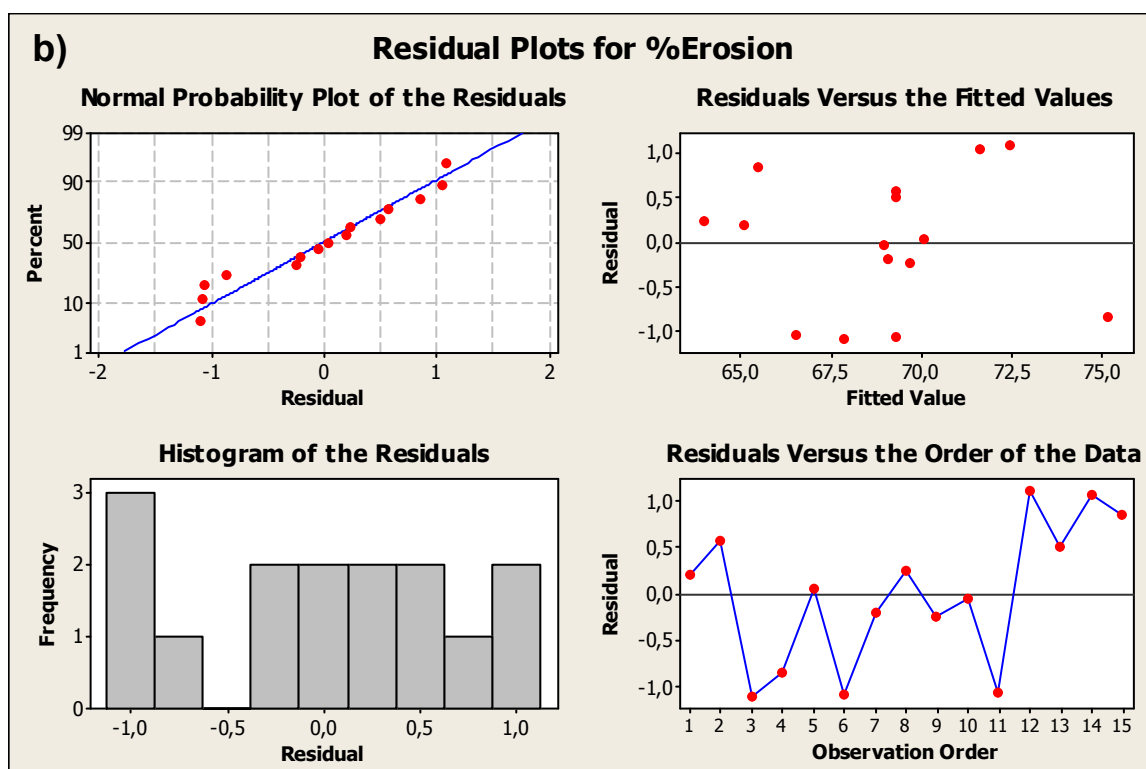
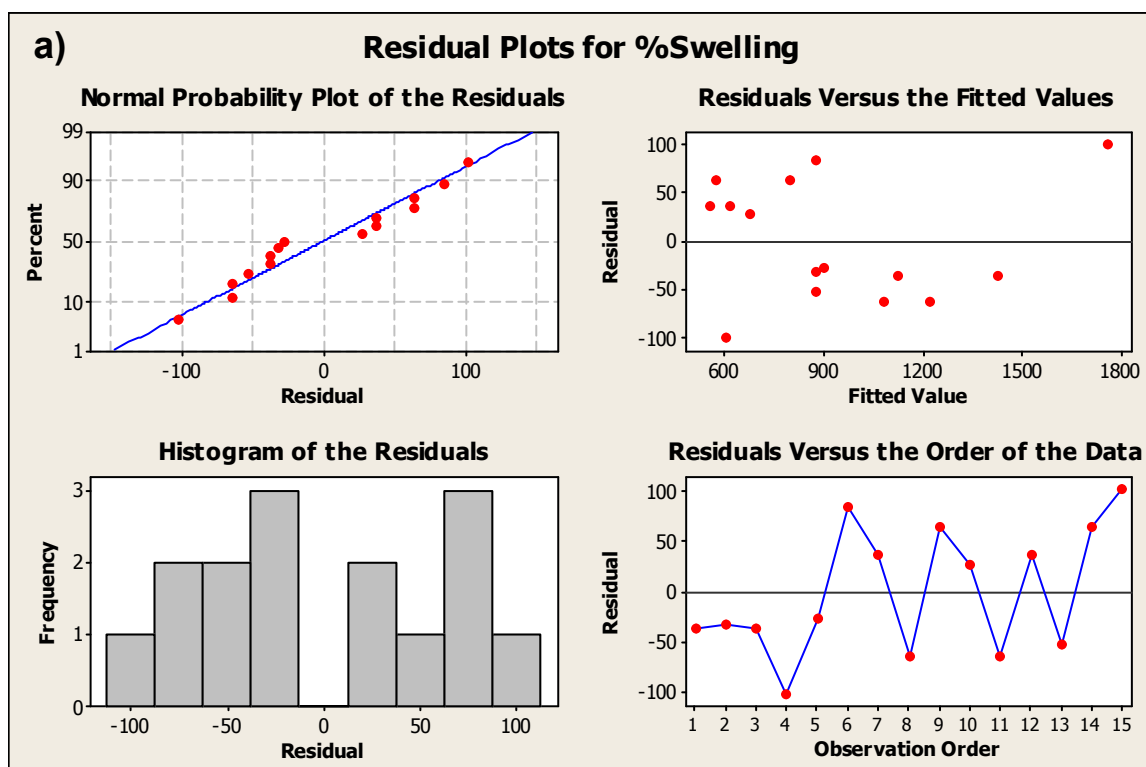
Equation 4.10

$$BSA \ (MDT) = 9.86 + 0.809[Gellan:Xanthan] + 14.1[CaCl_2] - 0.0672[PMMA]$$

Equation 4.11

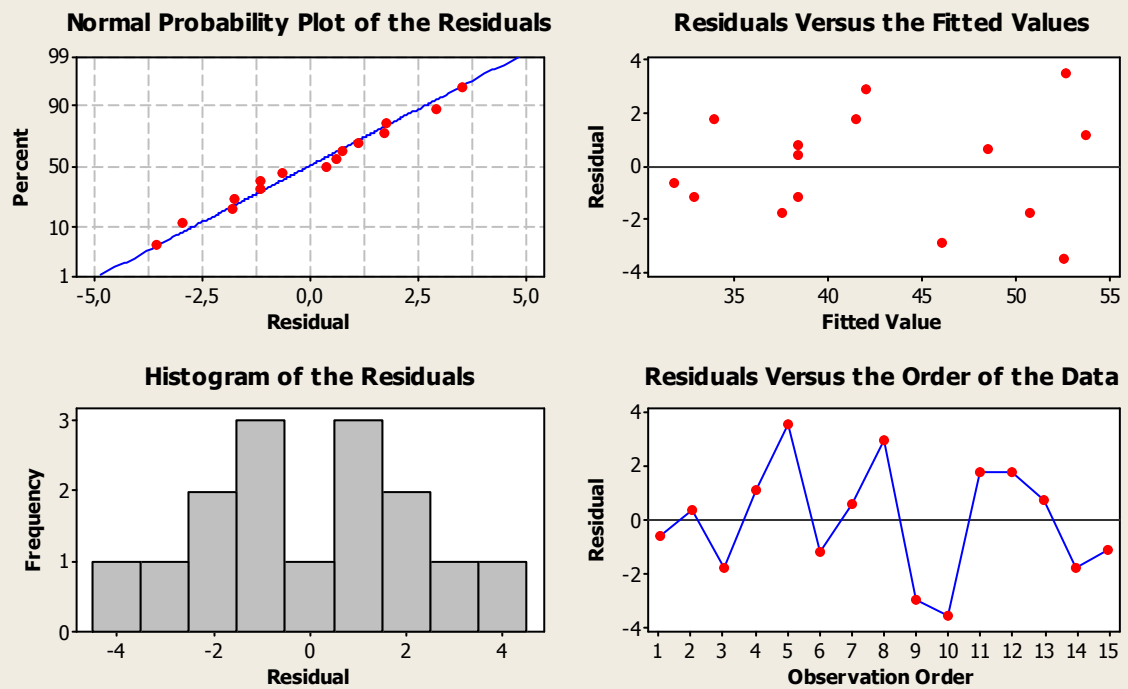
$$\text{Diclofenac Sodium (MDT)} = 6.26 + 0.139[\text{Gellan:Xanthan}] + 31.2[\text{CaCl}_2] - 0.0050[\text{PMMA}]$$

Equation 4.12



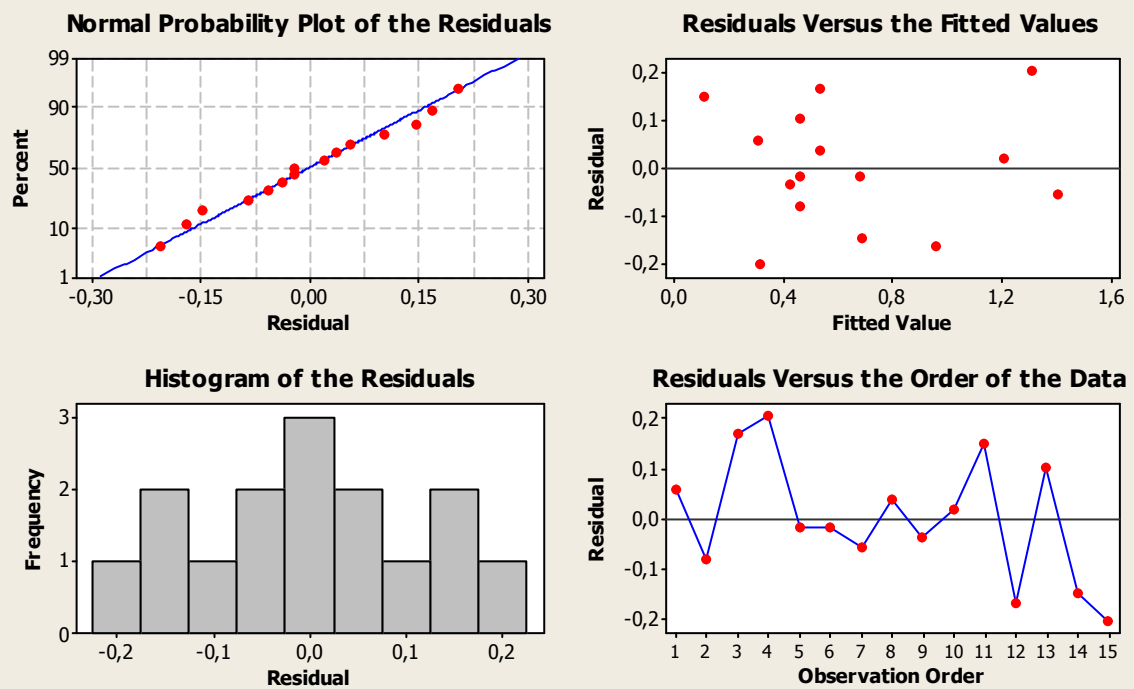
c)

Residual Plots for %Matrix Resilience



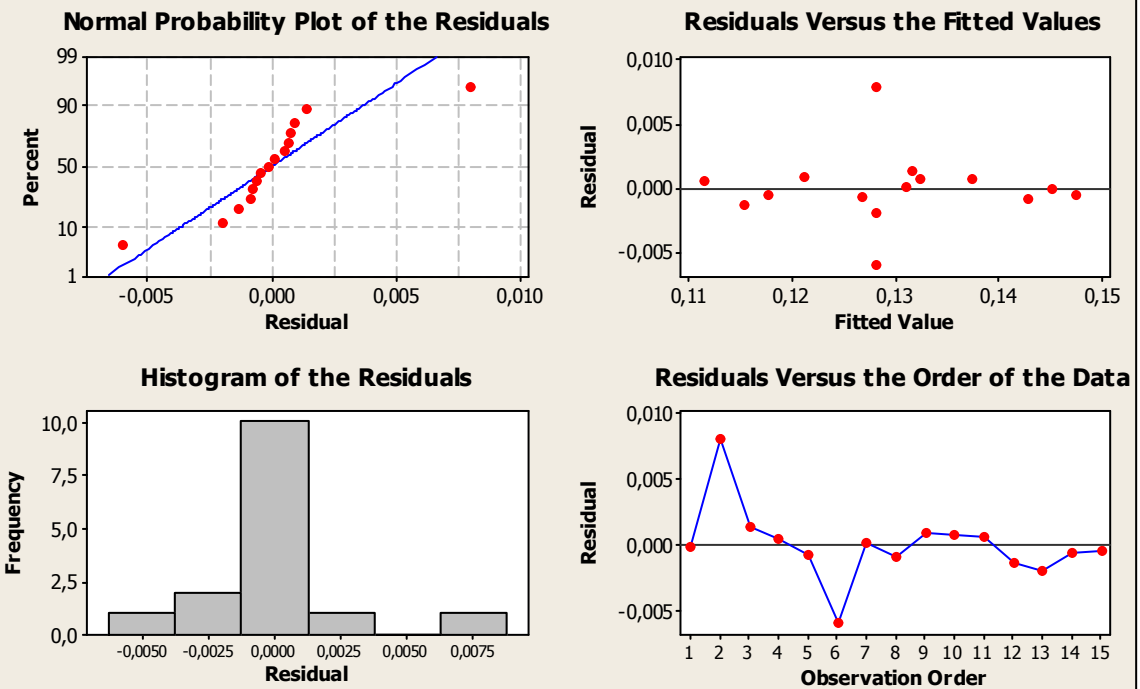
d)

Residual Plots for Deformation Modulus



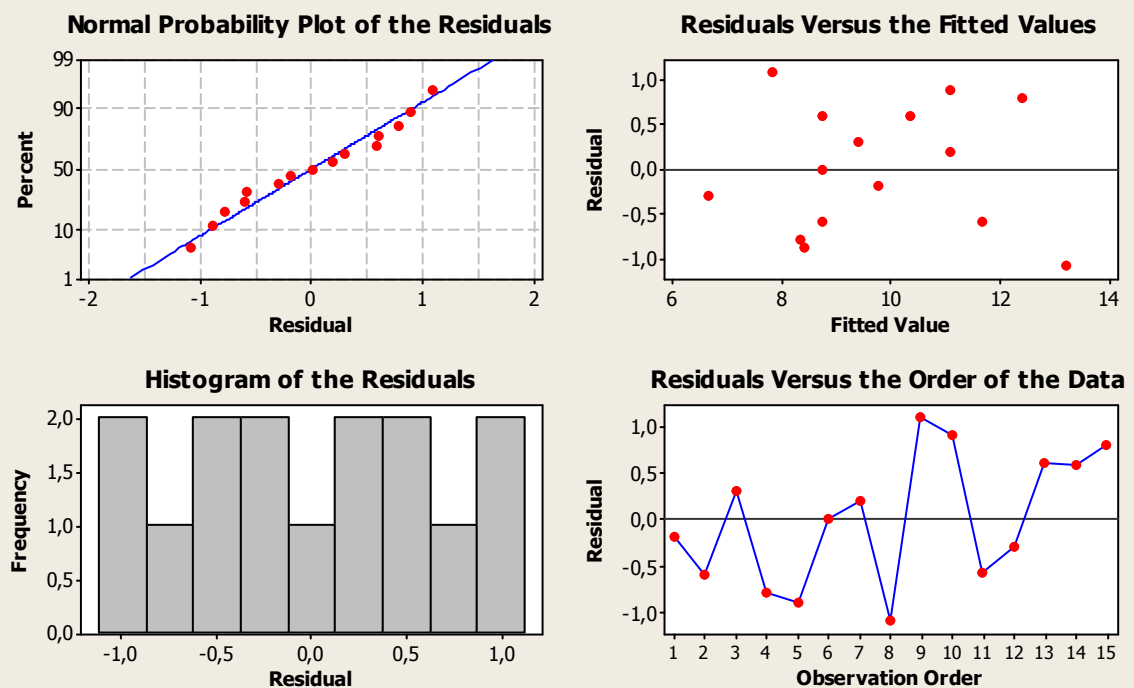
e)

Residual Plots for Fracture Energy



f)

Residual Plots for BSA MDT



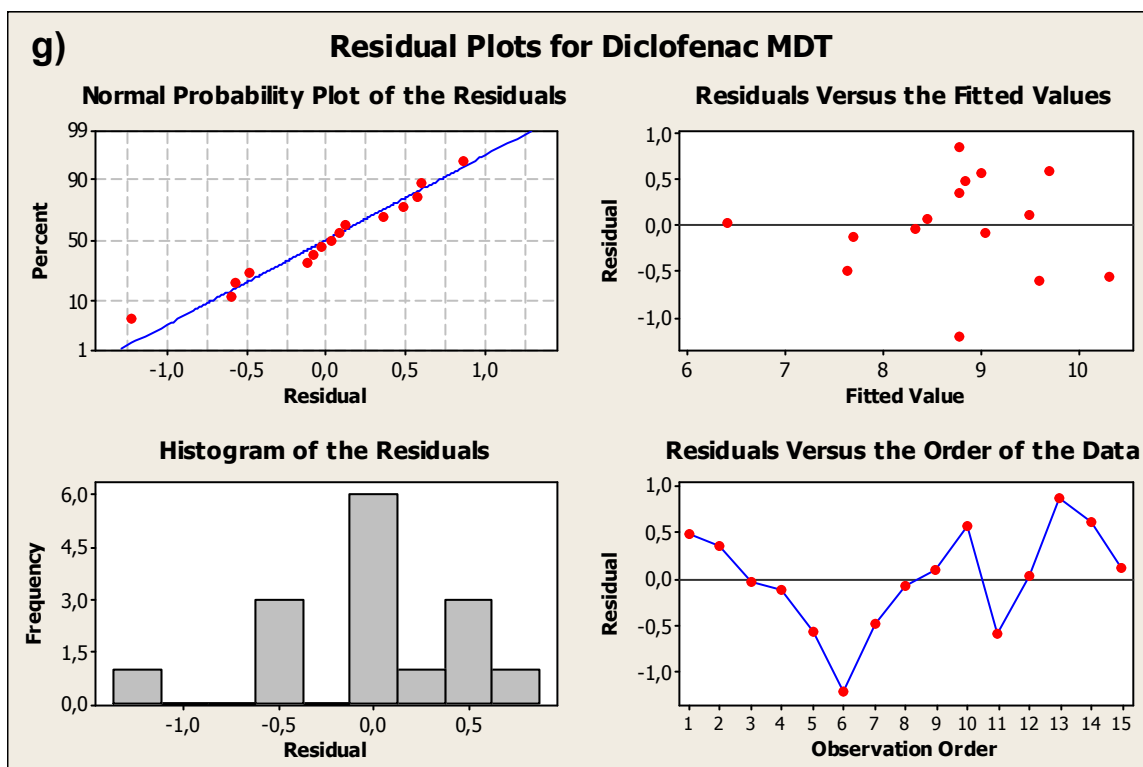


Figure 4.12: Residual plots for the responses a) %swelling b) %erosion c) %matrix resilience d) deformability modulus e) fracture energy f) BSA MDT and g) diclofenac sodium MDT.

4.3.4. The effect of hydrogel polymer composition on the hydrodynamic and mechanical properties of the matrix *in vitro*

Swelling and erosion studies provide insight into the dominant drug release mechanism and the resultant changes in mechanical properties by determining the degree of water-uptake into the matrix network. The overall change in size of the system (Figure 4.13) induced by swelling is an important factor to consider for implantable scaffolds, including its erosion properties, as these characteristics affect the mechanical strength of the implant and the *in vivo* performance. The presented data in Figure 4.13 shows the percentage swelling and erosion over a period of time for the different gellan-xanthan gum hydrogel formulations containing varying quantities of pristine PMMA particles and crosslinker. All polysaccharide polymers are hydrophilic materials with great propensity for swelling via their water-absorbing properties (Coviello *et al.*, 2006). The gradual absorption of water leads to various changes in the polymer network encompassing processes of hydration, conversion of the matrix from glassy to rubbery states, polymer chain relaxation and disentanglement eventually leading to disintegration. These intrinsic factors and the accumulation of water molecules amid the polymer chains create changes in the mechanical properties of the matrices over time. From the textural profiles obtained, it is noted that the ratios of

gellan:xanthan gum and the crosslinker concentration influence mainly deformation and fracture energy of the hydrogel matrices whereas pristine PMMA particle concentration largely affected matrix resilience properties. The concentrations of gellan, xanthan, PMMA particles and CaCl_2 affects the water-absorptive capacity of the hydrogel networks due to the various types of crosslinks formed and the unique gelling properties of the polysaccharide gums. Increase in pristine PMMA particle content reduces the swelling capacity of the polymer matrix due to the formation of pores leading to the potential continuous inflow and outflow of water through the pores. Swelling was enhanced with increased concentrations of CaCl_2 crosslinker in the gellan-xanthan formulations, which further increased the size of the hydrated conduit in terms wall thickness. The intermolecular and intramolecular calcium-carboxylate complexes formed between gellan-xanthan, gellan-gellan and xanthan-xanthan chains upon crosslinking promote enhanced holding of the network thereby increasing the hydrogel's water-holding capacity. Upon *in vitro* swelling and gap enlargement between the polymer chains forming the matrix network, the previously crosslinked bivalent calcium ions may escape being replaced with the weaker monovalent ions, potassium and sodium, from the dissolution media thereby unrestricting its water-uptake ability due to weak electrostatic shielding causing greater repulsion between the anionic carboxylates on the polymer side chains. Furthermore, it may be possible that an excessive concentration of CaCl_2 disrupts the structured conformation of specifically gellan gum leading to a comprised network structure (Tang *et al.*, 2001) thus permitting unhindered water penetration.

Likewise, formulations containing higher xanthan gum ratios underwent greater swelling with less erosion occurring over time. This is due to the inherent swelling and erosion characteristics of the individual polymers. Although some xanthan chains may be partially interlaced with those of gellan forming intermolecular gellan-xanthan crosslinks, the xanthan chains forming weak intramolecular xanthan-xanthan crosslinks dissociate and absorb water to initiate swelling. The hydrogen-bond dissociation and slippage of small calcium ions from xanthan chains may lead to the lack of electrostatic shielding causing increased repulsion between negatively charged pyruvate and glucuronate moieties resulting in polymer chain expansion as water-uptake increases.

It is noted that the enhanced swelling associated with increase in CaCl_2 concentration and the xanthan gum ratio produced hydrogels of lower resilience and deformation but with higher fracture energies as shown in Figure 4.14. This suggests that increased water-uptake and swelling resulting from accumulation of entrapped water within the matrix decreases matrix resilience and deformation while increasing fracture energies. This may be attributed to the changes occurring in mainly xanthan gum during water-absorption and swelling.

Xanthan gum does not form rigid 3D gels as gellan gum does, instead it undergoes changes in viscosity modulated by temperature and ionic strength of the gel solution. Increasing crosslinker concentrations only thicken the gel solution inhibiting its flow properties. Further penetration of water and potential loss of calcium ions from the single xanthan helices and chains initiates the change of the gel from a viscous to a more fluid state. As a result, matrix resilience is lowered due to filling of pores with xanthan gel and excess entrapped water. The hydrogel network in its swollen state is more flexible due to the viscous gel-sol state of xanthan gum according to the lowered deformation values, however, since xanthan gum is pseudoplastic (Pongjanyakul and Puttipipatkachorn, 2007; Dário et al., 2011) in nature it flows upon application of stress via a transient decrease in viscosity thus requiring higher energies to slightly deform in order to flow but not actually causing rupturing of the gel system. In contrast, increased gellan gum ratios inhibited water-uptake and swelling in addition to improving resilience properties. However, such formulations were stiff as presented by the higher deformation values due to the instantaneous formation of a solid gel-structure via establishment of junction zones when crosslinked with mono- and divalent ions at temperatures above 90°C. Compared to xanthan, gellan required less energy to initiate change in shape but it easily reverts to its original shape upon compression and decompression forces reflected by its enhanced resilience. However, it contributed towards the formation of a stiffer and less flexible system producing brittle matrices that were easily broken as indicated by the low fracture energies required to initiate rupture.

It was further noted that formulations containing higher ratios of gellan gum and calcium chloride, 4 parts gellan to 1 part xanthan and 0.1%^{w/v} crosslinker, displayed high fracture energies and low matrix flexibility due to the formation of a rigid gel network following conformation of an orderly molecular structure upon crosslinking. Increased gellan and calcium quantities produce brittle hydrogel matrices which crack upon application of a force due to resistance of the force rather than distributing the applied energy throughout the matrix to undergo plastic deformation. Formulations 1 and 15 undergoing the greatest degree of swelling (absorbing a maximum of 0.312mL and 0.428mL of water, respectively) were least resilient and the deformation modulus and fracture energies indicated a brittle matrix, particularly F15 which was observed to crack during compression from the textural probe. F1 was least prone to cracking compared to F15 due to its higher ratio of xanthan gum however; both formulations contained the maximum crosslinker concentration. Xanthan modifies the hardness of the hydrogel matrix offering a method for achieving a fine balance between flexibility and rigidity.

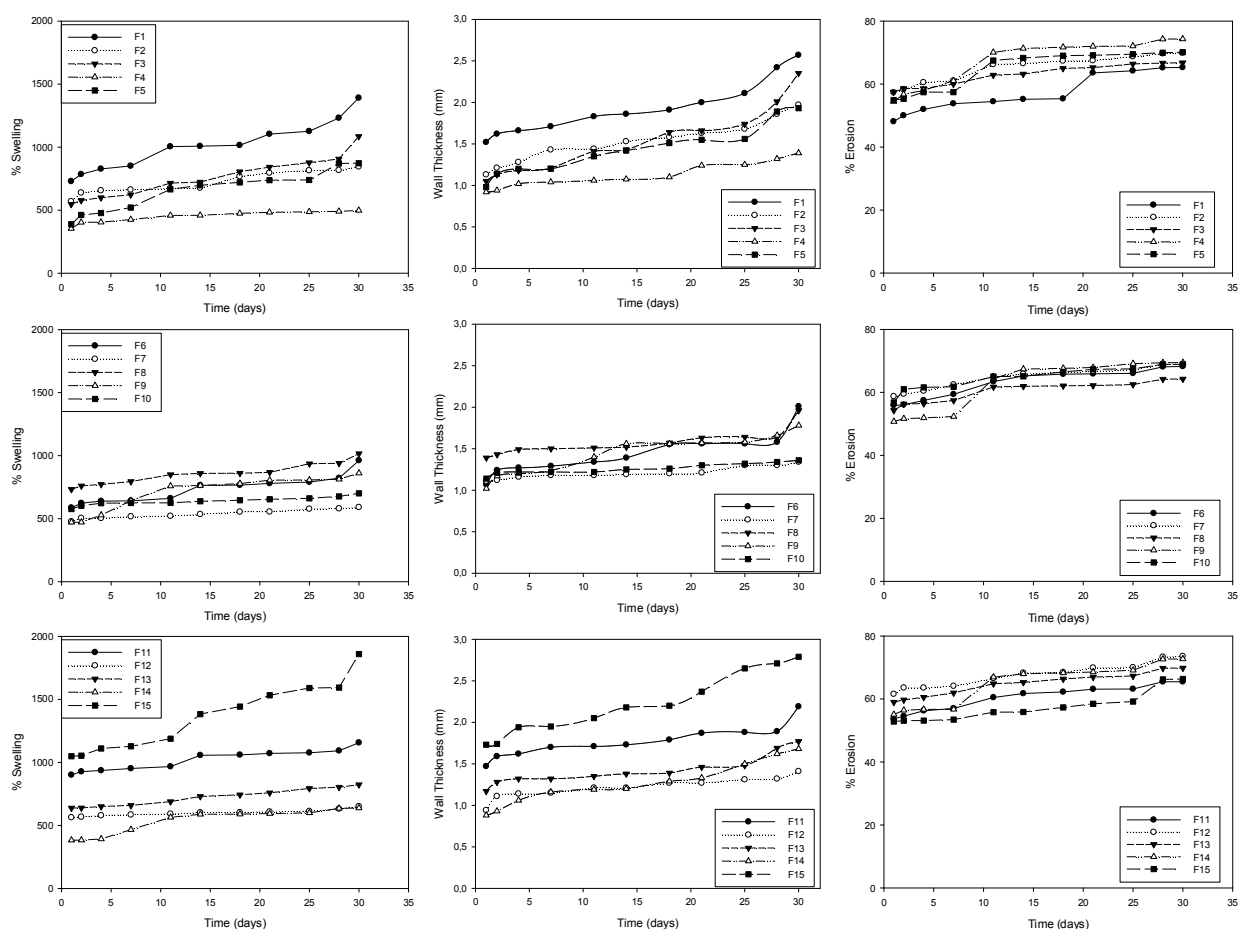


Figure 4.13: Water-uptake properties of Gel-Xan IPNs showing %swelling (top), wall thickness of the conduits (middle) and %erosion (bottom) over a study period of 30 days in PBS pH 7.4.

Erosion of the polymer matrix decreases with increased CaCl_2 crosslinker concentrations due to the formation of rigid crosslinks in certain areas of the hydrogel however the inclusion of pristine polymer particles had opposing effects on the erosion properties of matrices. The erosion rates of the gellan-xanthan gum matrices appeared steady; however, higher pristine polymer particle content increased the initial and final stages of erosion. The observed erosion characteristics may be attributed to the differences in ionic charge of the individual polymers and the ionic interactions taking place between the polysaccharide matrix and the intercalated pristine PMMA particles at pH 7.4. With both the gellan-xanthan combination and PMMA being anionic polymers, ionic attraction interactions are not possible and repulsion occurs between the similarly charged compounds. The actions of repulsion and further influx of water into the gellan-xanthan matrices results in the dissolution and subsequent release of a large number of PMMA particles thereby contributing to the hastened erosion rate signified by mass loss of the matrix. F4, F5, F12 and F14 exhibited the highest erosion rates and resilience due to the loss of PMMA particles from the matrix resulting in less polymer material remaining at the various sampling points. Subsequent to

erosion and loss of polymer mass, the lessened quantity of polymer matrix remaining is unable to absorb further water and undergo swelling compared to the slower eroding blends containing less PMMA particles, hence the stabilizing swelling profiles. As erosion continues, deformation and fracture energy decrease as the matrix loses bulk weight and weakens, thus indicating loss of rigidity of the network. A weakening matrix network requires less energy to cause a break in the system hence the declining fracture energies.

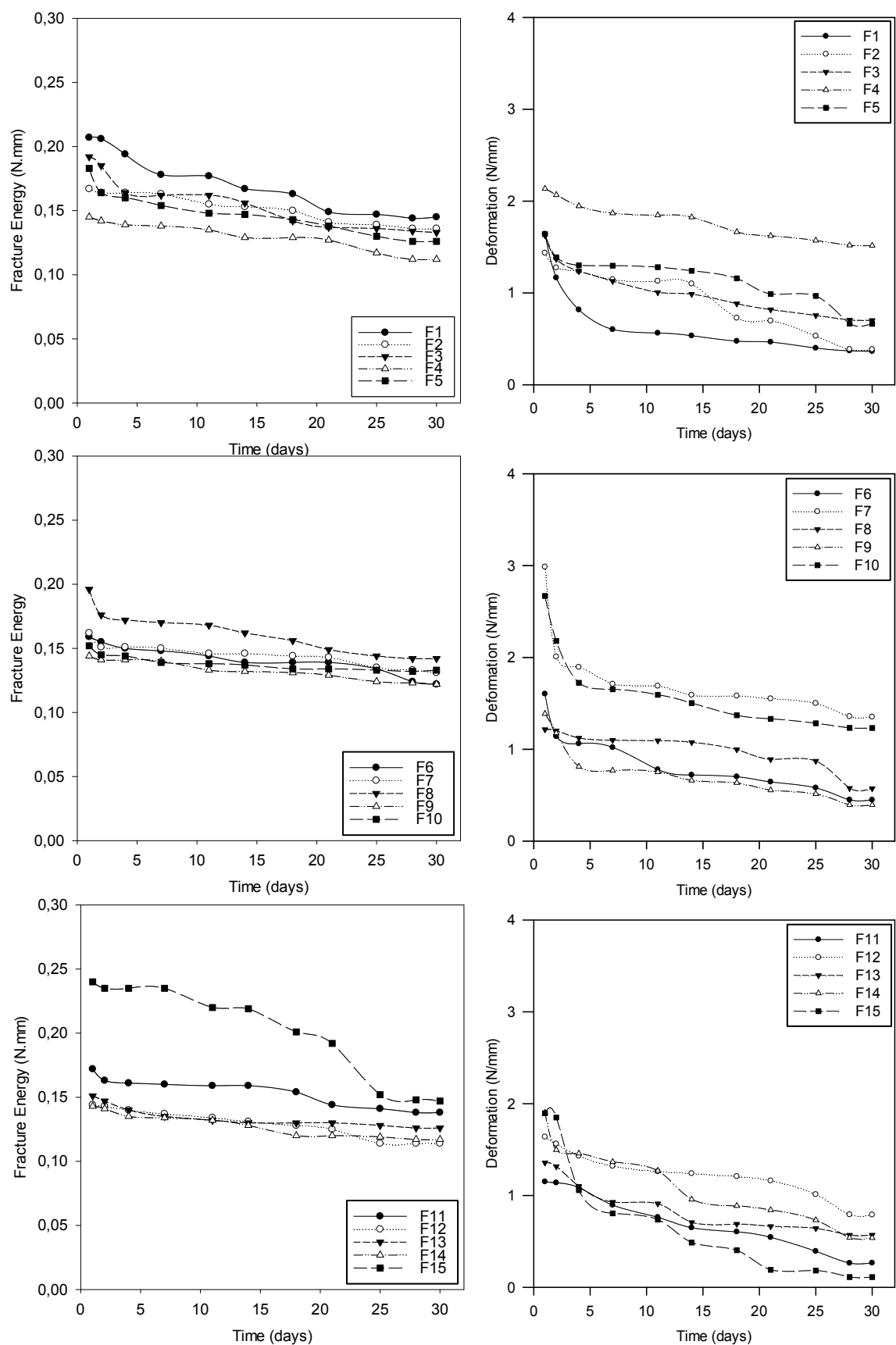


Figure 4.14: Texture analysis depicting fracture energy (left) and deformation (right) of the experimental formulations performed at various time-points post-dissolution in PBS pH 7.4.

4.3.5. Pristine polymer particle intercalation, hydrogel water-absorption analysis and formation of gelled-core microenvironment

The crosslinked gellan-xanthan gum hydrogel formulations were able to sustain the release of the model protein, BSA (Figure 4.15), up until 15-20 days. Thereafter, no further BSA was detectable. Formulations 2, 4, 5, 6 and 9 released between 45-50% of the total incorporated protein whereas F1, F3, and F13 released more than 50% and F7, F8, F10, F11 and F14 releasing between 60-67% of the total protein. At the end of 15 days, F12 released the lowest amount of protein, 37%, and F15 released the highest amount of 75%. Formulation 15, releasing the highest amount of BSA also exhibited the fastest release rate which corresponds to its high degree of water-uptake and swelling. Formulation 12 displayed the slowest release rate conferred by the equally slowed rate of water penetration and swelling. Similar release kinetics were observed with the release of diclofenac sodium however, the release of diclofenac sodium from all the formulations was significantly retarded with only 14-20% of the total drug being released over 30 days as shown in Figure 4.15.

Drug release from controlled-release systems occurs primarily via three mechanisms: diffusion-controlled, swelling-controlled and chemically-controlled (Fu and Kao, 2010; Siepmann and Peppas, 2011). The inclusion of pristine PMMA particles assisted in reducing the drug release rates for both BSA and diclofenac sodium. The slowed release rates imparted by the intercalation of pristine PMMA particles are seen particularly in F4 and F9 where drug release is dependent on water penetration into the matrix, hydration of the PMMA particles and subsequent dissolution of PMMA out of the matrix. The release rate of F4 corresponds to the reduced water-uptake and swelling. Formulations 1, 3, 5 and 15 containing the highest concentration of crosslinker displayed faster release rates which were mostly due to the enhanced swelling of the matrix, particularly F1 and F15 which underwent the greatest swelling and size change. Faster drug release rates in the presence of increased CaCl_2 concentrations may be attributed to the formation of intramolecular complexes between calcium ions and the pyruvate moiety of xanthan gum leading to collapse of the sidechains into the polymer backbone thereby decreasing the hydrodynamic volume of the polymer chains when a rigid orderly conformation is adopted. This intramolecular interaction and chain conformation results in the formation of large network pores which facilitate water and drug diffusion through the matrix (Jaipal *et al.*, 2013). Moreover, the exchange of excess calcium ions with weaker monovalent ions present in the dissolution medium and the pronounced electrostatic repulsion between anionic side chains of the polymers may have resulted in uncontrolled swelling thus allowing the escape of drug molecules via diffusion through the enlarging meshwork between the polymer chains of the gel matrix as swelling and network expansion continued (Baumgartner *et al.*, 2008; Jaipal *et al.*, 2013). The

erosion associated with the inclusion of pristine polymer particles did not affect the drug release rates, since upon the dissolution of the particles drug molecules remained within the gel matrix and slowly diffused out through the pores as the hydration interface expanded. Decreased drug release rates were noted with formulations containing higher ratios of xanthan gum. In these cases, the viscosity change of xanthan gum associated with its water-uptake and swelling combined with the pH-responsive particulate leaching contributed to the formation of gelled-filled channels and pore structures. These gel-filled spaces retarded the diffusion-driven movement of drug molecules out of the matrix thus retaining the drug particles in a gel-suspension within these voids.

Mathematical modelling was performed to assist the ascertainment of the drug release mechanism and its correlation to the observed swelling and erosion utilizing bioactive release as a function of time. Mathematical modelling results shown in Tables 4.7 and 4.8 for BSA and diclofenac sodium, respectively, show that the drug release kinetics of the incorporated model drugs is best described by the Higuchi equation. This indicates that the prevailing drug transport mechanism is diffusion-based where a rate limiting boundary layer may further influence drug release rates. The chemical composition of F15, however, ascribed its release of BSA to the First-Order model.

The diffusional exponent, derived from n of the Korsmeyer-Peppas equation, determines the type of diffusional release whereas the Peppas-Sahlin model identifies the contribution of Fickian diffusion (F) and Case-II relaxational (R) release of the system by calculation of the R/F ratios. The release of BSA presented with various changes in the n -values across different formulations of varying polymer compositions whereas that of diclofenac sodium release was maintained between the ranges of 0.32-0.44. This indicates that variances in polymer and crosslinker composition produced no significant change in the drug transport mechanism of diclofenac sodium which is considered a purely Fickian diffusional release. The fairly constant R/F ratios in the 0.011-0.014 range and the negligible K_2 values of 0.0001 compared to the K_1 values (0.006-0.010), listed in Table 4.8 for diclofenac sodium release, derived from the Peppas-Sahlin equation confirms Fickian diffusional release.

Regarding the release of BSA, of the 15 experimental formulations, 11 formulations presented with n -values below 0.45 indicating Fickian diffusion. Formulations presenting smaller n -values, particularly F2 (0.1464), F4 (0.1345), F5 (0.1718), F9 (0.2243) and F14 (0.2755) contained higher concentrations of pristine PMMA particles (0.5-0.75g/g) correlating to diffusion of drug molecules from an increasing series of porous microenvironment structures and expansion of the surrounding matrix attributed to swelling. The temporary

storage and release of drug molecules from within these gel-filled porous reservoirs produced near zero-order release profiles from Day 5 onwards. Similar n -values were noted for formulations comprising higher xanthan ratios and intermediate crosslinker and pristine particle concentration signifying the effect of water-uptake, swelling and viscosity change associated with xanthan gum on the diffusion-based transport of drug molecules. In contrast, formulations comprising increased ratios of gellan gum with intermediate calcium concentrations and low (0.25g/g) pristine PMMA particle intercalation exhibited higher n -values within the $0.45 < n < 0.89$ range. This was specifically noted with formulations F8, F10, F11 and F15 with n -values of 0.5897, 0.6263, 0.5084 and 0.7592, respectively. This suggests that decreasing PMMA particle concentrations and increasing gellan and calcium concentrations lead to a gradual shift in the drug release mechanism from an anomalous/non-Fickian transport towards Case-II transport encompassing predominantly polymer relaxation ensuing erosion as compared to the former anomalous transport featuring a coupled diffusion-polymer relaxation mechanism of drug transport (Fu and Kao, 2010). This dual mechanism translates to the processes of polymer chain disentanglement and subsequent relaxation following the decrease in electrostatic shielding between carboxylate groups as water penetration into the matrix enhances thereby leading to gelling of particularly xanthan gum whereas gellan gum undergoes erosion of the matrix. The Peppas-Sahlin model validates the shift in the drug release mechanism brought about by decreased PMMA-particle intercalation and increased gellan and calcium concentrations as presented by the R/F ratio, K_1 and K_2 values in Table 4. Overall, K_1 values were greater than K_2 values therefore, indicating Fickian diffusional release as the predominant drug release mechanism. The R/F ratio varied among the different formulations for BSA release where higher R/F values indicate increased contribution of the Case-II relaxational release mechanism. As noted earlier, formulations F1, F8, F10, F11 and F15 comprising a lower degree of PMMA-particle intercalation (0.25g/g) and higher calcium concentrations presented with R/F ratios in the 0.040-0.042 range. Formulations F3, F6, F7, F12 and F13 comprising 0.5g/g PMMA particles exhibited slightly lower R/F values between 0.031-0.036 and formulation F4 and F5 containing 0.75g/g PMMA displayed the lowest value of 0.028 and 0.016, respectively. The gradual decrease in R/F ratios upon increasing PMMA particle concentration promotes the dominance of Fickian diffusional release over Case-II relaxational release.

Additionally, it was noted that although pristine polymer particle intercalation indeed slowed drug release rates, this effect was minute for the release of diclofenac sodium compared to the differences observed with the BSA release. This phenomenon could be ascribed to the difference in the aqueous solubility of BSA ($\approx 40\text{mg/mL}$) and diclofenac sodium ($\approx 20\text{mg/mL}$).

Therefore, diclofenac sodium may be subject to a dissolution-controlled drug release which is typically attributed to the low solubility of drugs in the matrix environment. In this instance, the release of diclofenac sodium is dependent on its local dissolution of drug particles within the matrix before diffusion of dissolved drug molecules out of the matrix occurs. In essence, drug dissolution within the matrix should be rapid compared to drug diffusion out of the matrix. Furthermore, the significantly smaller molecular size of diclofenac sodium, compared to that of BSA, leads to an increased likelihood of its occupation and entrapment within the microenvironment spaces, thereby delaying its release out of the matrix. It can be ascertained that the control of drug release from the pristine polymer intercalated gellan-xanthan IPNs occurs via two steps: 1) the initial dissolution of intercalated particles which make way for drug molecules in the matrix to pass via diffusion 2) the resulting formation of gelled macroporous network which temporarily store drug molecules and inhibits its movement out of the matrix. Likewise, the diffusion-driven movement of drug molecules through the matrix occurs along a concentration gradient via a two-step process: 1) the movement of drug from the matrix into the empty large porous structures 2) the movement of drug out of these structures and into the surrounding media.

4.3.6. Optimization of Box-Behnken experimental design and assessment of *in vitro* response outcomes

Minitab® V15 statistical software was employed to generate the optimal variables and response outcomes based on the results of the Box-Behnken experimental design. As shown in Figure 4.16a, it was predicted that a gellan:xanthan ratio of 73:27, CaCl₂ concentration of 0.05^{w/v} and low PMMA particle intercalation of 0.25g would produce the optimal P⁴[Gel-Xan]IPN formulation featuring ideal BSA fractional release, diclofenac MDT, swelling, erosion and compatible mechanical properties at the end of the 30-day study period. The optimised P⁴[Gel-Xan]IPN hydrogel was similar in composition to F10, with 65.93% BSA and 19.51% diclofenac sodium released over 20 and 30 days, respectively (Figure 4.16b). At the end of the study, a swelling ratio of 677.04% and erosion of 68.79% with 42.01% resilience were noted whereas ideal deformation of 1.26N/mm and fracture energy of 0.173N.mm were obtained (Figure 4.16c-d). This demonstrated the ability of the optimised P⁴[Gel-Xan]IPN hydrogel in providing an attuned system for combined desirable drug release kinetics and durable textural properties, thereby conforming to the predicted response outcomes.

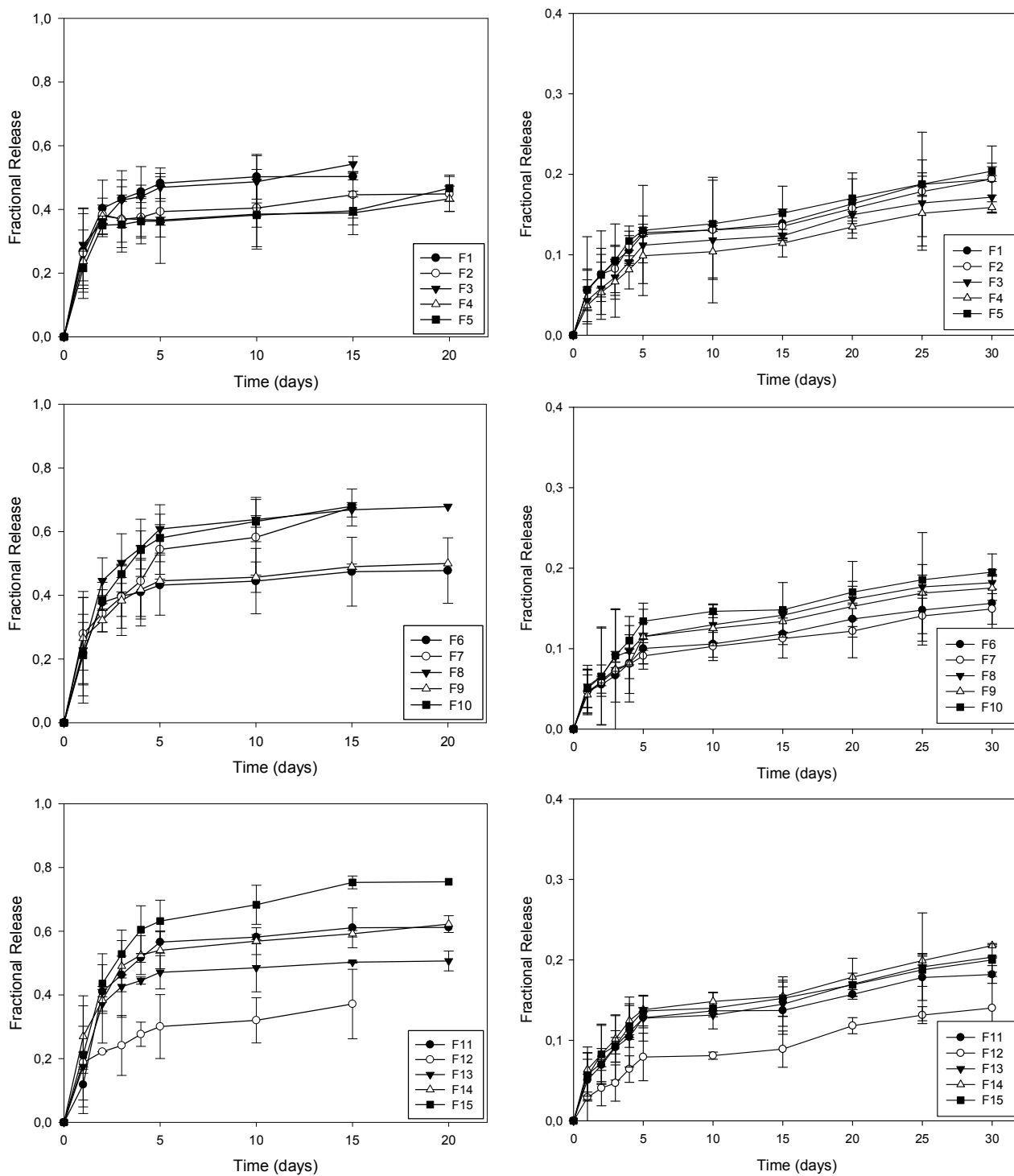


Figure 4.15: *In vitro* drug release profiles of BSA (left) and diclofenac sodium (right) in PBS pH 7.4.

Table 4.7: Kinetic modelling results of BSA release from the PMMA-intercalated IPN gellan-xanthan hydrogel conduits.

Formulation	Zero-Order	First-Order	Higuchi		Korsmeyer-Peppas	Peppas-Sahlin		Hopfenberg		Hixson-Crowell		Best-fit model
	R ²	R ²	R ²	R ²	n	R ²	K ₁	K ₂	R/F Ratio	R ²	R ²	
1	0.508	0.555	0.663	0.774	0.209	0.828	0.060	0.0025	0.042	0.532	0.539	Higuchi
2	0.632	0.680	0.743	0.805	0.146	0.747	0.021	0.0006	0.029	0.656	0.664	Higuchi
3	0.734	0.790	0.852	0.917	0.217	0.874	0.047	0.0017	0.036	0.762	0.772	Higuchi
4	0.440	0.478	0.526	0.601	0.135	0.488	0.014	0.0004	0.028	0.459	0.465	Higuchi
5	0.490	0.530	0.550	0.604	0.172	0.501	0.013	0.0002	0.016	0.510	0.517	Higuchi
6	0.510	0.570	0.643	0.715	0.208	0.701	0.035	0.0011	0.032	0.540	0.550	Higuchi
7	0.868	0.923	0.943	0.953	0.345	0.950	0.068	0.0022	0.032	0.898	0.907	Higuchi
8	0.587	0.698	0.735	0.927	0.590	0.852	0.104	0.0042	0.040	0.643	0.661	Higuchi
9	0.682	0.722	0.816	0.903	0.224	0.895	0.052	0.0042	0.081	0.702	0.709	Higuchi
10	0.674	0.787	0.814	0.970	0.626	0.901	0.106	0.0042	0.040	0.731	0.750	Higuchi
11	0.484	0.586	0.635	0.665	0.508	0.778	0.119	0.0050	0.042	0.534	0.551	Higuchi
12	0.875	0.896	0.948	0.974	0.250	0.943	0.029	0.0009	0.031	0.886	0.889	Higuchi
13	0.437	0.499	0.580	0.657	0.280	0.682	0.050	0.0017	0.034	0.468	0.478	Higuchi
14	0.600	0.684	0.738	0.822	0.276	0.770	0.050	0.0016	0.032	0.642	0.656	Higuchi
15	0.646	0.801	0.786	0.961	0.759	0.861	0.116	0.0046	0.040	0.725	0.751	First-Order

Table 4.8: Kinetic modelling results of diclofenac sodium release from the PMMA-intercalated IPN gellan-xanthan hydrogel conduits.

Formulation	Zero-Order	First-Order	Higuchi	Korsmeyer-Peppas	Peppas-Sahlin					Hopfenberg	Hixson-Crowell	Best-fit model
	R ²	R ²	R ²	R ²	n	R ²	K ₁	K ₂	R/F Ratio	R ²	R ²	
1	0.901	0.913	0.952	0.960	0.342	0.928	0.007	0.0001	0.014	0.907	0.909	Higuchi
2	0.881	0.892	0.931	0.941	0.333	0.902	0.007	0.0001	0.013	0.887	0.889	Higuchi
3	0.879	0.891	0.944	0.952	0.389	0.924	0.008	0.0001	0.013	0.885	0.887	Higuchi
4	0.899	0.909	0.957	0.958	0.401	0.935	0.007	0.0001	0.014	0.904	0.906	Higuchi
5	0.883	0.897	0.947	0.954	0.349	0.920	0.008	0.0001	0.012	0.890	0.893	Higuchi
6	0.906	0.915	0.964	0.969	0.357	0.950	0.007	0.0001	0.014	0.910	0.912	Higuchi
7	0.923	0.930	0.973	0.981	0.324	0.951	0.006	0.0001	0.014	0.927	0.928	Higuchi
8	0.897	0.909	0.966	0.972	0.355	0.953	0.009	0.0001	0.012	0.903	0.905	Higuchi
9	0.879	0.891	0.951	0.960	0.402	0.939	0.009	0.0001	0.011	0.885	0.887	Higuchi
10	0.833	0.848	0.919	0.931	0.377	0.901	0.010	0.0002	0.020	0.841	0.843	Higuchi
11	0.837	0.851	0.917	0.933	0.349	0.894	0.008	0.0001	0.012	0.844	0.846	Higuchi
12	0.927	0.933	0.958	0.959	0.443	0.942	0.006	0.0001	0.011	0.930	0.931	Higuchi
13	0.909	0.921	0.956	0.960	0.350	0.932	0.008	0.0001	0.013	0.915	0.917	Higuchi
14	0.892	0.906	0.946	0.955	0.330	0.916	0.008	0.0001	0.013	0.899	0.902	Higuchi
15	0.857	0.871	0.920	0.930	0.333	0.889	0.008	0.0001	0.013	0.864	0.867	Higuchi

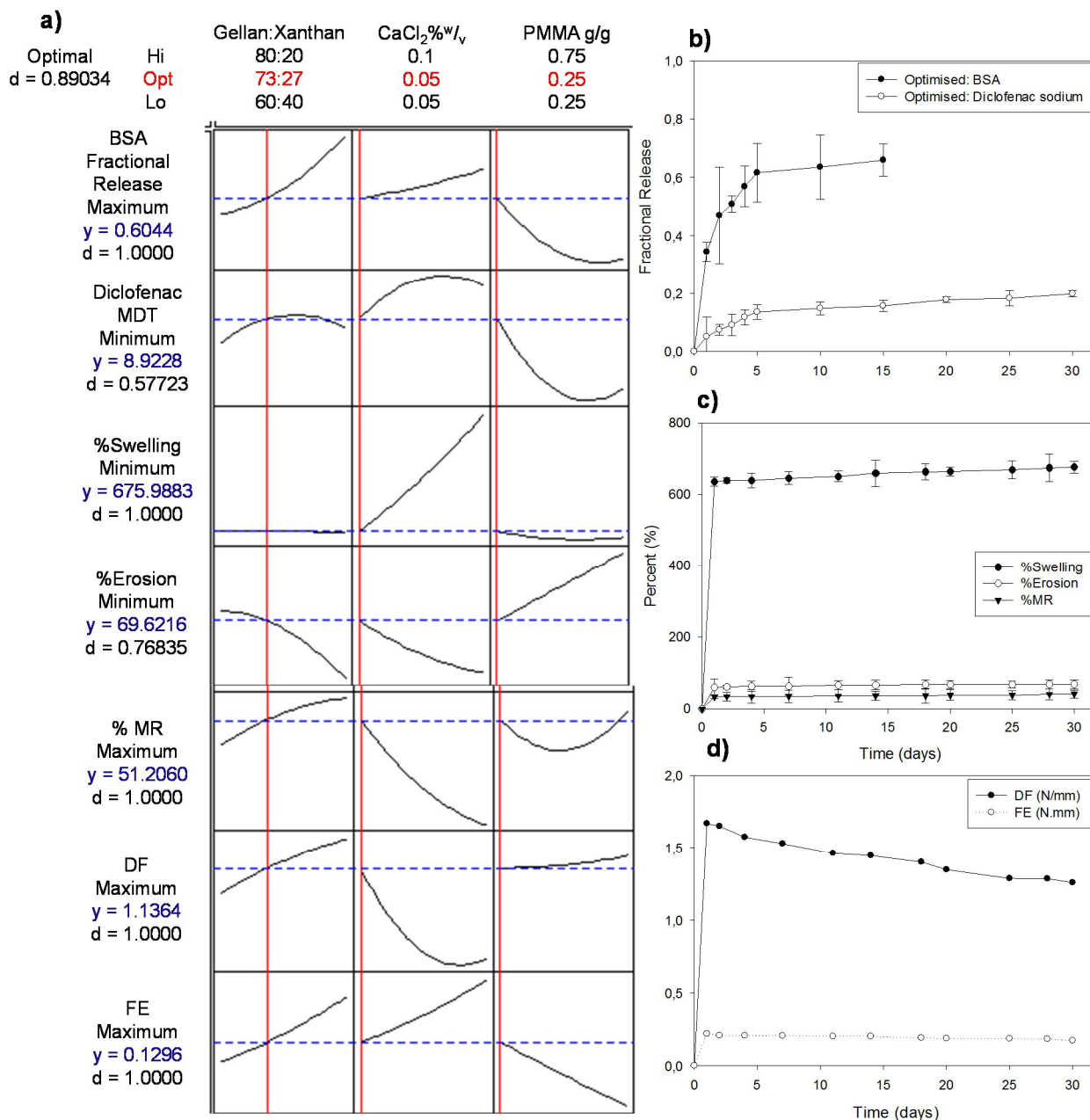


Figure 4.16: Statistical optimization and response parameter outcomes of the P4[Gel-Xan]IPNs: a) desirability plots depicting the variables required for the optimised formulation and response outcomes for the optimised Gel-Xan IPN hydrogel b) *in vitro* drug release profiles c) %swelling, %erosion and %matrix resilience and d) textural analysis depicting deformation and fracture energy conducted over 30 days of dissolution in PBS pH 7.4.

4.4. Concluding Remarks

Utilizing heat for the transformation of polysaccharide gums from random coils to ordered helical structures combined with divalent metal ions for the physical crosslinking of xanthan moieties to that of gellan, via targeting of the carboxylate functional group, yields the successful formation of an IPN through a series of intramolecular and intermolecular

crosslinking mechanisms with metal ion chelation being the primary crosslinking method. Incorporating the technique of pristine polymer particle intercalation, using an appropriate pH-responsive polymer, is capable of producing zero-order release kinetics of protein and small molecular weight compounds via a tuneable combination of Fickian diffusional and Case-II relaxational release, dependent on the level of particle intercalation and hydrodynamic properties of the matrix. The *in situ* particulate leaching mechanism preceding the pH-responsive dissolution of the intercalated particles results in the formation of a porous microchannel network allowing for improved matrix resilience via formation of void space and controlled drug release modulated by the intermediate step of particle dissolution. Furthermore, integrating the concept of particle intercalation with an IPN swellable system provides a strategy for the simultaneous manipulation of matrix resilience properties whereas crosslinking of a weak gelling agent with a rigid gelling gum assists in adjusting scaffold pliability and stiffness for enhanced tissue compatibility of various soft and connective tissue types in addition to extending drug release rates for long-term drug delivery via resistance to diffusion-driven transport of drug molecules.

The study involving the intercalation of pristine polymer PMMA particles within the anionic crosslinked gellan-xanthan gum polysaccharide matrices shows the advantages of decreased swelling, prolonged drug release and enhanced mechanical properties specifically matrix resilience and flexibility imparted by the pH-responsive dissolution of the particles leading to the formation of a porous network and channels. Furthermore, the tuning of matrix rigidity and flexibility was attainable via variation of xanthan gum composition in the hydrogel formulation. In addition, the ionic crosslinking of the hydrogel blends forming an interpenetrating network, as validated by FTIR spectrometry, provides the appropriate attributes of a tissue repair material offering a matrix network of suitable mechanical properties for tissue compatibility and the concurrent sustained release of proteins and small molecular weight drug molecules. The variables and responses investigated in this study necessitate an extended phase of formulation optimisation, based on the current findings, utilizing appropriate neurotrophic factors and subsequent analysis of the conduit's regenerative potential in an *in vivo* peripheral nerve injury model. The results of the study show promise for the development of drug-delivering long-term tissue repair implantable scaffolds for the restoration of damaged tubular organ structures such as nerves, blood vessels and intestinal tissues.

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CHAPTER 5

DESIGN, FORMULATION AND STATISTICAL OPTIMIZATION OF ELECTROSPUN NEURAL GUIDANCE CUE SCAFFOLDS

5.1. Introduction

The technique of electrospinning for the design and fabrication of nanofibrous scaffolds for drug delivery and tissue engineering applications has acquired swift popularity over the years. Its maintained appeal for the fabrication of tissue regeneration scaffolds emanates from its ability to produce highly porous fibrous meshes of high surface-area-to-volume ratio and improved resemblance to the architectural properties of native extracellular matrix (ECM) in addition to its ability to encapsulate a diverse range of pharmaceutical drug molecules for the design of site-specific and controlled-release drug delivery strategies (Chew *et al.*, 2007; Masaeli *et al.*, 2013 and Souza *et al.*, 2014).

The electrospinning process consists of the basic setup, as depicted in Figure 5.1. A metal nozzle is connected to high voltage source and a syringe pump loaded with the polymer solution. The application of a high voltage current is required to surpass a specific critical value of the resulting electric field. When the electrostatic force exceeds the surface tension of the polymer solution, a fine jet of the polymer solution is emitted from the nozzle tip towards a grounded collector. Evaporation of the solvent from the polymer jet results in the deposition of fibers onto the collector. A cylindrical rotating collector is generally used for the acquirement of aligned fibers whereas a static collector leads to the formation of randomly-orientated nonwoven fibers (Pan *et al.*, 2006).

The polymer utilized for the electrospinning of fibrous films in this study was Poly(3-hydroxybutyric acid-co-3-hydroxyvaleric acid) (PHBV). PHBV is a polyhydroxyalkanoate (PHA) belonging to the class of naturally-derived biodegradable polyesters since it is bio-synthesized by microorganisms that use it as an intracellular energy source (Masaeli *et al.*, 2013). The most commonly utilized PHAs are poly(3-hydroxybutyrate) and poly(3-hydroxyvalerate) which are often blended together in various ratios for modification of the physicochemical and physicomechanical properties of the resulting PHBV copolymer. Although various studies have demonstrated its promising potential as non-cytotoxic scaffolds for neural, skin and bone tissue engineering; its high crystallinity, rigidity and brittleness further narrows its process-ability qualities for techniques such as film and

injection moulding, extrusion, blown-film manufacturing and fibre spinning (Masaeli *et al.*, 2013 and Wei *et al.*, 2015).

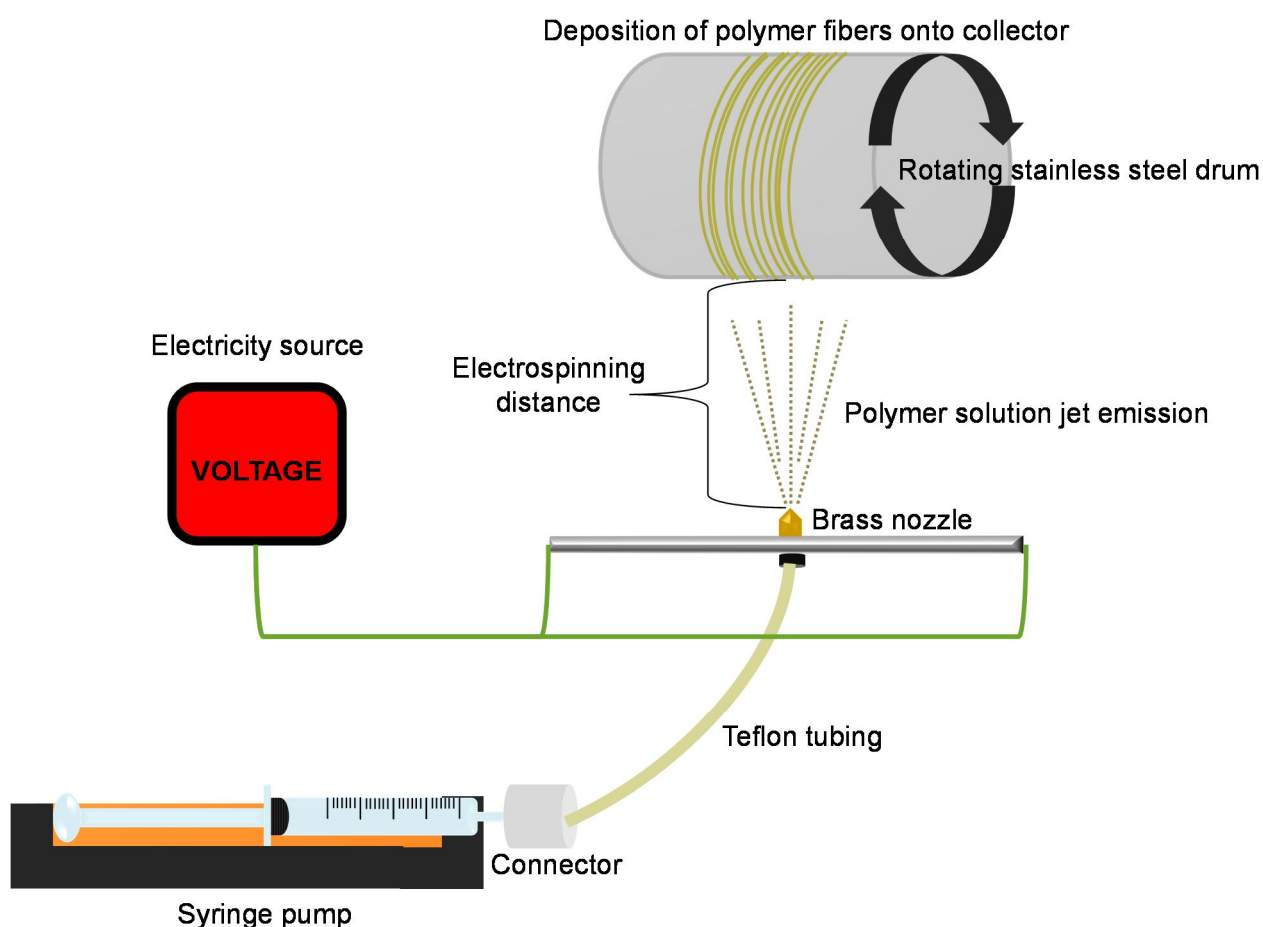


Figure 5.1: Schematic representation of the electrospinning setup and process for the collection of aligned fibers.

PHBV, being hydrophobic, requires modification and blending with other constituents to enhance its limited cell adhesion and proliferation properties (Pompe *et al.*, 2007 and Masaeli *et al.*, 2013). Therefore magnesium, oleic acid and N-acetyl-L-cysteine (NAC) were incorporated to function as therapeutic and chemical cues for the design of neuronal guidance scaffolds for improved neuronal biocompatibility.

The presence of magnesium ions may have potential for improving functional recovery after peripheral nerve injury via its neuroprotective properties. It was shown that increased consumption of magnesium as a dietary mineral improved functional recovery after sciatic nerve crush injuries (Pan *et al.*, 2011). Vennemeyer *et al.*, 2014a, demonstrated the benefit of magnesium salts in improving cell proliferation and neurite extension in primary mouse

neuronal stem cell cultures. In addition to its neuroprotective effects, the inclusion of magnesium could provide an electrical stimulus cue to assist nerve regeneration and muscle innervation (Vennemeyer *et al.*, 2014b). An interesting study investigating the biodegradation and regeneration potential of magnesium wire filaments across the rat sciatic nerve gap injury model, reported adequate biocompatibility with mild inflammation and improved tissue regeneration while possibly slowing the rate of muscle atrophy (Vennemeyer *et al.*, 2014b).

To decrease the extent of inflammation in response to implantation of a foreign solid material and to assist with formulation preparation and elimination of chemical incompatibilities, magnesium ions were introduced into the scaffold system as a magnesium-oleate (MgOI) salt utilizing oleic acid. Oleic acid, known by the systematic name of octadecanoic acid, forms a large percentage weight composition of the sciatic nerve lipids (45% weight in the rat sciatic nerve) and myelin fatty acids (Berry *et al.*, 1965; Norton and Cammer, 1984 and Sastry, 1985). Furthermore, it has been investigated for its role as a neurotrophic factor in the central nervous system (Velasco *et al.*, 2003).

Similarly, NAC has shown potential as a neuroprotective and growth-promoting agent in peripheral nerve injuries and as an adjunct therapy to pain management strategies in nerve injuries and neuropathies (Hart *et al.*, 2004; Welin *et al.*, 2009; Karalija *et al.*, 2012; Panesar, 2012 and Scheib and Höke, 2013). It has further, shown prospect as an effective enhancer of neuronal differentiation via increased expression of N-cadherin and activation of Akt (Noh *et al.*, 2012).

The present study utilizes the technique of electrospinning for the fabrication of PHBV aligned fibrous films for application as potential neuronal guidance scaffolds in peripheral nerve injuries. The blending of MgOI and NAC were further employed as modifiers of chemical, physical and mechanical properties of the electrospun PHBV fibers whilst contributing to the dual function as therapeutic and chemical cues of the scaffold. Figure 5.2 depicts the 3 ways in which the electrospun fibrous sheets can be implemented for the design or improvement of nerve repair devices. The resulting fibrous films were analysed for chemical, thermal and crystallinity transitions, mechanical properties, erosion, bioactive release and neuronal proliferative effects in response to variations in MgOI and NAC concentrations.

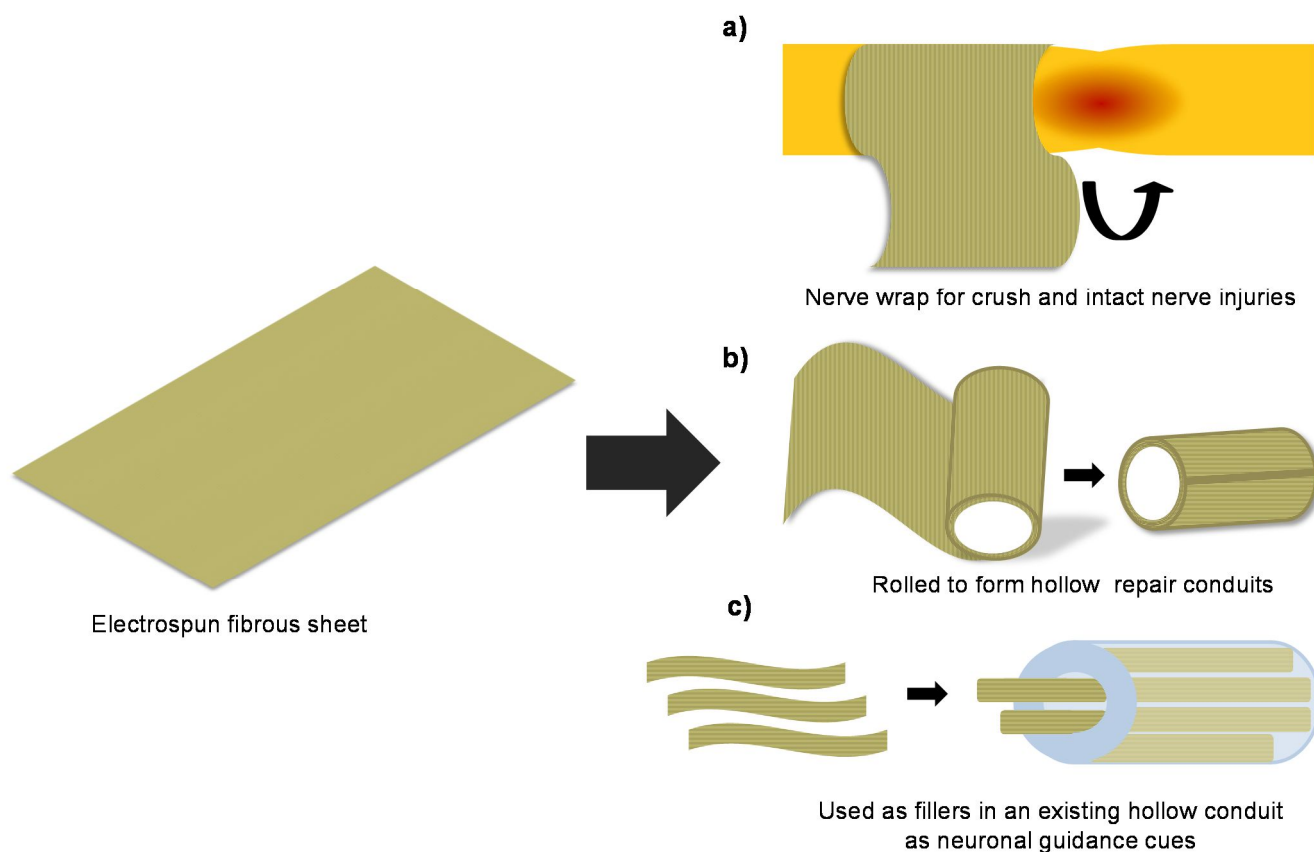


Figure 5.2: Representation of the different ways of implantation of the NAC loaded PHBV-MgOI electrospun fibrous films: a) utilized as a wraps or dressings with a tissue glue sealant for the protection of crush injuries where the nerve is unbroken b) electrospun sheets can be rolled and thermos-sealed into multi-ply hollow conduits of various sizes for the repair of transected nerve injuries c) electrospun sheets can be cut into various sizes to be used as liners or fillers in an existing hollow conduit for the introduction of physical neuronal guidance cues.

5.2. Materials and Methods

5.2.1. Materials

Poly(3-hydroxybutyric acid-co-3-hydroxyvaleric acid) (PHBV) (PHV content 12mol%) natural origin, N-Acetyl-L-cysteine (NAC) ($\geq 99\%$ TLC), 2,2,2-Trifluoroethanol ($\geq 99\%$ GC) and dialysis tubing cellulose membrane (molecular weight cut-off = 14000Da) were purchased from Sigma Aldrich, Steinham, Germany. Sodium hydroxide pellets, chloroform, oleic acid vegetable (extra pure) and Span 80 were purchased from Merck, Darmstadt, Germany. Magnesium sulphate heptahydrate CP ($\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$), methanol and acetone were purchased from Associated Chemical Enterprises, Southdale, Johannesburg, South Africa. Ethanol (99% absolute) was purchased from LabChem, Edenvale, Johannesburg, South Africa. Milipore water was used for washing of preparations. Dulbecco's Modified Eagle Medium (DMEM), Donor Equine Serum (DES) and Fetal Bovine Serum (FBS) were procured from Hyclone, Separations, South Africa. Pencillin/Streptomycin/Amphotericin B (P/S/AB)

solution and the Roche Cell Proliferation Kit I was purchased from Lonza, USA and Sigma-Aldrich, USA, respectively.

5.2.2. Determination of the upper and lower variable limits for construction of a Box-Behnken experimental design

For investigations into formulation optimization, a 3-factor Box-Behnken experimental design was constructed utilizing independent variables for fibre fabrication and the corresponding upper and lower limits for successful electrospinning processes. Electrospinning of preliminary formulations, varying in the chemical constituent concentrations, allowed the identification of the formulation variables and limits and optimization of the electrospinning parameters: flowrate, distance, rotation speed and voltage. Preliminary investigations revealed that PHBV polymer concentration and concentrations of MgOI (as grams per gram of PHBV) and NAC incorporation influenced the quality outcome of the electrospinning process. Although NAC was used as a bioactive and therapeutic agent, its incorporation into the fibrous films affected the mechanical properties, thus, its effects had to be considered for obtaining films of adequate strength. The electrospinning potential of the trial formulations, listed in Table 5.1, was used to determine the upper and lower variable limits. The aim was to keep the variable limits within the set electrospinning parameters. Therefore, the detected ideal electrospinning parameters were kept constant for the fabrication of fibers from the entire Box-Behnken experimental solutions. This was done as to prevent variations in formulation properties ascribed by alteration of the electrospinning parameters. The following electrospinning and design criteria were adhered to during determination of the variable limits and optimisation of the electrospinning parameters:

- Physical and chemical stability of the formulation constituents in solution over the duration of electrospinning.
- Tensile strength and flexibility of the electrospun films.
- Smooth and unproblematic removal of the electrospun film from the collecting sheet.
- Elimination of solution sputtering and nozzle blockage.
- Minimisation of the deposition frequency of randomly orientated fibers.

Table 5.1: List of preliminary formulation compositions and electrospinning parameters

Formulation Composition	Electrospinning Parameters (Flowrate; Distance; Voltage; Rotation)
5%^{w/v} PHBV, 0.5g/g MgOI in chloroform	3mL/hour; 155mm; 24.0kV; 850rpm 3mL/hour; 130mm; 24.0kV; 1000rpm 3mL/hour; 155mm; 24.0kV; 1200rpm
10%^{w/v} PHBV, 1g/g MgOI in chloroform	2mL/hour; 135mm; 24.0kV; 1000rpm

5% ^{w/v} PHBV, 0.2g/g MgOI in chloroform 10% ^{w/v} PHBV, 0.5g/g MgOI in chloroform	2mL/hour; 130mm; 24.0kV; 1000rpm
5% ^{w/v} PHBV, 0.1g/g MgOI, 4% ^{w/v} NAC 5% ^{w/v} PHBV, 0.6g/g MgOI, 4% ^{w/v} NAC 5% ^{w/v} PHBV, 0.6g/g MgOI, 2% ^{w/v} NAC All in 3:2 chloroform:trifluoroethanol	2mL/hour; 150mm; 25.0kV; 1200rpm

The trial formulations above assisted in the determination of the upper and lower variable limits as well as the detection of ideal electrospinning parameters suited for the various formulations lying within the range of the identified limits. Table 5.2 lists the variables, the identified limits and the effect on electrospinning of the samples utilizing fixed electrospinning parameters of: 2mL/hour flowrate, 150mm distance, 25.0kV voltage and 1200rpm rotation speed.

Table 5.2: The identified variables and the upper and lower limits for generation of a Box-Behnken experimental design

Variable		Effect
PHBV	Lower limit: 5% ^{w/v}	Concentrations <5% ^{w/v} produced sparse fibers with thin and fragile film formation.
	Upper limit: 10% ^{w/v}	Concentrations >10% ^{w/v} resulted in increased viscosity of the polymer solution leading to overflowing at the nozzle tip and reduced electrospinning potential.
MgOI	Lower limit: 0.1g/g	Concentrations <0.1g/g produced brittle and friable fibrous films that could not be removed from the collecting sheet without tearing.
	Upper limit: 0.6g/g	Concentrations >0.6g/g conferred poor electrospinning properties to the solution, often separating out and collecting at the nozzle tip causing blockages thus resulting in sputtering and spraying of the solution rather than spinning of fibers. Anything higher would precipitate out of solution as 0.6g/g was the solubility limit.
Bioactive Loading		
NAC	Lower limit: 0% ^{w/v}	NAC-free formulations imparted increased tensile strength of the fibrous films.
	Upper limit: 4% ^{w/v}	Concentrations >4% ^{w/v} resulted in solution stability issues and precipitation of MgOI in addition to decrease in tensile strength of the films. However, NAC-loading enhanced conductivity and electrospinning of the

5.2.3. Fabrication of aligned guidance cues

5.2.3.1. Synthesis of magnesium-oleate complex

The magnesium-oleate (MgOleate) complex was synthesised using a method adapted from Ghosh *et al.*, 2013, for metal-oleate preparation using oleic acid and methanol. Briefly, 4.93g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and 12.7mL of oleic acid were dissolved in 100ml methanol. After 30 minutes of magnetic stirring, 100mL of a 0.4mol/L sodium hydroxide solution was slowly added (to avoid unwanted magnesium hydroxide precipitation) and stirred for a further 3 hours for reaction completion to yield an oily white layer containing the MgOleate complex. The oily liquid was separated from the solvent, placed into dialysis tubing and dialysed against deionized water for 3 days followed by 3 washings with ethanol and acetone for the removal of any unreacted constituents. The resulting MgOleate precipitate, a yellow wax-like solid, was obtained by overnight drying in a vacuum oven at 100°C and then stored in an airtight glass container until further use.

5.2.3.2.. Electrospinning procedure for fibre fabrication

The polymer solutions for electrospinning were prepared by dissolving PHBV pellets in a solvent mixture of chloroform and trifluoroethanol (for NAC solubilisation) in a 3:2 ratio under continuous magnetic stirring at 60°C to prepare 5-10%^{w/v} concentration solutions followed by the addition of MgOleate in the range of 0.1-0.6g/g PHBV and NAC in concentrations of 0-4%^{w/v} of polymer solution. For fabrication of the films, 5mL of the PHBV-MgOleate solutions with or without NAC were loaded into a syringe for electrospinning (NanoSpinner24, Inovenso, Turkey) onto Span 80-coated aluminium sheets (to facilitate effortless film-removal) attached to a rotating drum set at 1200rpm, using a voltage of 25kV, a flowrate of 2mL/hour and a nozzle distance of 150mm. The resulting films measured 8cm×30cm and were cut into 0.5cm×3cm strips for further analysis. A total of 15 experimental formulations, varying in PHBV, MgOleate and NAC concentrations, were synthesised employing a 3-factor Box-Behnken design utilizing Minitab® statistical software (V15, Minitab Inc., PA, USA) as listed in Table 5.3.

Table 5.3: Formulations obtained via the Box-Behnken experimental design

Formulation	PHBV (% ^{w/v})	MgOleate (g/g PHBV)	NAC (% ^{w/v})
1	5	0.35	4
2	10	0.35	4
3	7.5	0.35	2
4	7.5	0.35	2
5	5	0.6	2

6	5	0.35	0
7	5	0.1	2
8	7.5	0.35	2
9	7.5	0.6	4
10	10	0.1	2
11	7.5	0.1	4
12	7.5	0.6	0
13	10	0.35	0
14	10	0.6	2
15	7.5	0.1	0

5.2.4. Response surface analysis as per Box-Behnken design and formulation optimization

Response surface analysis of the 15 design formulations were carried out employing Minitab® statistical software (V15, Minitab Inc., PA, USA). The *in vitro* performance of the experimental formulations were evaluated according to the response outcomes of water-uptake, erosion and tensile strength to elucidate the influence of polymer concentration, metal-oleate content and bioactive loading on the structural integrity of the electrospun films. Statistical optimization of the results obtained from the Box-Behnken experimental design predicted the optimal quantities of each chemical component required to obtain the ideal formulation based on the responses.

5.2.5. Characterization of the electrospun aligned films

5.2.5.1. Detection of vibrational molecular changes of the chemical constituents and electrospun fibers

FTIR spectroscopy was used to confirm the synthesis of MgOleate and detect the molecular transitions and changes in crystallinity indices, of the experimental formulations, arising from interactions between PHBV, MgOleate, NAC and the overall electrospinning process. Samples were analysed over an FTIR spectra of wavelengths between 4000-600cm⁻¹ using a PerkinElmer Spectrum 2000 ATR-FTIR (PerkinElmer 100, Llantrisant, Wales, UK) spectrometer fitted with a single-reflection diamond MIRTGS detector.

5.2.5.2.. Thermophysical characterization of the electrospun fibers

The thermal transitions upon heating and cooling of the electrospun fibers were determined using a Temperature Modulated Differential Scanning Calorimeter (TMDSC) (Mettler Toledo, DSC, STARe System, Swchwerzenback, ZH, Switzerland). Samples of 8mg were sealed in aluminium crucibles and heated or cooled over a temperature range of 0-300°C and 200-0°C, respectively, at a temperature change rate of 10°C/min. DSC curves were obtained by plots of sample weight over sample temperature. Differences in the heat flow, phase

transitions and crystallization temperatures of the formulations provided insight into the effects of polymer/metal-oleate interaction and electrospinning. A measure of crystallinity was determined using Equation 5.1.

$$\%Crystallinity = [\Delta H_m - \Delta H_c]/\Delta H_m^\circ \quad \text{Equation 5.1}$$

Where the heats of melting, ΔH_m , and cold crystallization, ΔH_c , are in terms of J/g and ΔH_m° is the heat of melting of the 100% crystalline reference polymer.

The determination of change in sample weight as a function of temperature was performed using a Thermogravimetric analyser (TGA) (PerkinElmer, TGA 4000, Llantrisant, Wales, UK). Samples were heated at a rate of 10°C/min from 30-900°C under continuous nitrogen purging. Thermograms were generated as percentage weight vs. temperature and analysed using the Pyris™ software. Correlation of DSC and TGA data assists in determining the effect of MgOleate incorporation in relation to polymer thermal degradation properties.

5.2.5.3. X-ray diffraction analysis

Sample crystallinity and PHBV crystallite size were determined using X-ray diffraction measurements (Rigaku MiniFlex 600, Tokyo, Japan) computed into the %Crystallinity Index equation (Equation 5.2) and the Scherrer equation (Equation 5.3), respectively.

$$Crl = I_{16}/I_{Total} \times 100 \quad \text{Equation 5.2}$$

Where Crl reflects the %crystallinity index of the sample, I_{16} is the intensity of the peak close to $2\theta = 16^\circ$ and I_{Total} is the sum of the intensities of all the crystalline peaks of PHBV.

$$\pi = K\lambda/\beta \cos\theta \quad \text{Equation 5.3}$$

Where π is the particle size of single crystallites, K is the shape factor of value 0.9 for spherical particles, λ is the X-ray wavelength of 1.54 for CuK α X-ray source, β is the line broadening width (FWHM) at half maximum intensity measured in radians and $\cos\theta$ is the Bragg angle.

5.2.5.4. Tensile strength analysis

Determination of the mechanical properties of the fibrous films before and after dissolution, at pre-determined sampling points was conducted using a Texture Analyser (TAXT.plus Stable Microsystems, Surrey, UK) fitted with two clamps and a 5kg load cell. A 0.5cm×3cm

film strip was fixed at a distance of 10mm between the two clamps and a pre-test, test and post-test speed of 0.5mm/s with a 0.5N trigger force was applied to the sample over a distance of 20mm. The force (N) at breaking point of the film sample was computed via generation of a Force-Distance textural profile. For the determination of tensile strength (MPa), Equation 5.4 was employed, where film dimensions of length, width and thickness were measured using a digital Vernier calliper (Krafft, DV150GW, Schoellerstr Düren, Germany). The deformability modulus (DM) (MPa) was determined from the slope of the generated Force-Distance curve whereas toughness of the sample (N.mm) was calculated from the area under the curve.

$$\text{Tensile Strength} = \frac{\text{Force at break point}}{\text{Cross-sectional area of sample}} \quad \text{Equation 5.4}$$

5.2.5.5. Scanning Electron Microscopy for visualisation of fibre morphology and size and Energy Dispersive X-ray Spectroscopy for elemental analysis

Evaluation of surface morphology, fibre alignment, measurement of fibre size and image capturing was performed under different magnifications using an FEI Nova NanoLab 600TM Scanning Electron Microscope (SEM) (FEI Company, Hillsboro, OR, USA). The samples were further analysed for elemental composition using SEM-Energy Dispersive X-ray Spectroscopy (EDS).

5.2.5.6. Water-uptake and erosion studies

Water-uptake and erosion studies were performed by submersing 0.5cm×3cm film samples in 1mL of PBS pH 7.4 contained in glass vials. An orbital shaking incubator (LM-530-2, MRC Laboratory Instruments Ltd., Hahistadrut, Holon, Israel) maintained at 37°C and set at 50rpm was used for sample agitation. Samples were removed at predetermined time intervals, excess moisture was carefully removed by blotting with tissue paper and the change in mass was measured for determination of moisture absorption. After 72 hours of drying time at 40°C, for complete removal of residual moisture, the film samples were weighed for determination of the eroded mass. Equations 5.5 and 5.6 were employed for the calculation of water-uptake and erosion, respectively.

$$\% \text{ Water-uptake} = \frac{W_h - W_i}{W_i} \times 100 \quad \text{Equation 5.5}$$

$$\% \text{ Erosion} = \frac{W_f - W_i}{W_i} \times 100 \quad \text{Equation 5.6}$$

Where W_i is the initial weight of the film samples, W_h is the weight of the samples after hydration and W_f is the weight of the final eroded samples.

5.2.5.7. *In vitro* release studies and kinetic modelling

In vitro release studies of NAC from the electrospun films were performed using 0.5cm×3cm samples containing either 0.5mg or 1mg NAC. Samples were placed in glass vials containing 1mL PBS pH 7.4 and stored in an orbital shaking incubator. At pre-determined time intervals, 0.5mL of dissolution media was removed for sample analysis and replaced with an equal fresh volume of media for maintenance of sink conditions. Samples were analysed for the quantity of NAC released using UV-spectrophotometry set at $\lambda_{max} = 212\text{nm}$ (Specord40, Analytik Jena, AG, Germany). The results were analysed using WinASPECT® Spectroanalytical Software (Analytik Jena AG, Jena). Further insight into the drug-release mechanisms was obtained via kinetic modelling of the drug-release profiles using the Zero-order, First-order, Higuchi, Hixson-Crowell, Korsmeyer-Peppas and Peppas-Sahlin models as described in Chapter 4 Table 4.4.

5.2.5.8. PC12 Cell culture and MTT proliferation assay

Rat adrenal gland pheochromocytoma PC12 mixed adherent/suspension cell line from Cellonex (Separations, South Africa) was cultured in tissue culture treated (TPP) T-75 flasks using DMEM supplemented with 10% ν DES, 5% ν FBS and 1% ν P/S/AB solution in a humid 5% CO₂ atmosphere at 37°C. The culture medium was replaced at 75% every 2 days.

For the detection of cell proliferation and cyto-compatibility of the electrospun fibers, the MTT-based Roche Cell Proliferation Kit I was utilised. Electrospun film samples of size 1cm×1cm were sterilized under UV light for 12 hours before overnight incubation in 400 μ L culture medium containing 10% ν DES, 5% ν FBS and 1% ν P/S/AB in a 48-well plate maintained at 37°C at 5% CO₂. PC12 cells were seeded onto the film samples at a density of 2×10^4 cells/well and incubated for 72 hours. Thereafter, 40 μ L MTT solution was added to each well followed by a further 4-hour incubation period after which 400 μ L solubilising agent was added to dissolve the formazan crystals. The entire well contents were aspirated off, placed into 2mL Eppendorf tubes and centrifuged at 2000rpm for 5 minutes to separate suspended cells. The resulting supernatant was transferred into a 96-well plate and measured for absorbance at 550nm, with a background subtraction at 690nm, using a multi-plate reader (BioTek, USA).

5.3. Results and Discussion

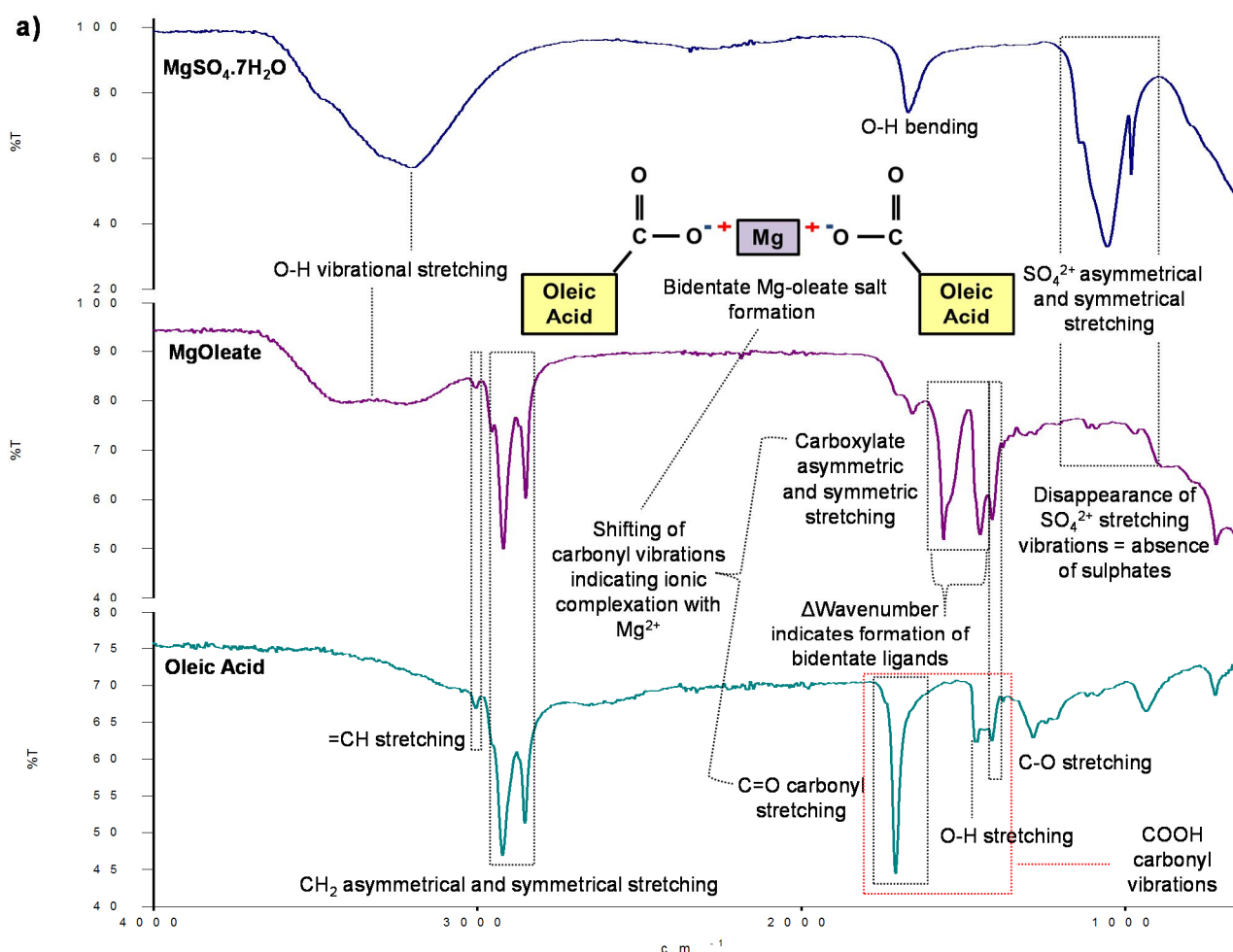
5.3.1. Validation of MgOleate complex synthesis

FTIR spectroscopy (Figure 5.3a) of the primary reagents, oleic acid and $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, were studied to elucidate the transitional vibrational shifts corresponding to the molecular composition of the resulting metal-oleate salt. Oleic acid presented with bands at 3004cm^{-1} , 2922cm^{-1} and 2853cm^{-1} corresponding to $=\text{CH}$ stretching in the $\text{C}=\text{CH}$ group, $-\text{CH}_2$ asymmetrical and symmetrical stretching, respectively. The carbonyl $=\text{COOH}$ groups of oleic acid presented with peaks at 1709cm^{-1} , 1463cm^{-1} and 1284cm^{-1} relating to $\text{C}=\text{O}$ carbonyl stretching, O-H band and C-O stretching, respectively. Bands at 1412cm^{-1} , 935cm^{-1} and 723cm^{-1} were indicative of CH_3 groups, out-of-plane O-H stretching and CH_2 rocking, respectively (Xu and Hu, 2012). Peaks indicating water of crystallisation in the heptahydrate region of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ appeared at 3202cm^{-1} and 2343cm^{-1} representing O-H vibrational stretching whereas O-H bending emerged at 1670cm^{-1} . Asymmetrical and symmetrical stretching vibrations of sulphate produced peaks at 1056cm^{-1} and 982cm^{-1} , respectively (Zhao *et al.*, 2006; Ovalles *et al.*, 2009). The FTIR spectrum (Figure 5.3a) of the synthesised MgOI complex revealed that the metal-oleate salt formed as a result of Mg^{2+} ions binding to the free carbonyl groups of oleic acid as indicated by the formation of new peaks occurring at 1561cm^{-1} and 1452cm^{-1} representing carboxylate asymmetrical and symmetrical stretching, respectively, and the shift of a weakened C-O stretching peak to 1308cm^{-1} (Ross and Lemay, 1985; Labidi and Iddou, 2007). The numerical difference between the carboxylate asymmetrical and symmetrical peak wavenumbers suggest the formation of a bidentate ligand between Mg^{2+} ions and COO^- groups in MgOI as described by Bronstein *et al.*, 2007.

TGA analysis further confirmed the formation of the MgOI complex by studying the thermal degradation patterns of oleic acid and $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ as shown in Figure 5.3b. Oleic acid presented with 2 phases of degradation: the first ranging from $310.91\text{--}326.72^\circ\text{C}$ corresponding to the thermal decomposition of carboxylates and the second phase occurring from $400\text{--}800^\circ\text{C}$ indicating thermal cracking of the hydrocarbon chains (Manzi-Nshuti *et al.*, 2009). The thermal degradation of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ occurred in 4 stages: the first stage at 97.88°C indicative of water loss and the second (147.08°C) and third (287.82°C) stages attributed to the decomposition of the organic sulphate region of the compound. The fourth stage occurring between $300\text{--}800^\circ\text{C}$ presented with no change in weight loss and thus indicated the inorganic magnesium component forming 50% of the sample mass. Likewise, the thermal decomposition of the organic and inorganic constituents of MgOI occurred in 3 steps (Figure 5.3b): the first step occurring over $342.63\text{--}375.63^\circ\text{C}$ was indicative of carboxylate decomposition and the second step at 452.59°C suggested thermal cracking of

hydrocarbons. A shift to higher temperatures is noted in the first step corresponding to carboxylate degradation which could be attributed to the formation of ionic complexation with Mg^{2+} ions thereby increasing thermal stability (Figure 5.3b). The first 2 steps involved organic decomposition of the compound whereas the last step, 550-850°C, presenting with minimal change in weight indicated the inorganic magnesium residue at 9.1% of the total sample mass. DSC analysis established the melting point of MgOI as 128.34°C with the onset of thermal degradation at 270.58°C. The high thermal stability of MgOI indicates the presence of elemental magnesium.

Both FTIR and TGA analysis confirm the synthesis of a magnesium-oleate salt complex where unreacted reagents and sulphate or sodium residues are undetectable. Samples subject to EDS analysis further revealed the presence of only magnesium in the electrospun PHBV-MgOI fibers.



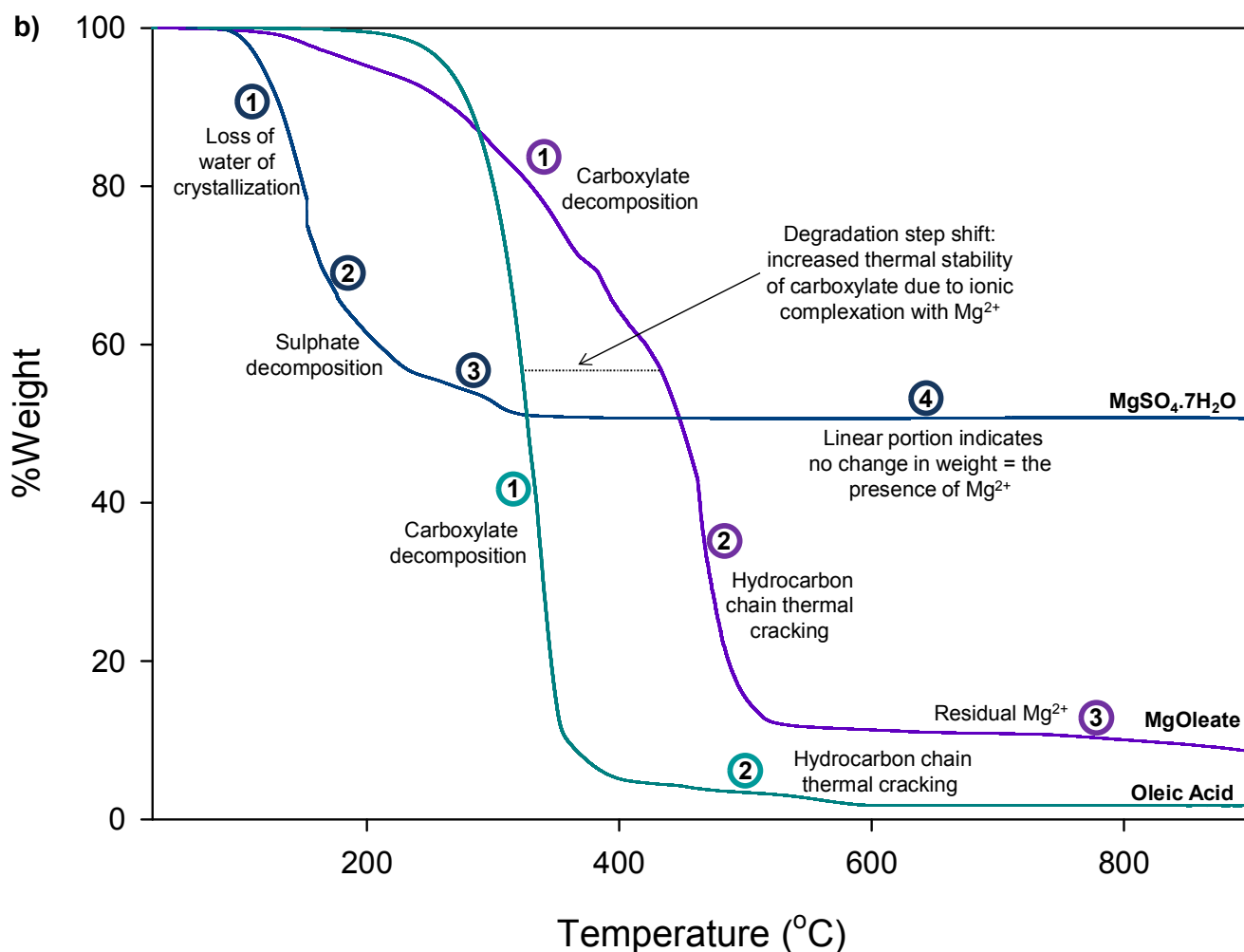


Figure 5.3: Validation of MgOI synthesis a) FTIR spectra showing the composition of the reagents used to synthesize MgOI and the mechanism of ionic bidentate complexation and b) TGA thermograms showing the presence and increased thermal stability of the synthesized MgOI compared to its primary constituents.

5.3.2. Assessment of vibrational molecular and thermophysical transitions of PHBV fibers upon MgOI and NAC incorporation

FTIR spectroscopy of the experimental design formulations validated the PHBV-MgOI blend where the molecular composition of the electrospun fibers remained similar to that of the parent constituents, PHBV, MgOI and NAC (Figure 5.4). Formulations containing NAC presented with a broad band at 3370cm^{-1} indicative of N-H stretching and weak bands at 2547cm^{-1} and 1916cm^{-1} where the former is attributed to S-H stretching (Picquart *et al.*, 1998). Bands at 2927cm^{-1} were indicative of PHBV symmetrical $-\text{CH}_2$ stretching and MgOI asymmetrical stretching whereas the weak peak at 2854cm^{-1} represented MgOI $-\text{CH}_2$ symmetrical stretching vibrations (Ross and Lemay, 1985; Labidi and Iddon, 2007; Cheng *et al.*, 2009). Peaks at 1378cm^{-1} were attributed to $\text{CH}_2\text{-CO}$ vibrations of PHBV (Cheng *et al.*,

2009). Asymmetrical and symmetrical stretching of the magnesium-carboxylates groups of MgOI are assigned to peaks at 1564cm^{-1} and 1454cm^{-1} where the latter could represent scissoring vibration of $-\text{CH}_2$ groups of PHBV as well (Ross and Lemay, 1985; Labidi and Iddon, 2007; Cheng *et al.*, 2009). The high intensity peak at 1720cm^{-1} is attributed to the $\text{C}=\text{O}$ vibration of the carboxylic groups of PHBV and reflects the highly crystalline composition of the polymer (Cheng *et al.*, 2009). It was noted that the intensity of this crystalline peak decreased upon increased MgOI addition compared to formulations containing less MgOI, as shown in Figure 4: F5 and F7, thus suggesting a change in the crystalline nature of the polymer.

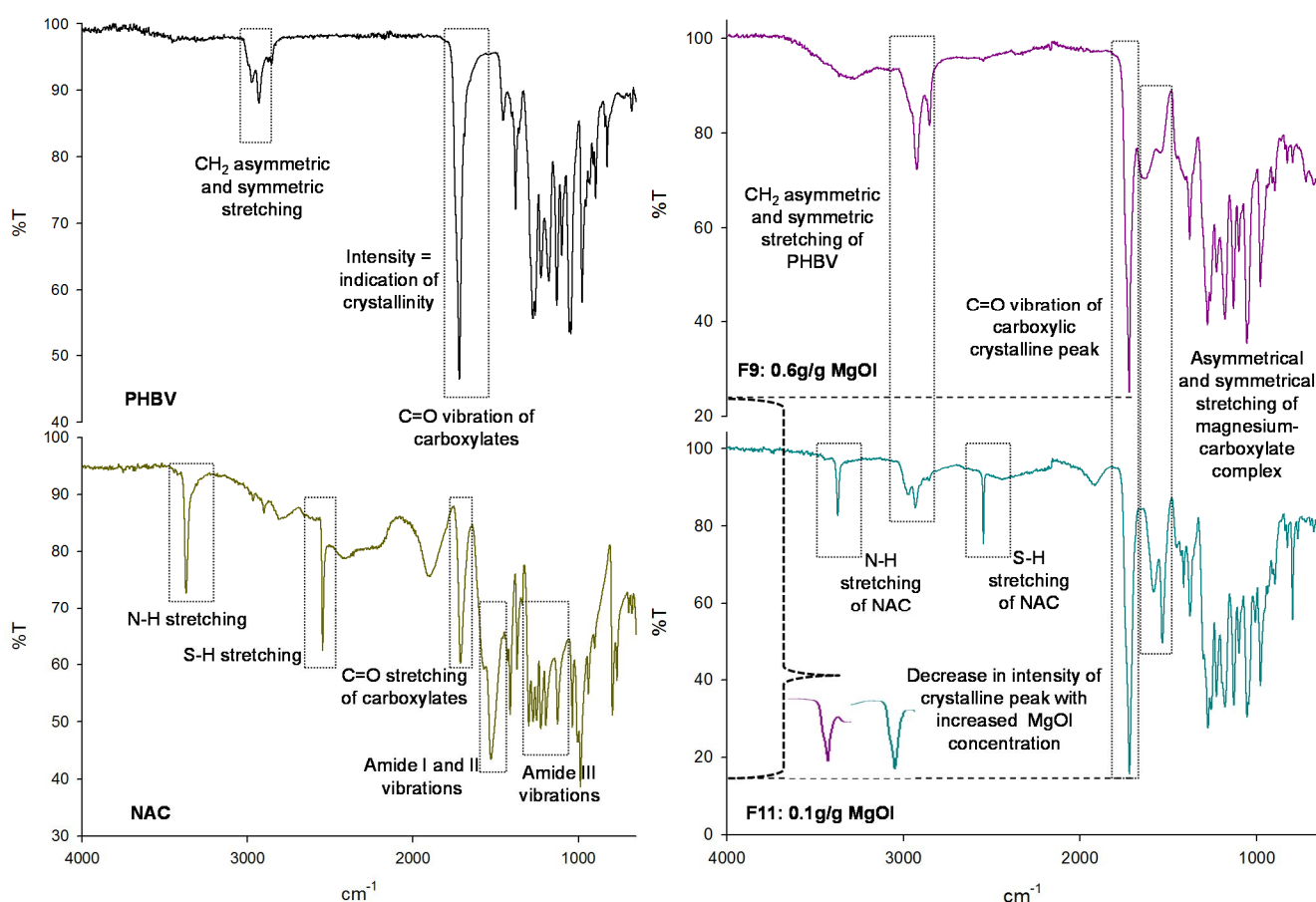


Figure 5.4: FTIR spectra of pristine PHBV and NAC and F5 and F7 showing molecular compositions and transitions upon blending of PHBV with MgOI and NAC.

The blending of MgOI with and incorporation of NAC in the PHBV polymer base did not induce any notable structural molecular changes in the final electrospun film fibers. However, DSC and TGA analysis revealed thermophysical transitions relating to melting points, onset of degradation and overall thermal decomposition when upon the addition of MgOI and NAC.

TGA thermograms depicted the mass degradation of the experimental formulations occurring in 2 major steps. The first step occurring at 230°C suggested the degradation of NAC and appeared only in formulations containing NAC since F6, F12 (shown in Figure 5.5), F13 and F15 free of NAC were absent of this peak. It was further noted that F14 containing the maximum variable concentrations of PHBV and MgOI and only 1%^{w/w} NAC did not present this peak. Similarly, F3, F4 and F5 containing 1%^{w/w} NAC presented with weak shouldering peaks ranging from 233°C and 236°C. The major decomposition peak indicative of PHBV degradation at 298.36°C (in pure PHBV) was significantly decreased upon MgOI addition, ranging from 254.82-282.53°C. This suggested that formulations containing higher concentrations of MgOI possessed lower thermal stability, as seen in Figure 5.5 with F9 (265.23°C) and F12 (254.49°C), attributed to the decrease in crystallinity as noted in FTIR analysis of the PHBV carboxylate peak (Figure 5.4) whereas stability was improved upon NAC addition and decrease in MgOI as noted in F11 (282.53°C) containing 2%^{w/w} NAC (Figure 5.5). These results corresponded to that obtained from DSC analysis.

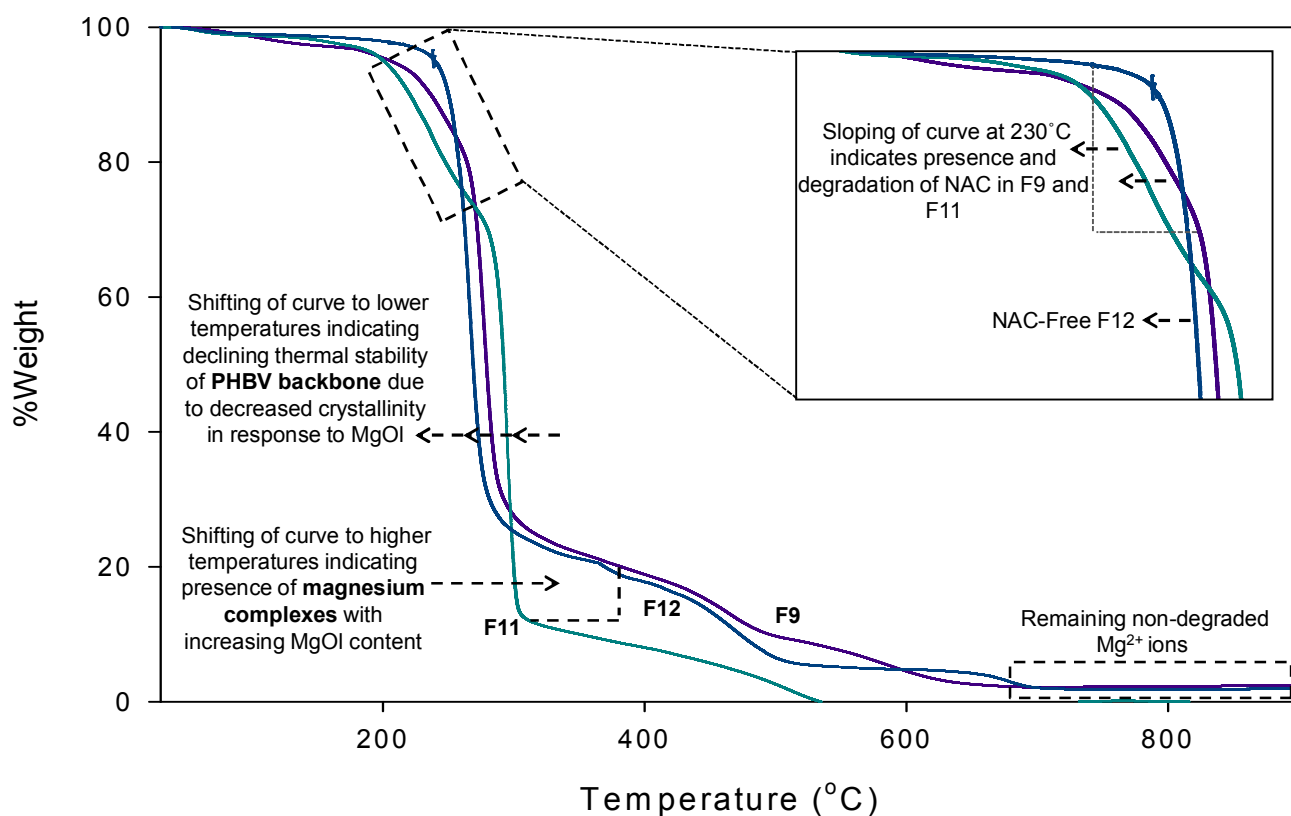


Figure 5.5: Representation of TGA curves depicting the transitions in thermal degradation properties of the formulations upon incorporation of MgOI and NAC-loading into the electrospun PHBV fibers.

DSC curves, shown in Figure 5.6, of the experimental formulations depicted the emergence of 2 distinct endothermic peaks corresponding to PHBV melting and degradation. In some cases, an initial weaker endothermic peak at around 120°C was noted, which could be attributed to the melting of either MgOI or the hydroxyvaleric acid co-polymer in PHBV. The melting points (T_m) of the parent constituents were established as 154.71°C, 128.34°C and 111.67°C for PHBV, MgOI and NAC, respectively. The T_m and degradation temperature (T_d) of pure PHBV peaking at 282.54°C was noted to decrease after the addition of MgOI as indicated in the comparisons in Figure 5.6c-f of selected formulations composed of the lower and upper limit of MgOI. Changes in PHBV content of the film fibers presented no change in the T_m and T_d . It was noted that for formulations composed of 5%^{w/v} (F5 and F7) and 10%^{w/v} (F14 and F10) PHBV, increase in MgOI concentration resulted in an increased T_m (151.61°C and 149.59°C; 150.14°C and 148.22°C, respectively) and decreased T_d (244.55°C and 253.49°C; 243.72°C and 256.15°C, respectively) whereas those composed of 7.5%^{w/v} PHBV resulted in the decrease of both T_m and T_d , as seen with F9 (141.59°C and 248.98°C, respectively) and F11 (146.25°C and 261.35°C, respectively). As shown in Figure 5.6b, formulations containing NAC demonstrated decreases in T_m compared to the NAC-free varieties (F9: 141.59°C and F12: 142.58°C; F11: 146.25°C and F15: 151.57°C; F1: 142.56°C and F6: 149.59°C; F2: 142.42°C and F13: 151.77°C) which could be attributed to the lower T_m of NAC, however, such formulations tolerated higher T_d temperatures making the NAC-loaded fibers more heat stable. The experimental formulations presented with a T_m in the range of 141-152°C and a T_d ranging between 206-262°C. The blending of MgOI with PHBV resulted in the emergence of the glass transition temperature (T_g), as shown throughout Figure 5.6, at approximately 3°C whereas the T_g of PHBV in Figure 5.6a is absent due to its probable occurrence at temperatures lower than 0°C. In addition to the effects of MgOI and NAC, it was further revealed that the electrospinning process too impacted the thermal properties of the formulations. A sample of pure PHBV (7.5%^{w/v}) electrospun fibers were determined as having a T_m and T_d of 152.90°C and 264.82°C, respectively, being considerably lower than that of neat unmodified PHBV pellets. This sample of pure electrospun PHBV was further utilized as a comparative to evaluate the effects of electrospinning on formulation crystallinity.

The thermophysical transitions, in conjunction with FTIR analysis, suggest an alteration of the crystalline properties of the polymer rather than chemically-induced changes as indicated by the weakened thermal stability of the experimental formulations and the absence of major molecular vibrational shifts.

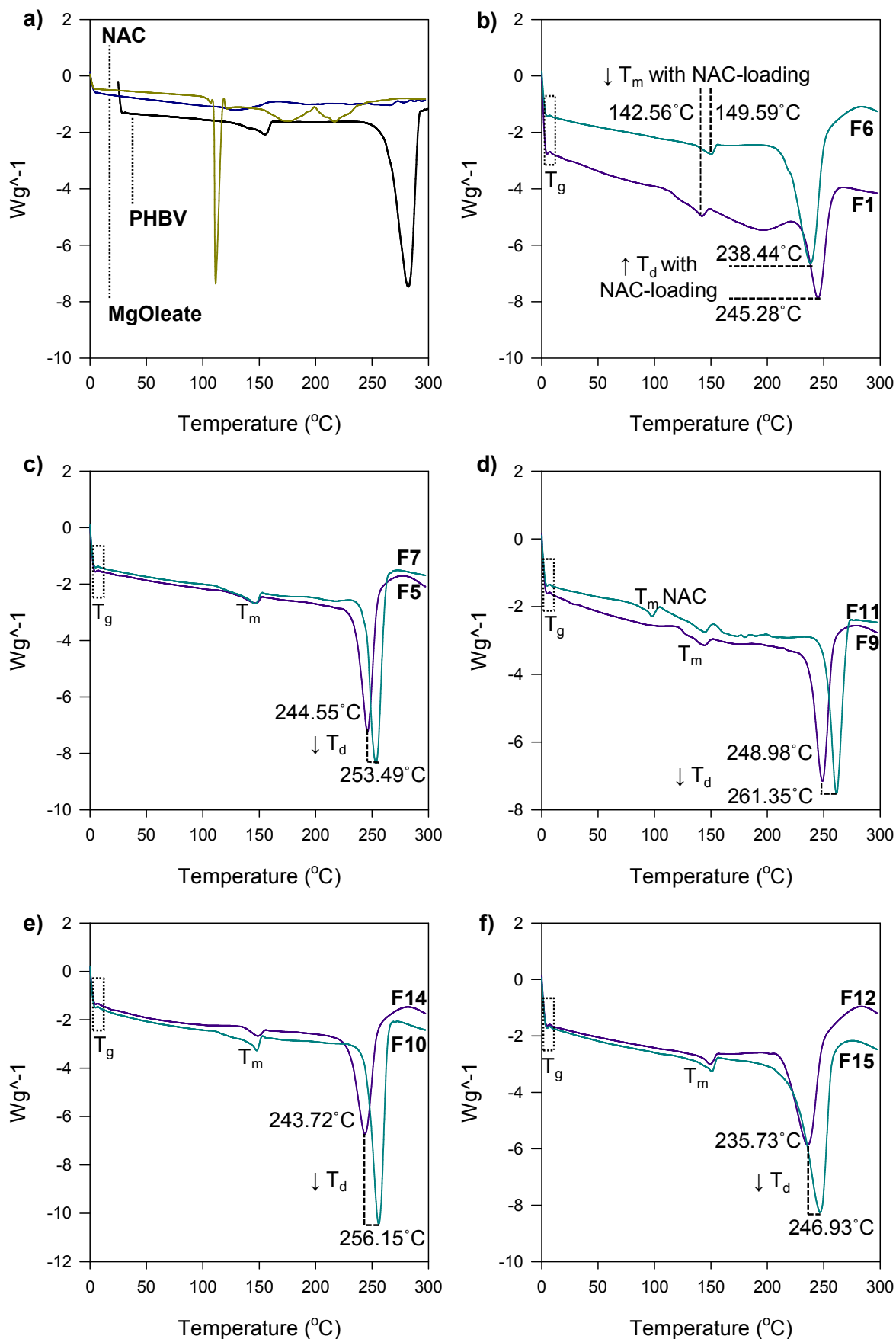


Figure 5.6: DSC thermograms of a) the parent constituents and representation of the changes observed with b) NAC-loading (F1) and c-f) increase in MgOI from 0.1g/g (F7, F11, F10 and F15) to 0.6g/g (F5, F9, F14 and F12).

5.3.3. The effect of MgOI, NAC and electrospinning on PHBV fibre crystallinity

FTIR spectroscopy was used to determine the changes in crystallinity imparted by the addition of MgOI and NAC and the overall electrospinning process by noting the changes in intensity of various crystalline and amorphous peaks of PHBV. It has been established that the bands appearing at 1720cm^{-1} , 1278cm^{-1} , 1230cm^{-1} and 980cm^{-1} are characteristic of crystalline states of PHBV whereas amorphous bands are attributed to signals appearing at 1740cm^{-1} and 1185cm^{-1} (Li *et al.*, 2004; Buzarovska *et al.*, 2009; Wei *et al.*, 2015). The peaks appearing at 1382cm^{-1} and 1453cm^{-1} are considered insensitive to the composition and crystallinity of PHBV blends and hence are used as normalising peaks when determining the Crystallinity Index (CI), as an indication of the degree of crystallinity, of the various formulations (Li *et al.*, 2004; Luo *et al.*, 2007; Sridhar *et al.*, 2013). The ratios of the absorbance peaks at $1382\text{cm}^{-1}/1185\text{cm}^{-1}$ (Bloembergen *et al.*, 1986; Li *et al.*, 2004; Sridhar *et al.*, 2013) and $1453\text{cm}^{-1}/1185\text{cm}^{-1}$ (Luo *et al.*, 2007) provided a measure of the amorphous composition of the formulations. The extent of crystalline content was determined by the ratios of the absorbance peaks at $1720\text{cm}^{-1}/1740\text{cm}^{-1}$ and $1230\text{cm}^{-1}/1453\text{cm}^{-1}$ (Wei *et al.*, 2015).

It is noted that process of electrospinning yields changes in the crystalline state of PHBV. The results obtained show minimal amorphous change between PHBV pellets, in its raw unprocessed form, and electrospun fibrous films (Δ 0.039-0.044). However, the CIs determined from the $1720\text{cm}^{-1}/1740\text{cm}^{-1}$ and $1230\text{cm}^{-1}/1453\text{cm}^{-1}$ bands demonstrate increased crystallinity of the polymer upon electrospinning (3.048 and 3.614, respectively) compared to unprocessed PHBV (2.605 and 3.365, respectively) as shown in Figure 5.7a. Pure electrospun PHBV presented with the highest FTIR-derived CI in comparison to the experimental formulations. Likewise, it was noted that the experimental formulations presented with minimal CI changes with regards to the amorphous bands in contrast to the crystalline bands which exhibited significant variations to the addition of MgOI and NAC. Formulations containing NAC presented with higher CI values, corresponding to particularly the $1230\text{cm}^{-1}/1453\text{cm}^{-1}$ bands, compared to those without NAC, therefore suggesting increased crystallinity of the formulations in response to its addition. Contrasting, the CI values corresponding to the $1720\text{cm}^{-1}/1740\text{cm}^{-1}$ crystalline region of PHBV depict a decrease in CI with NAC addition, thus indicating decreased crystallinity. However, it is a possibility that these sets of bands may be subject to signal interference emanating from the amide group at $1220\text{-}1310\text{cm}^{-1}$ or the C=O carbonyl stretching of NAC at 1712cm^{-1} , thereby resulting in opposing observations. Increased concentrations of MgOI resulted in profoundly decreased crystallinity, as indicated by the CI values, in both the $1720\text{cm}^{-1}/1740\text{cm}^{-1}$ and $1230\text{cm}^{-1}/1453\text{cm}^{-1}$ set of bands, thereby, indicating a shift in the polymer to a more

amorphous state in the formulations upon MgOI incorporation. The decrease in crystallinity, noted from the $1720\text{cm}^{-1}/1740\text{cm}^{-1}$ and $1230\text{cm}^{-1}/1453\text{cm}^{-1}$ bands, respectively, resulting from MgOI increase was particularly noted in the formulations highlighted in Figure 5.7b-e: b) F7 (2.45 and 3.13) and F5 (2.18 and 2.44); c) F11 (2.28 and 3.50) and F9 (1.97 and 2.33); d) F10 (2.61 and 3.17) and F14 (2.10 and 2.23); e) F15 (2.85 and 3.23) and F12 (2.40 and 2.02).

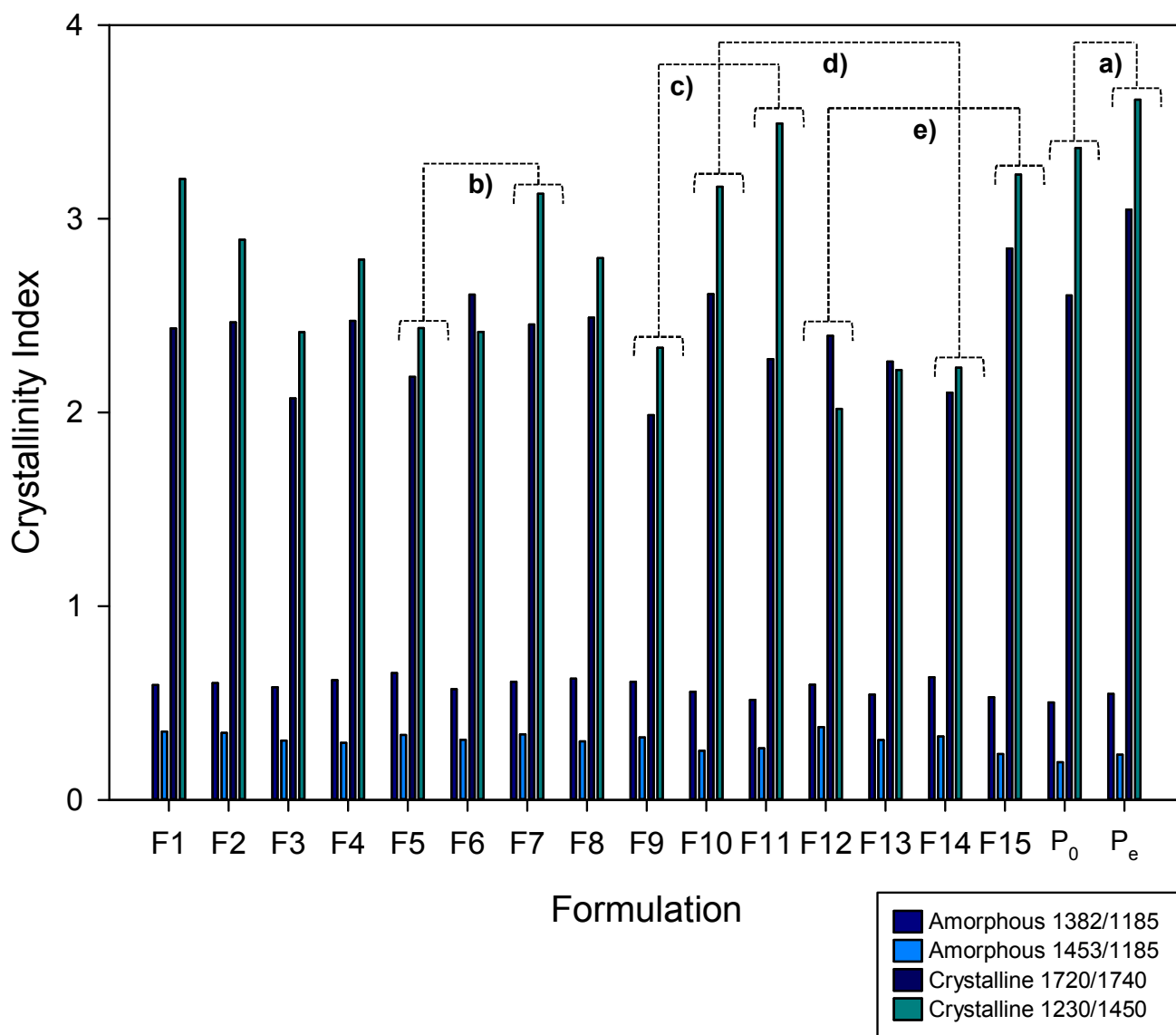


Figure 5.7: Comparison of crystallinity transitions observed from FTIR analyses depicting increased CIs resulting from electrospinning a) P₀ (unprocessed PHBV) and P_e (pure electrospun PHBV) and upon increased MgOI concentration from 0.1g/g to 0.6g/g, respectively b) F7 and F5, c) F11 and F9, d) F10 and F14 and e) F15 and F12.

DSC was employed to further study the crystallinity transitions induced by MgOI and NAC as established by FTIR characterisation. Although the incorporation of MgOI in the present study was intended to function as potential neuro-protectant for injured and regenerating neuronal cells, previous research has shown the ability of oleic acid and metal-oleate salts in the altering of mechanical properties of PHAs via its effect as nucleating agents which accelerate crystallization rate. Upon introduction of a nucleating agent, the increased nucleation density and crystallization rate of the polymer results in reduced spherulite size and thus may induce subsequent changes in crystallinity (Kai *et al.*, 2005). DSC was used to obtain a quantitative measure of crystallinity of neat PHBV, pure electrospun PHBV and the PHBV-MgOI electrospun fibers with and without NAC. To evaluate changes in the crystallization rate, nonisothermal melt crystallization temperatures (T_{mc}) were recorded from the calorimetric curves generated from the subsequent cooling of the samples from a molten state. The %crystallinity was determined using the enthalpy of fusion of pure electrospun PHBV which was taken to be the 100% crystalline reference polymer since FTIR analysis established it to be more crystalline than unprocessed PHBV. This was further validated when DSC analysis revealed the enthalpy of fusion of electrospun PHBV as 25.5 whereas that of unprocessed PHBV was lower at 19.63, therefore, confirming that electrospinning indeed produces an increased crystalline state of the polymer. Using pure electrospun PHBV as the 100% crystalline reference, the %crystallinity of unprocessed PHBV was calculated to be 76.98%

In general, the results obtained from DSC analysis, depicted in Figure 5.8, showed that formulations exhibiting lower T_{mc} s in the range of 61-75°C presented with increased %crystallinity between 62-76%. Formulations characteristic of this observation were F11, F15 (containing 0.1g/g MgOI), F6 and F13 (containing 0.35g/g MgOI). In contrast, F1, F2, F3, F5 and F9 containing higher concentrations of MgOI (0.35-0.6g/g) presented with higher T_{mc} s in the 76-79°C range and lower %crystallinities between 41-58%. It is considered that high T_{mc} temperatures are indicative of faster crystallization (Kai *et al.*, 2005 and Qian *et al.*, 2007). Therefore, the results demonstrate the effects of increased crystallization rates with increased concentrations of MgOI as a potential nucleating agent. In addition to formulations containing higher MgOI concentrations exhibiting increased T_{mc} s and crystallization rates, decreased crystallinity was noted as indicated by both DSC and FTIR analyses.

Furthermore, DSC results were consistent with that of FTIR concerning the effect of NAC on the overall formulation crystallinity. According to the findings derived from calorimetric studies, the addition of NAC resulted in increased T_{mc} s equating to faster crystallization rates and a subsequent decrease in crystallinity of the resulting formulations. The 4 formulations in

the experimental design (F6, F12, F13 and F15) free of NAC were compared to formulations (F1, F9, F2 and F11) equivalent in PHBV-MgOI composition but containing 4%^{w/w} NAC, respectively. Formulations F6, F12, F13 and F15 presented with lower T_{mc} temperatures of 71.66°C, 63.48°C, 61.49°C and 67.34°C and increased %crystallinities of 74.04%, 50.59%, 62.67% and 76.18%, respectively. In comparison to the NAC-loaded derivatives, F1, F9, F2 and F11 featured increased T_{mc} temperatures of 77.31°C, 78.33°C, 78.14°C and 75.67°C with significantly reduced %crystallinities of 44.35%, 41.53%, 54.35% and 71.57%, respectively. These results indicate that the addition of NAC, like MgOI, promotes acceleration of crystallization rate leading to the probable formation of smaller sized PHBV spherulites and an apparent consequential decrease in crystallinity thereby producing a shift to a more amorphous state of the polymer in the synthesized electrospun PHBV-MgOI-NAC composite fibers. This effect induced by NAC suggests its possible action as a nucleating agent.

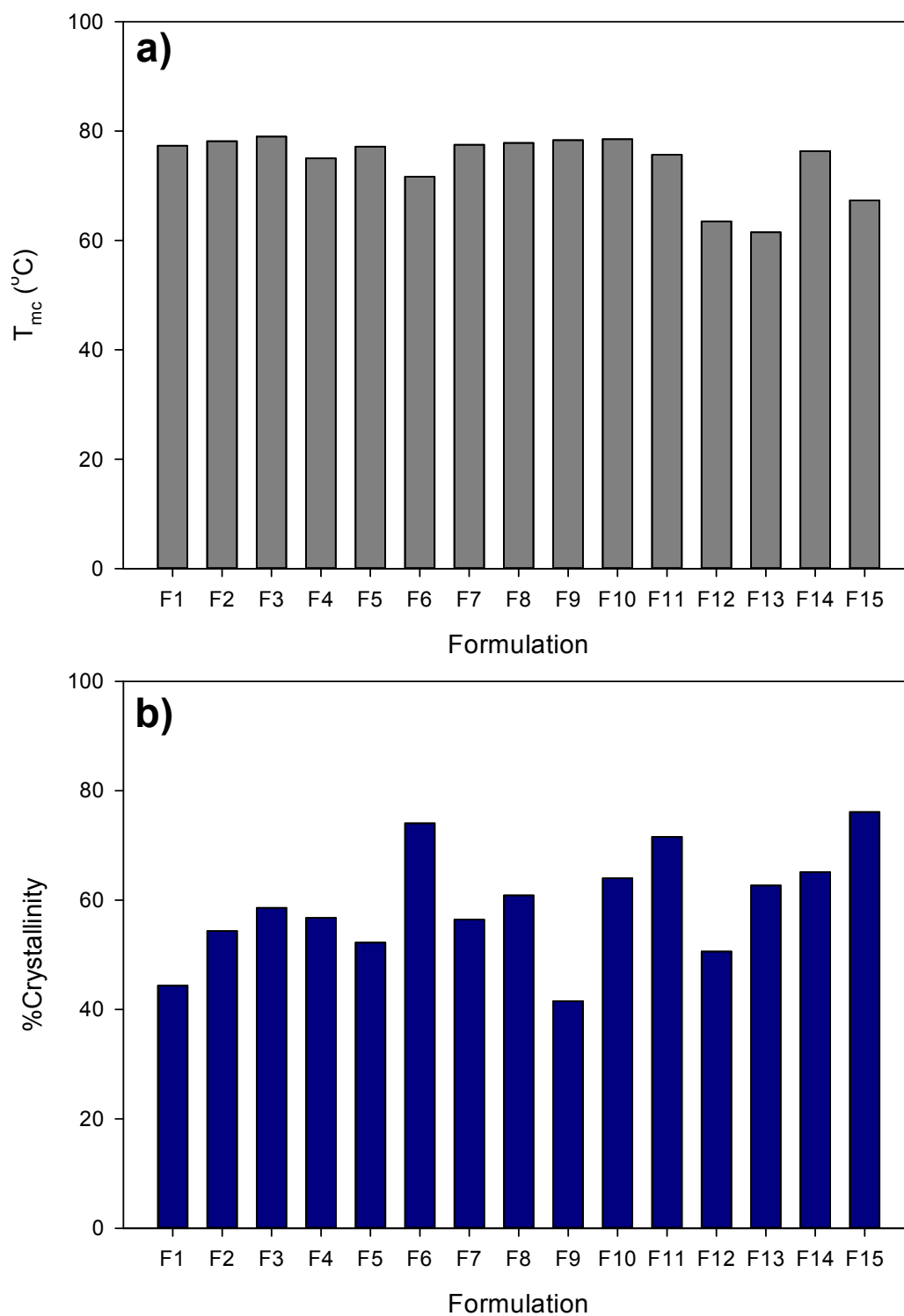


Figure 5.8: DSC analysis depicting a) changes in T_{mc} and b) %crystallinity utilizing pure electrospun PHBV as the crystallinity reference.

To further investigate the changes in PHBV crystallite size of the experimental formulations in response to MgOI and NAC incorporation, XRD analysis was conducted. The %crystallinity derived from the XRD peak intensity of $2\theta = 16^\circ$ was correlated to that determined from DSC. The XRD diffractograms in Figure 5.9 depicted the occurrence of 2 principal peaks characteristic of PHBV and the experimental formulation samples at $2\theta = 13^\circ$ and 16° , which correspond to the (110) and (020) planes, respectively. The $2\theta = 13^\circ$ peak, varying notably in response to changes in chemical composition, was used to calculate the crystallite size of the various samples.

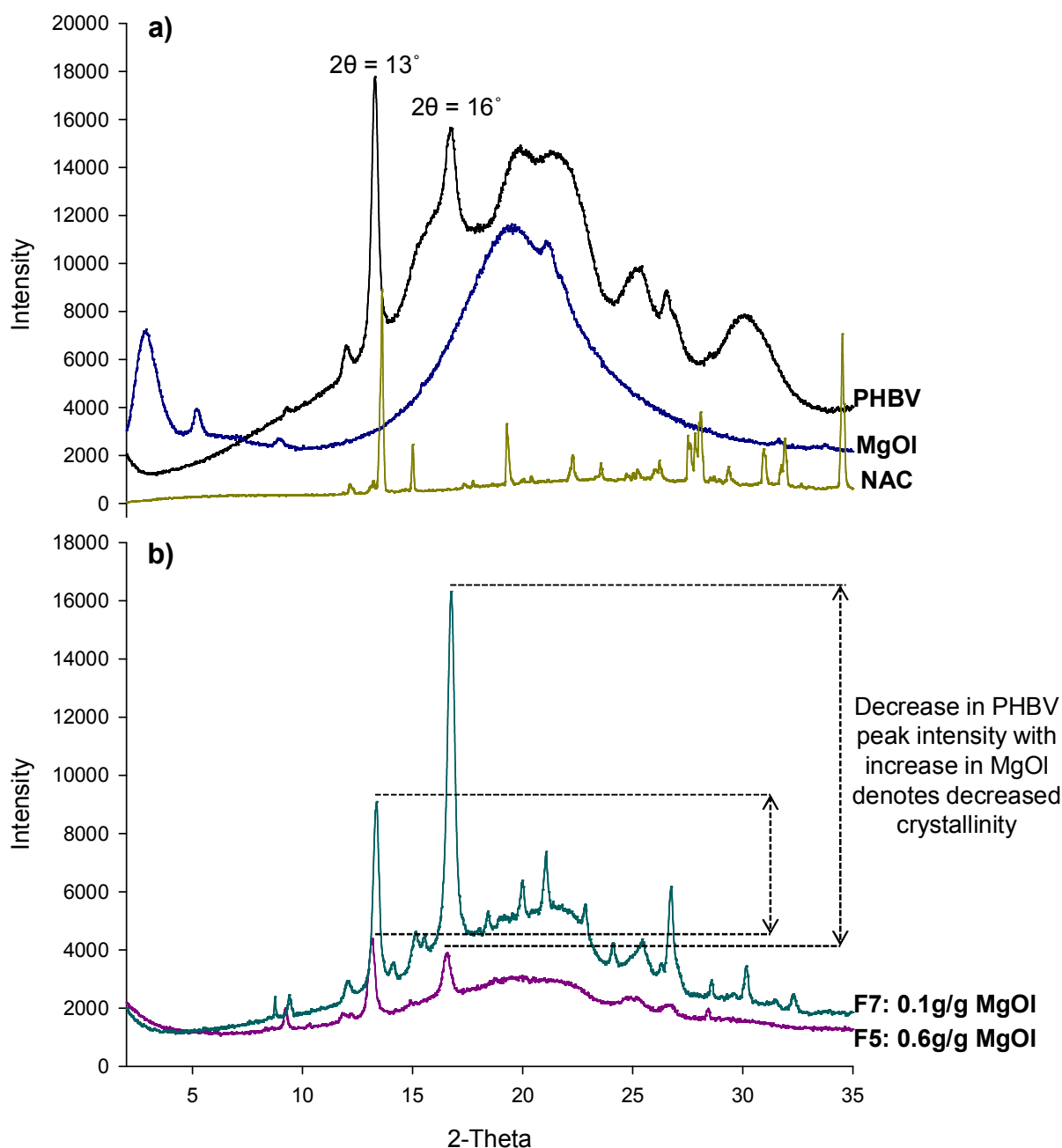


Figure 5.9: XRD diffractograms depicting a) analyses of the parent constituents and b) the decrease in crystallinity of PHBV upon increase in MgOI with reference to F5 and F7.

As listed in Table 5.4, pure electrospun PHBV presented with a slightly larger crystallites than neat PHBV of 4.50Å and 4.43Å, respectively. The electrospun composite fibrous films however, depicted generally larger crystallite sizes than that of both electrospun and neat PHBV with the exception of F5 (4.29Å), F9 (4.31Å) and F12 (4.30Å) all containing the maximum 0.6g/g MgOI concentration and exhibiting smaller sized crystallites. Formulations containing NAC presented with increased crystallite sizes compared to NAC-free formulations which could be attributed to the presence of large intact NAC crystal-like particles distributed throughout the PHBV-MgOI matrix and hence resulting towards the collective crystallite size encompassing both PHBV and NAC crystallites. Function as a nucleating agent, formulations containing 0.6g/g MgOI compared to those containing a 0.1g/g concentration demonstrated decreased crystallite size when PHBV and NAC concentrations were kept constant. Formulations F5, F9, F14 and F12 containing 0.6g/g MgOI had crystallite sizes of 4.29Å, 4.31Å, 4.75Å and 4.30Å, respectively. Decreased MgOI content (0.1g/g) in F7, F11, F10 and F15 resulted in increased crystallite sizes of 4.75Å, 4.99Å, 4.78Å and 4.60Å, respectively. The reduced crystallite sizes resulting from MgOI incorporation correlates to the increased crystallization rates elucidated from DSC nonisothermal melt crystallization studies. XRD analysis shown in Figure 8b depicts the decrease in crystallization observed, with regards to the crystalline peaks of PHBV at $2\theta = 13^\circ$ and 16° , when MgOI concentration is increased from 0.1g/g to 0.6g/g.

Table 5.4: List of XRD-derived %crystallinity and spherulite size of the experimental design

Formulation	%Crystallinity	Spherulite size (Å)
F1	42.41	4.66
F2	55.52	4.84
F3	58.01	4.64
F4	57.38	4.84
F5	47.25	4.29
F6	49.42	4.60
F7	64.38	4.75
F8	57.32	4.66
F9	48.89	4.31
F10	65.98	4.78
F11	64.01	4.99
F12	50.56	4.30
F13	46.72	4.54
F14	57.74	4.75
F15	52.15	4.60
Unprocessed PHBV	45.78	4.43
Pure electrospun PHBV	78.12	4.50

The infrared crystallinity indices and the %crystallinity calculated from the heat of fusion obtained from DSC both corresponded to the %crystallinity indices (%CrI) determined from XRD measurements. XRD %CrI, as listed in Table 5.4, indicated decreased crystallinity

upon increased MgOI concentrations. Reductions in crystallite size were congruent with decline in %crystallinity. On the contrary, The inclusion of NAC resulted in larger sized spherulites, however, it was noted that a combination of higher MgOI concentrations (0.35-0.6g/g) and lower PHBV concentrations (5-7.5%^{w/v}) with 4%^{w/w} NAC lead to decreased %CrI whereas lower MgOI concentrations (0.1-0.35g/g) and higher PHBV concentrations (7.5-10%^{w/v}) with 4%^{w/w} NAC produced increased crystallinity of the electrospun fibrous films. This observation could suggest that NAC inclusion acts synergistically with MgOI to yield an overall decreased crystallinity of the PHBV composite fibers, although it is not yet ascertained whether NAC could be undertaking a nucleating function, due the noted pattern of increasing crystallite size. The distribution of NAC particles throughout the polymer matrix however, may affect the crystallinity of PHBV via disruption of its crystalline structure upon recrystallization and regrowth of its spherulites.

5.3.4. SEM visualisation of surface morphology and EDS analysis of PHBV-MgOI fibrous films

SEM imaging of the electrospun fibrous film surface morphology reveals the formation of thread-like nanofibers orientated in a random to linear assembly (as indicated by the red arrows throughout Figure 5.10). The random orientation of some of the fibers confers a “branching” characteristic to the overall appearance of the fibrous films. This “branching” arrangement results in the formation of inter-fibre interstices (as shown throughout Figure 5.10 by the green markings) thus creating a net-like appearance and imparting a certain degree of porosity to the film. The formation of a porous meshwork is favourable as it enhances permeability of the films for uninterrupted gaseous exchange and permeation of nutrients to the regenerating tissues. In the case of lining a hollow conduit with the fibrous films an open meshwork is necessary to allow the diffusion of bioactive agents from the conduit wall to the regenerating tissues. Furthermore, it allows the sprouting axons to traverse through the interstices and around each fibre thereby maximising axonal interaction with the fibers while mimicking the native architecture of fibrin deposition and ECM. The fairly linear orientation of the electrospun fibers could further improve directional growth of the axons towards the distal nerve stump and reduce the chances misdirection and inadequate innervation of the target tissues. Should this be adequately achieved, an improvement in functional recovery of the injured site is expected.

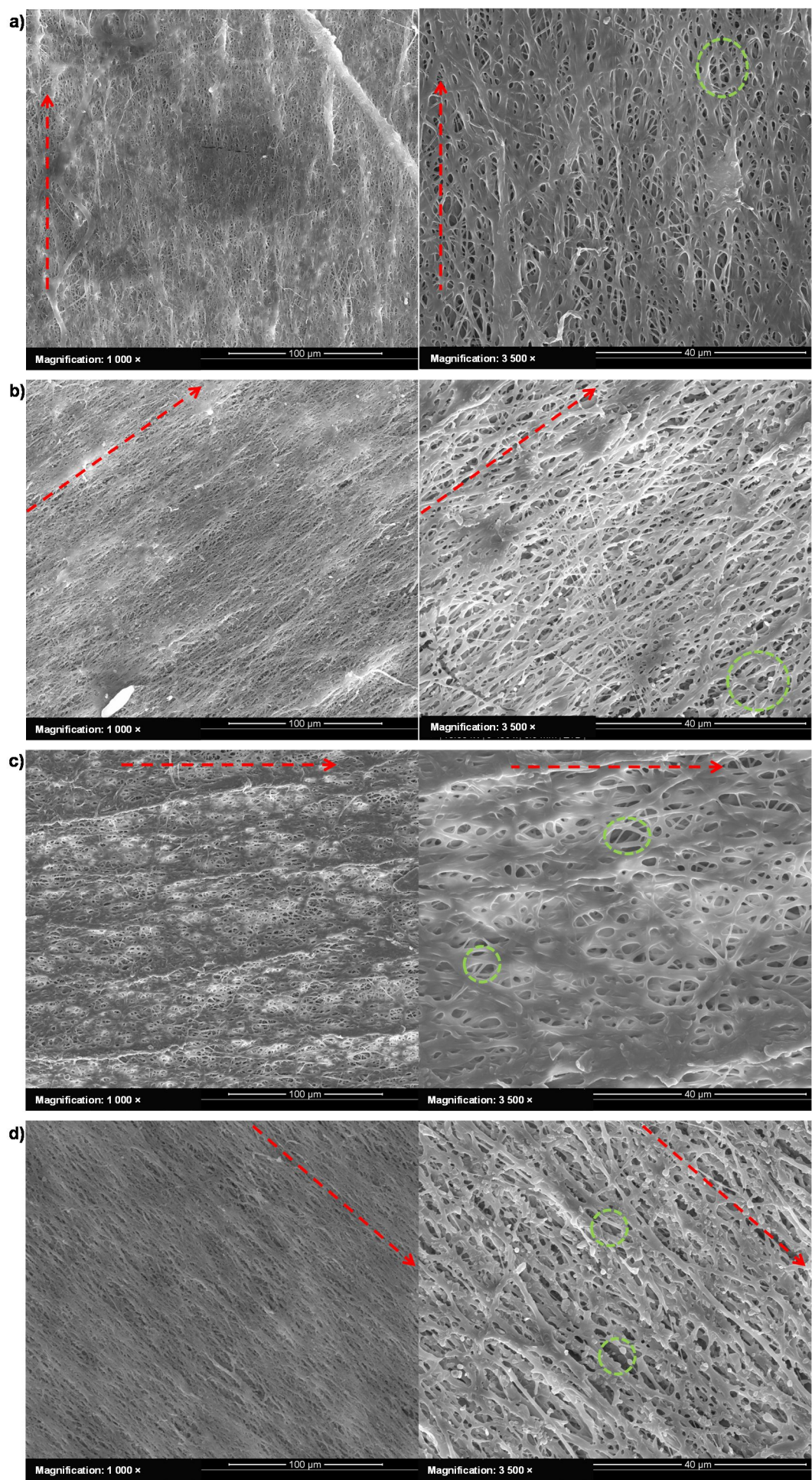
It is noted that increases in PHBV polymer concentrations from 5%^{w/v} to 10%^{w/v} (the lower and upper limits, respectively) result in the formation of fibers that are slightly thicker in diameter as noted with F7 and F10 in Figures 5.10b and 5.10f. Likewise, increased concentrations of MgOI appear to increase fibre diameter and thickness. The increase in

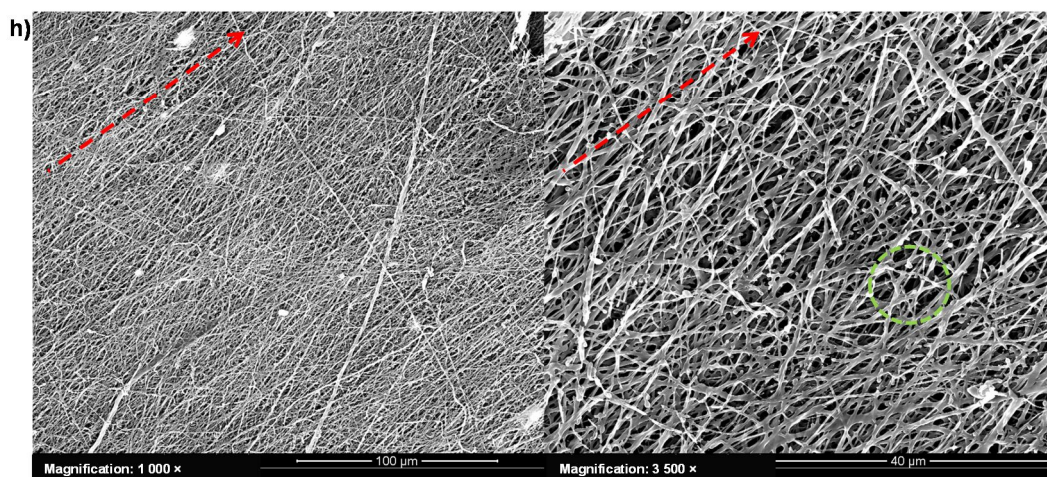
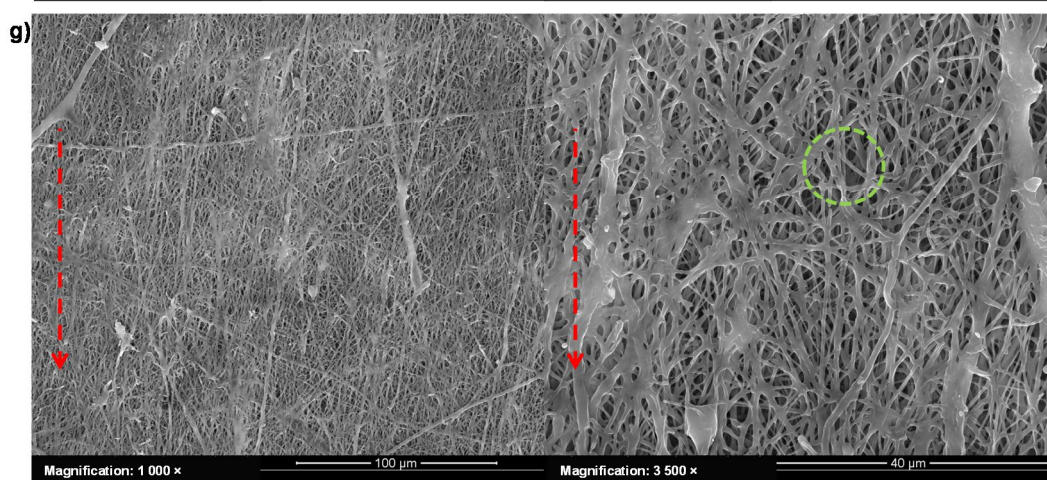
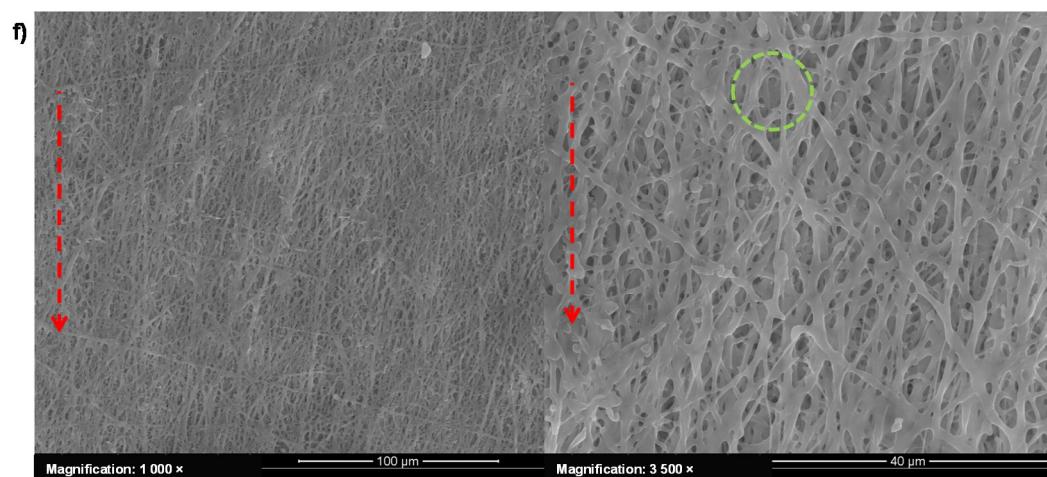
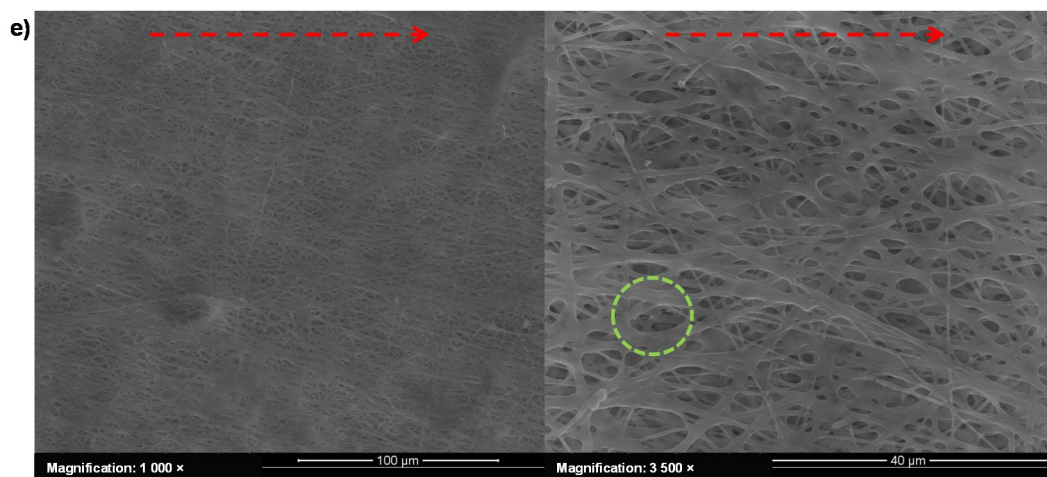
fibre diameter is attributed to the increase in viscosity of the electrospinning solution in response to the addition of increasing concentrations of polymer and MgOI thus affecting the stretching duration of the polymer jet and solvent evaporation (Tong and Wang, 2011). It is further noted that formulations containing 0.35g/g and 0.6g/g MgOI appear to coat the fibers thereby slightly increasing fibre size. Moreover, the upper limit of MgOI incorporation results in coating of the film surface creating a reduction in the number of visible open interstices. Another effect of MgOI on fibre electrospinning is the deposition and arrangement of the fibers. From Figures 5.10, it is evident that increasing MgOI content contributes to the formation of a greater number of randomly orientated fibers thus decreasing the linear directionality of the deposited fibers. Conversely, decreases in MgOI content results in the deposition of tighter packed fibers aggregating towards the formation of a linear unidirectional arrangement. The latter arrangement of the fibers would enhance controlled directional guidance of the regenerating axons whereas the former is desirable for the generation of porosity for enhanced permeability and tissue infiltration.

The incorporation of NAC is observed to affect fibre morphology and topography via induction of textural modification of the fibre surface. Formulations containing the maximum limit, 4%^{w/v}, concentration, of NAC impart a rough and spinous texture to the surface of the fibers whereas lower concentrations and absence of NAC contribute to the formation of smooth-surfaced fibers, as seen in Figure 5.10i indicated by the yellow arrows. A slightly roughened surface increases the total surface area available for cellular interaction and enhances cellular adhesion via provision of a topographical guidance cue; however, an excessively roughened surface with protruding jagged and sharp edges could result in unwanted cell damage and death.

Therefore, prudent adjustments in the concentrations of MgOI and NAC could assist in the attainment of appropriately suited topographical textured guidance cues and morphological properties of the electrospun fibrous films for enhanced cellular interaction.

Regarding the concentration of elemental magnesium in the electrospun fibers, via addition of MgOI, EDS analysis (Figure 5.11) revealed that formulations containing 0.35g/g and 0.6g/g MgOI contained between 1.4-1.7% weight magnesium. The detected carbon and oxygen emanate mostly from PHBV and oleic acid whereas sulphur detection stems from the inclusion of NAC. The EDS analysis confirms the successful incorporation of magnesium, as a therapeutic cue, in the electrospun fibrous films.





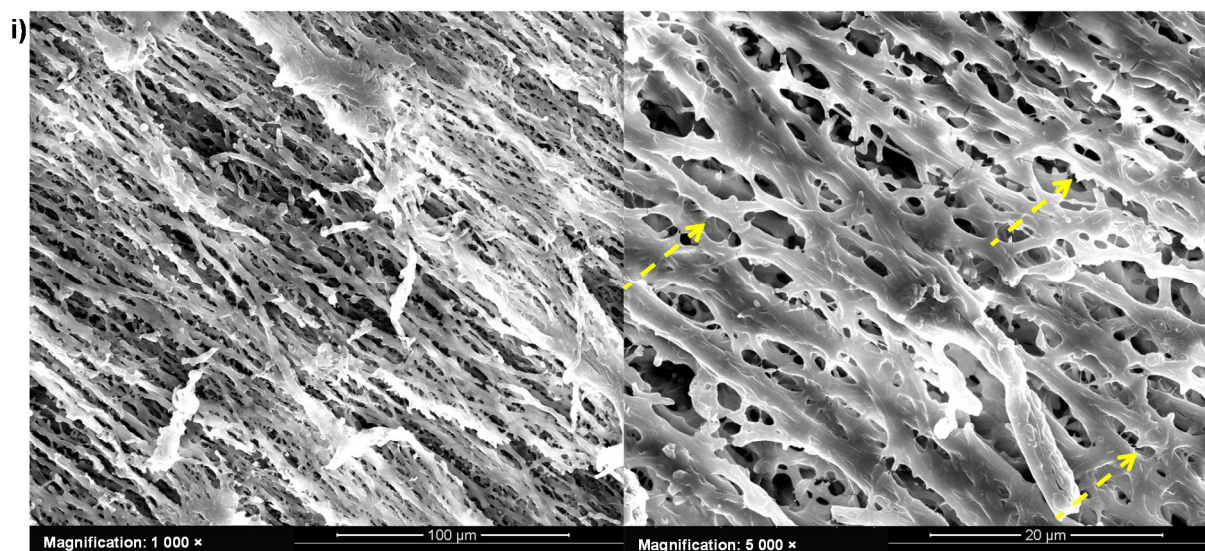


Figure 5.10: SEM surface morphology visualisation of selected electrospun formulations showing the changes in fiber morphology upon variations in MgOI at 1000× and 3500× magnification: 0.6g/g MgOI a) F5, c) F9, e) F14 and g) F12; 0.1g/g MgOI b) F7, d) F11, f) F10 and h) F15; depicting the effects of NAC-loading on fiber morphology at 1000× and 5000× magnification i) F1.

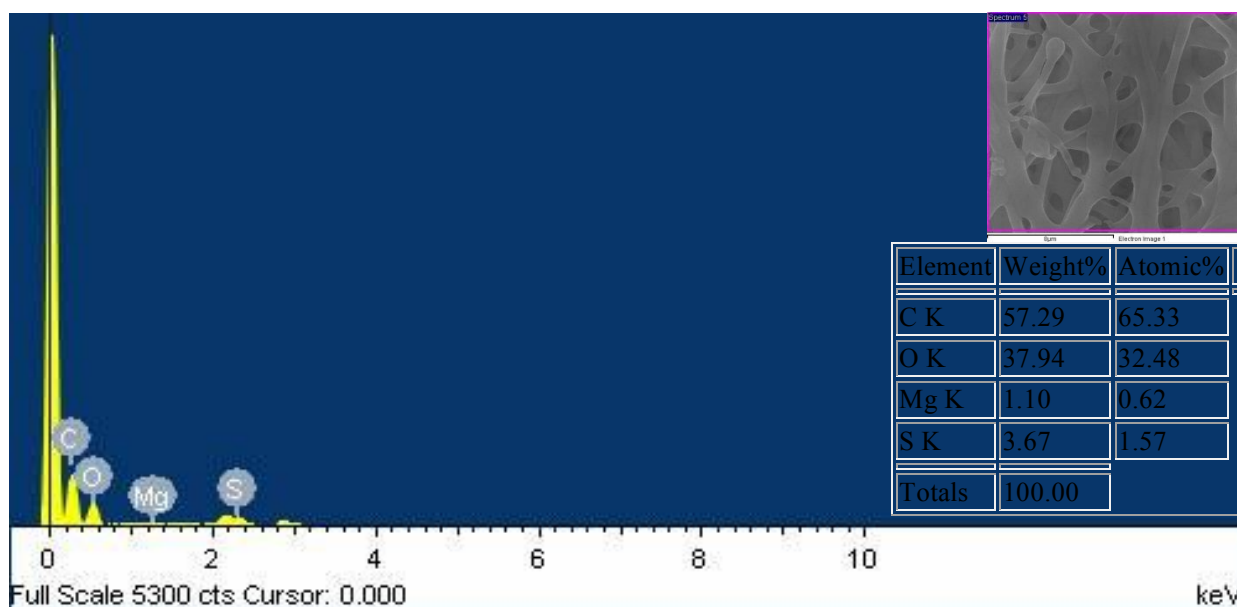


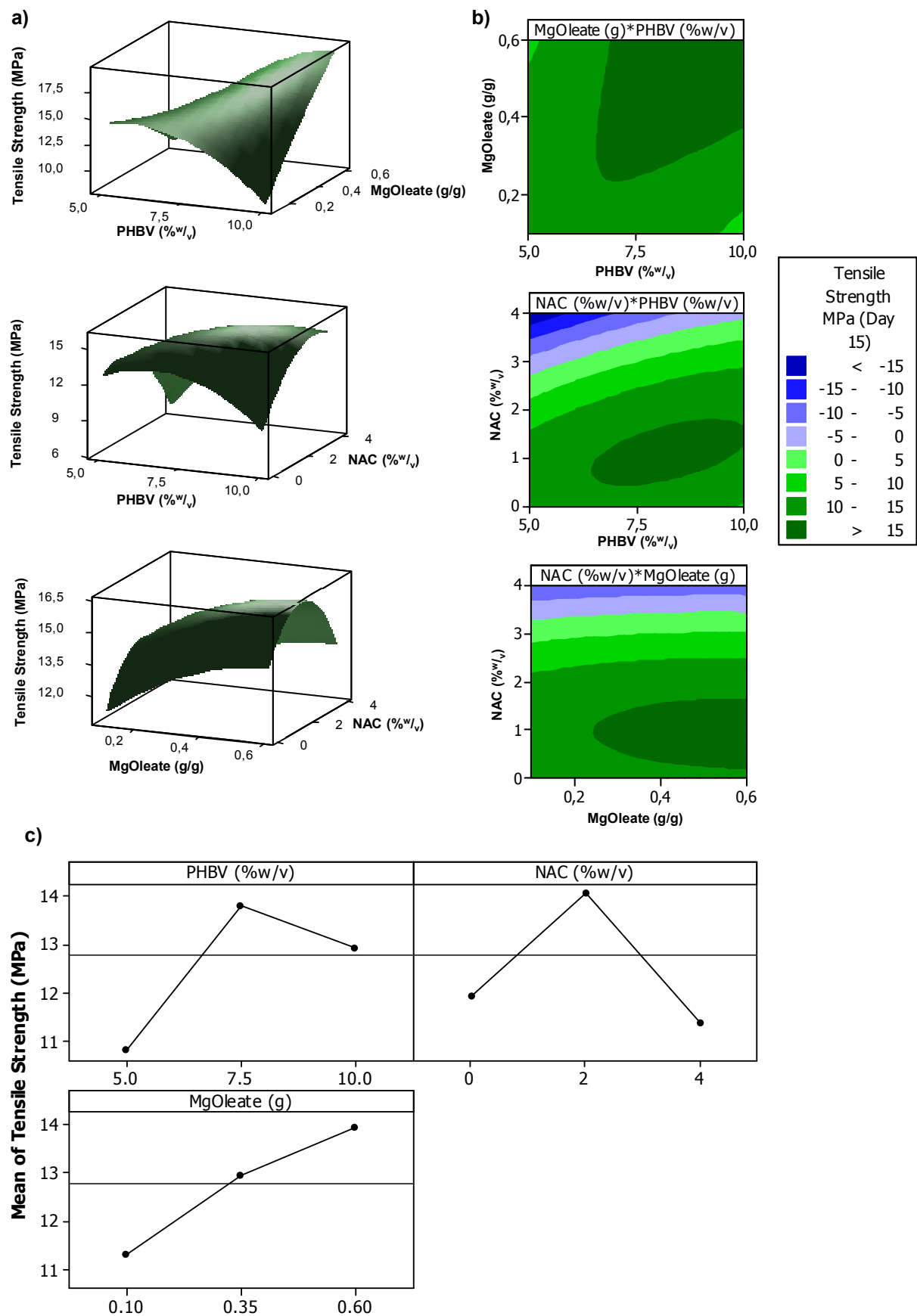
Figure 5.11: EDS representation of the elemental composition of F2 indicating the presence of elemental Mg from MgOI incorporation and the emergence of sulphur from NAC-loading.

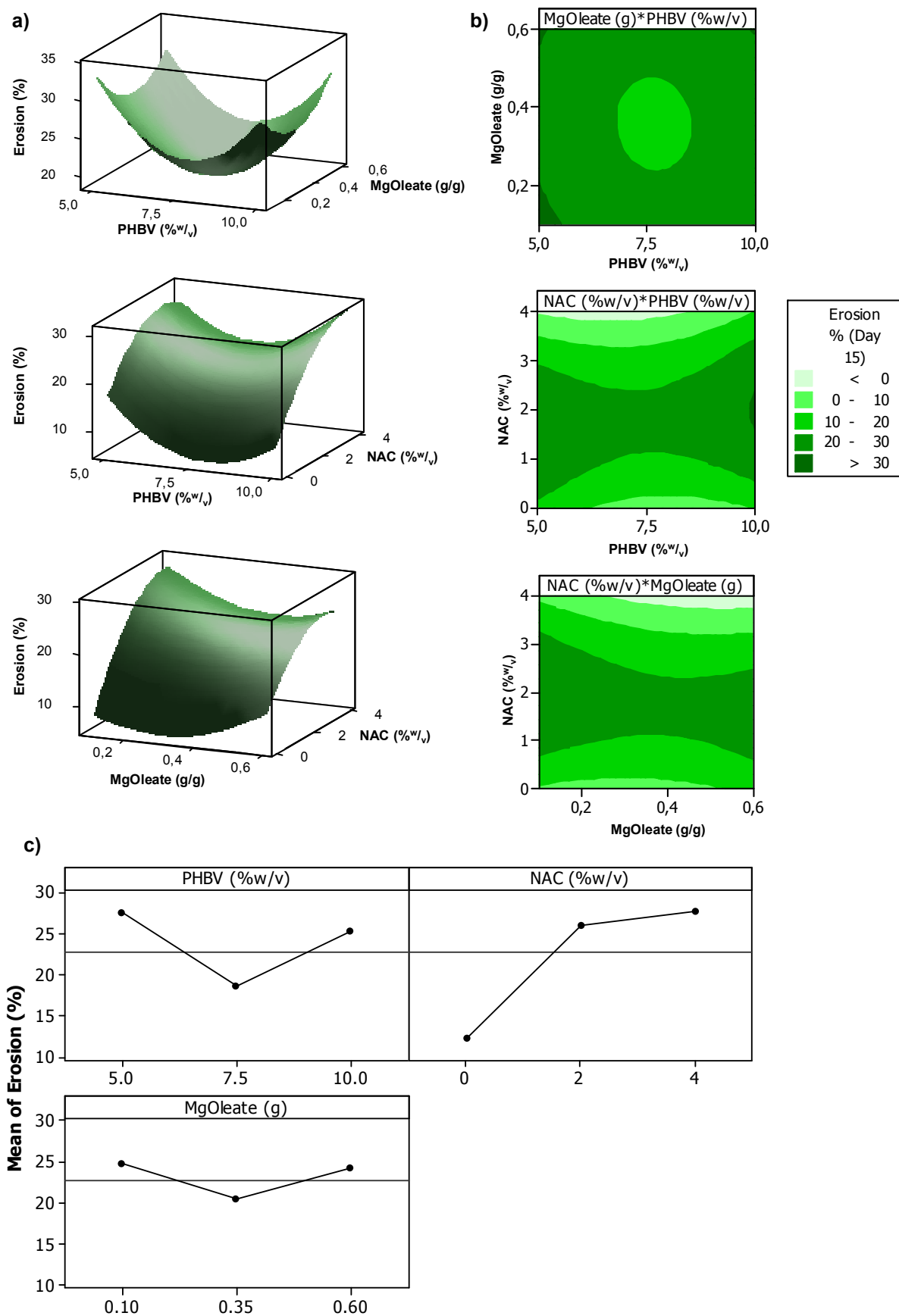
5.3.5. Response surface and statistical analysis of the Box-Behnken experimental design

Response surface and contour plots were utilized as representations of the functional relationships between the experimental independent variables and the measured response outcomes obtained from investigatory studies of the experimental formulations. Response analysis of tensile strength and %erosion were conducted with data collected from Day 15 of dissolution studies whereas that of %water-uptake was done after 24 hours of hydration.

As depicted in Figure 5.12, increase in PHBV polymer concentration with lower MgOI quantities produced a decrease in tensile strength after dissolution in PBS. Tensile strength improved upon combination with increasing quantities of MgOI due to a probable plasticising effect on the polymer. The effect of PHBV and NAC combinations on tensile strength presented a min\max bell-shaped response curve where the ideal NAC concentration for maximum improvement of tensile strength was 2%^{w/v} and anything below or above that concentration resulted in a decrease in tensile strength. A similar effect is noted with regards to the relationship of MgOI and NAC combinations, where enhanced tensile strength was observed with progressively increasing concentrations of MgOI and an optimal concentration of 2%^{w/v} of NAC.

The effects of PHBV and MgOI concentrations on the erosion properties of the electrospun fibrous films, shown in Figure 5.13, presented an inverted bell-shaped curve depicting 2 maxima and 1 minima point. This indicated that concentrations of PHBV and MgOI closer to the midpoint value of the upper and lower limits (7.5%^{w/v} PHBV and 0.35g/g MgOI) resulted in the largest minimisation effect of erosion rates. The inclusion of NAC leads to increased erosion, in relationship to PHBV and MgOI, due to greater dissolution of NAC molecules into the surrounding aqueous media compared to that of PHBV and MgOI, thus increasing the total %weightloss of the samples.





Higher concentrations of PHBV and lower concentrations of MgOI equates to increased water-uptake of the fibrous films after 24 hours immersion in PBS, as noted in Figure 5.14. Decreased hydration observed with this variable combination is attributed to the water-resistance properties of the wax-like MgOI. Concerning the effects associated with NAC-loading in combination with PHBV and MgOI, it is noted that increasing concentrations of the constituents lead to decreased water-uptake. NAC-loading produces a general decrease in water-uptake of the films irrespective of changes in PHBV and MgOI concentrations. NAC, present as crystalline water-soluble particles within the fibre matrix, solubilises and dissolves upon contact with the dissolution media whereas PHBV and MgOI are water-insoluble and have a propensity to absorb a certain amount of water to initiate hydrolytic degradation. It is revealed that enhancement of water-uptake properties of the formulations could be achieved via the implementation of lower MgOI concentrations and the exclusion of NAC from the formulations. Although, the experimental design model was constructed to investigate the effects imposed by NAC addition and exclusion on the response outcomes, the complete elimination of NAC from the optimised formulation is unlikely due to the necessity of its potential chemical and therapeutic effects on neuronal regeneration.

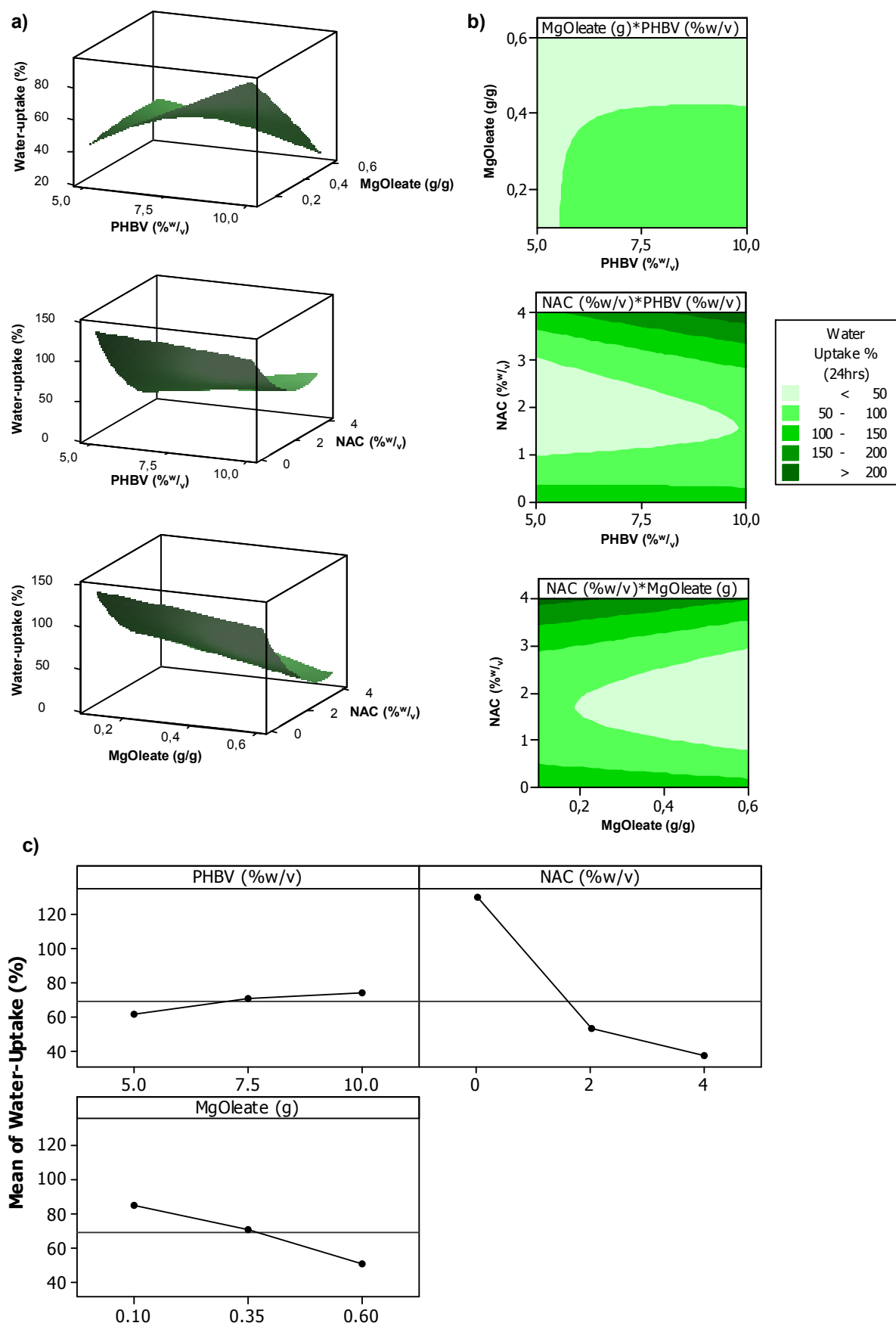


Figure 5.14: a) Response surface, b) contour and c) main effects plots depicting the effects PHBV, MgOI and NAC concentrations on %water-uptake.

Additionally, a complete ANOVA analysis (presented in Table 5.5) was executed on the formulation response outcomes of tensile strength, %erosion and %water-uptake with the intend of ascertaining the relationship between the response variables and the predictor variables. A p-value ≤ 0.05 was considered significant with regards to the effect observed between the response and variable factor. Apart from the significant effect noted for the NAC (NAC, $p = 0.001$; NAC*NAC, $p = 0.003$) and PHBV*MgOleate ($p = 0.02$) factor of %water-uptake, the other factors presented with no significant effect with regards to the selected formulation outputs.

Further statistical evaluation of the experimental model was obtained via the analysis of residual plots (Figure 5.15) to determine the uniformity and distribution of the data points attained for the selected experimental design responses. The normal probability plots of the residuals, pertaining to the responses of tensile strength and %erosion, depict a linear arrangement of the points thus, indicating normal distribution and lack of influence from external variations. However, the normal probability plot of %water-uptake features 3 distinct and interrupted arrangements of the points as opposed to a linear distribution thereby indicating the probable effect of some unaccounted variable. The residuals versus fitted plots for the 3 design responses depicted the random scattering, and no clustering, of points on either side of the zero line indicating an absence of trends. The histogram for %water-uptake presented a unimodal and symmetrical distribution of the residuals indicating the probability of a uniform distribution of the data set. The histograms relating to effects of tensile strength and %erosion depicted a lack of symmetry suggesting the occurrence of a random error of the data set. Regarding the scatter plot of the residuals in relation to the observation order, non-random error is observed for %water-uptake whereas tensile strength and %erosion were indicative of a random error as identified likewise from the obtained histograms.

Table 5.5: ANOVA analysis for the measured responses

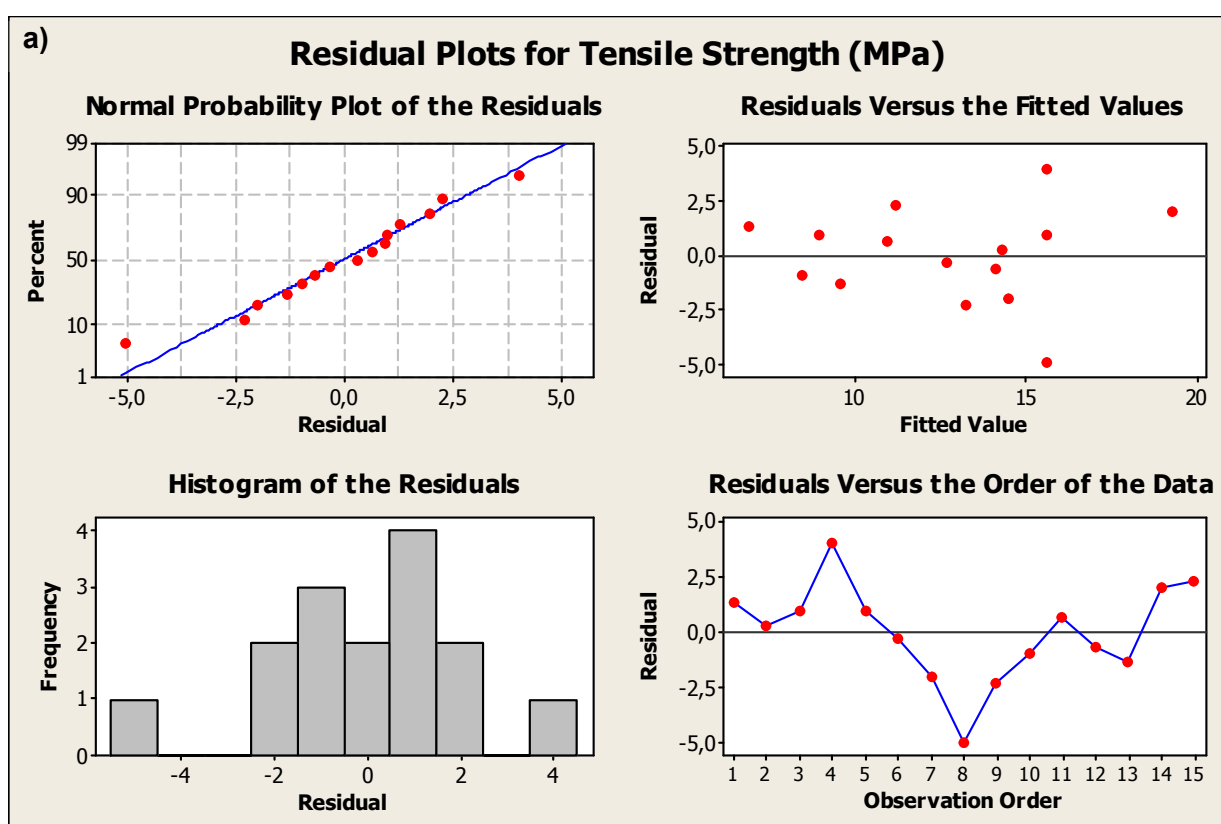
Response Variable	p-values		
	Tensile Strength	%Erosion	%Water-Uptake
Constant	0.563	0.045	0.251
PHBV	0.649	0.062	0.490
MgOleate	0.314	0.439	0.106
NAC	0.720	0.331	0.001
PHBV*PHBV	0.306	0.072	0.835
MgOleate*MgOleate	0.745	0.280	0.862
NAC*NAC	0.230	0.198	0.003
PHBV*MgOleate	0.077	0.822	0.020
PHBV*NAC	0.210	0.518	0.057
MgOleate*NAC	0.941	0.538	0.368

The complete regression equations generated for the responses of tensile strength, %erosion and %water-uptake are presented as follows:

$$\text{Tensile Strength (MPa)} = 8.04 + 5.25[\text{MgOleate}] + 0.423[\text{PHBV}] - 0.27[\text{NAC}] \quad \text{Equation 5.7}$$

$$\% \text{Erosion} = 18.17 - 1.1[\text{MgOleate}] - 0.44[\text{PHBV}] + 7.76[\text{NAC}] \quad \text{Equation 5.8}$$

$$\% \text{Water-Uptake} = 119 - 67.5[\text{MgOleate}] + 2.62[\text{PHBV}] - 46.3[\text{NAC}] \quad \text{Equation 5.9}$$



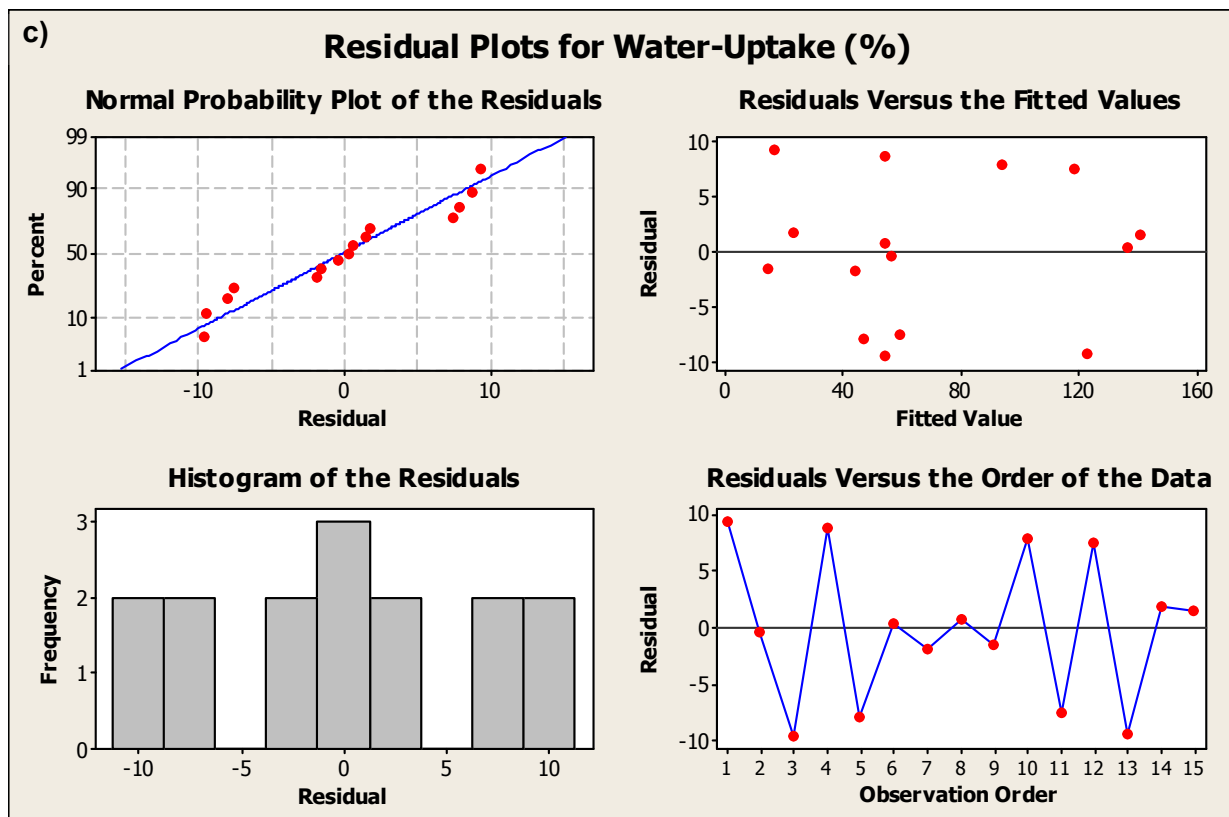
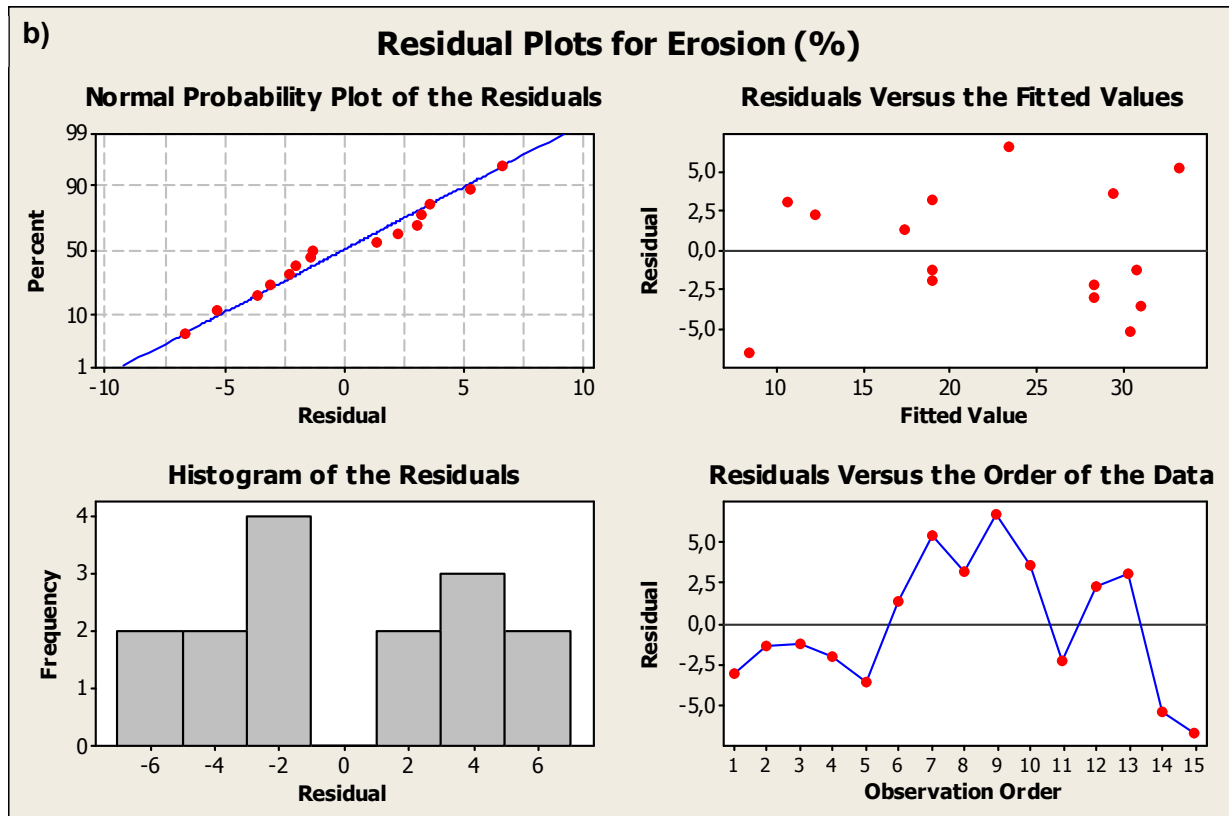


Figure 5.15: Residual plots for the responses a) tensile strength b) %erosion and c) %water-uptake.

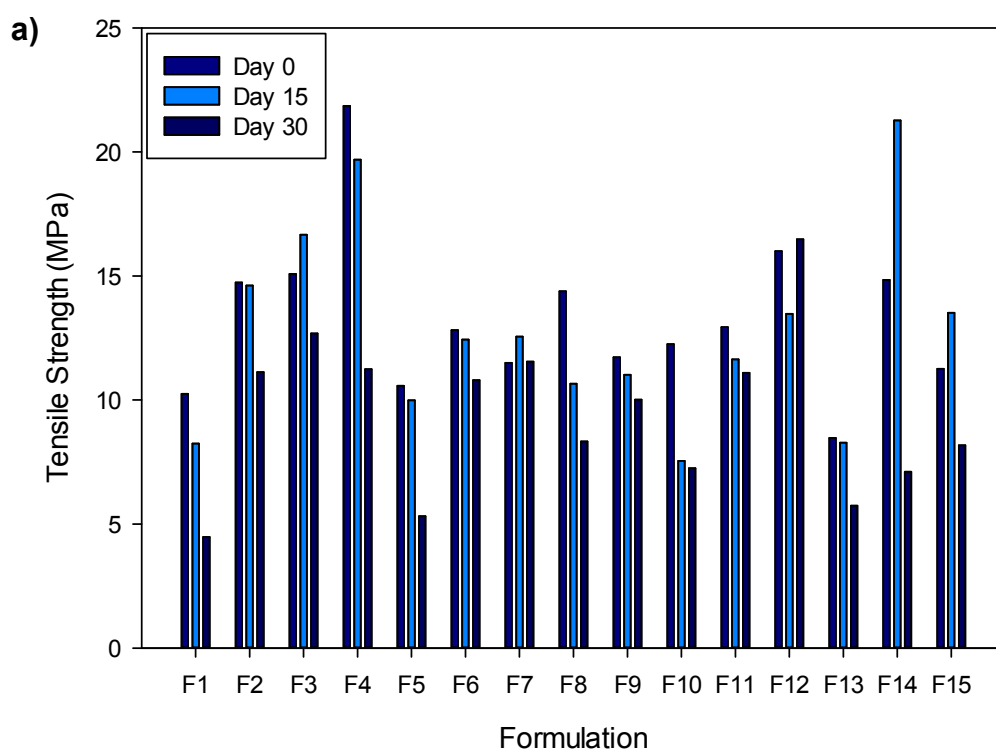
5.3.6. The effect of MgOI and NAC induced crystallinity transitions on the mechanical properties of PHBV fibers

The tensile strength of the NAC-loaded and PHBV-MgOI composite fibrous films is pertinent to the ability of the films to maintain its mechanical strength during physical handling, further processing and surgical implantation. Materials and devices intended for tissue repair should possess similar mechanical properties to that of the native tissue under repair for enhanced biocompatibility. Furthermore, such materials should be able to withstand mechanical strain without failure for the appropriate duration of time during which tissue healing and regeneration takes place. The 15 experimental formulations were assessed for tensile strength before dissolution and at 15 and 30 days post dissolution in PBS.

Direct comparison of the results, using selected formulations containing the upper (0.6g/g) and lower (0.1g/g) limits of MgOI, respectively (F5 and F7; F9 and F11; F14 and F10; F12 and F15) revealed that formulations containing less MgOI were slightly superior in tensile strength before and after immersion in PBS at days 15 and 30 (Figure 5.16a). It was further noted that, as depicted in the main effects plots in Figure 5.12, formulations composed of higher MgOI concentrations and zero to intermediate NAC concentrations (F10 and F14 (2%^{w/v}); F15 and F12 (NAC-Free)) tended to possess enhanced tensile strength, compared to those synthesized from lower MgOI and higher NAC concentrations (F7 and F5; F11 and F9), in the hydrated state as noted at days 15 and 30. As shown in Figure 5.12 of the surface response analysis and main effects plots, decreasing MgOI concentrations with a high NAC concentration of 4%^{w/v} results in compromised tensile strength of the formulations. Decline in tensile strength noted with increased MgOI may be attributed to the formation of a weakened, soft and rather amorphous matrix whereas declining mechanical strength (in unhydrated samples) observed with higher concentrations of NAC may be due to the result of a disrupted PHBV crystalline structure leading to the formation of inter-spherulitic cracks thereby contributing to a comprised mechanical resistance. In addition, upon hydration, NAC particles dissolve into the surrounding media leaving probable voids throughout the fibre architecture thus weakening its integrity. Nevertheless, the highly crystalline pure electrospun PHBV fibrous film was excessively brittle and could not be separated from the aluminium foil backing without coming off the foil in a powdery state. This is indicative of the need to attune the degree of crystalline and amorphous properties of a polymer as either overly crystalline or amorphous states may adversely impact desirable mechanical properties.

It is further noted that crystallinity is directly proportional to the deformability of the sample and its ability to absorb energy. A measure of deformation was taken from the slope of the

linear portion of the Force-Distance Curve indicating the samples' elastic extensibility over the 20mm stretch before undergoing permanent plastic deformation following failure at breaking point. The amount of energy absorbed by the sample to undergo deformation (N.mm) is termed as toughness of the sample. Samples of higher crystallinity (F4, F10, F13 and F15) presented with increased DMs (136.9MPa, 282.4MPa, 125.8MPa and 172.6Mpa, respectively) indicating a rigid, inflexible material whereas those samples with increased MgOI content demonstrated lower DM values corresponding to enhanced elasticity and flexibility where the sample dissipates energy to readily undergo deformation upon application of stress as opposed to resistance to deformation (Figure 5.16b). Formulations of low stiffness demonstrating a low DM presented with increased toughness (Figure 5.16c) as such materials are able to absorb increasing amounts of energy to deform rather than resisting the applied force resulting in immediate breakage. Although the crystalline samples composed of lower MgOI content exhibited higher tensile strength indicating strong fibers, the increased resistance to deformation indicated a brittle and inflexible matrix. Increased MgOI concentrations produced fibrous films that were slightly weaker in strength but tough and more flexible compared to those of low MgOI content. Elastic deformability and rigidity of the sample is essential in further processing of the fibrous films into various architectures such as rolled conduits and microchannel tubes. Brittle films would be challenging to manipulate as a result of easy and frequent breakage of the sample.



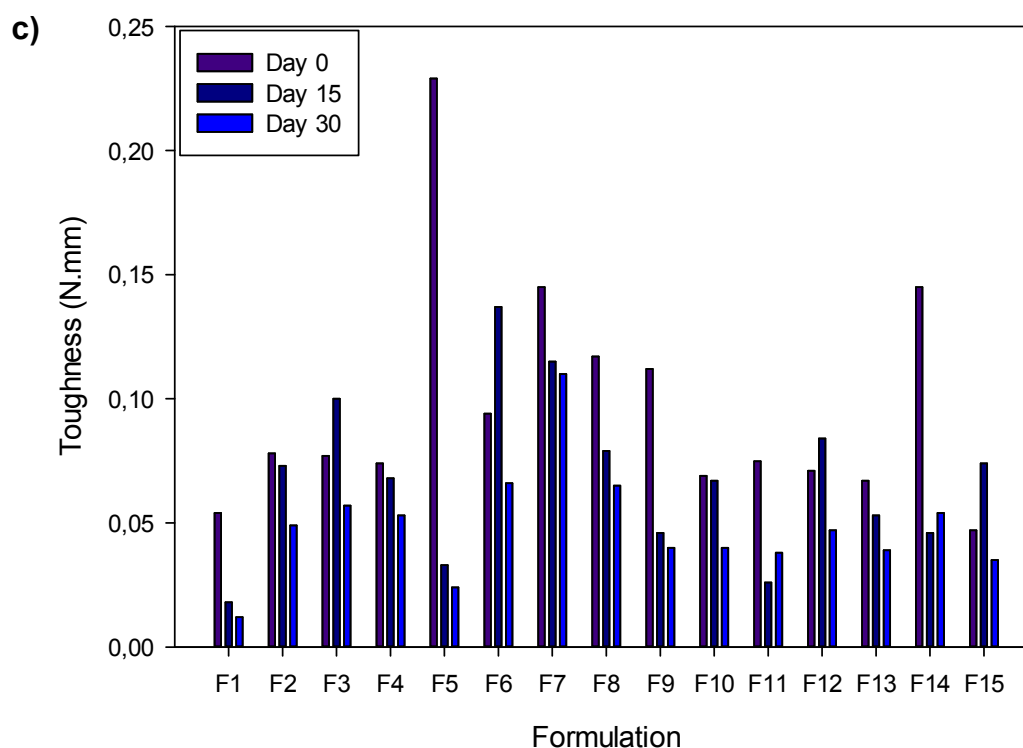
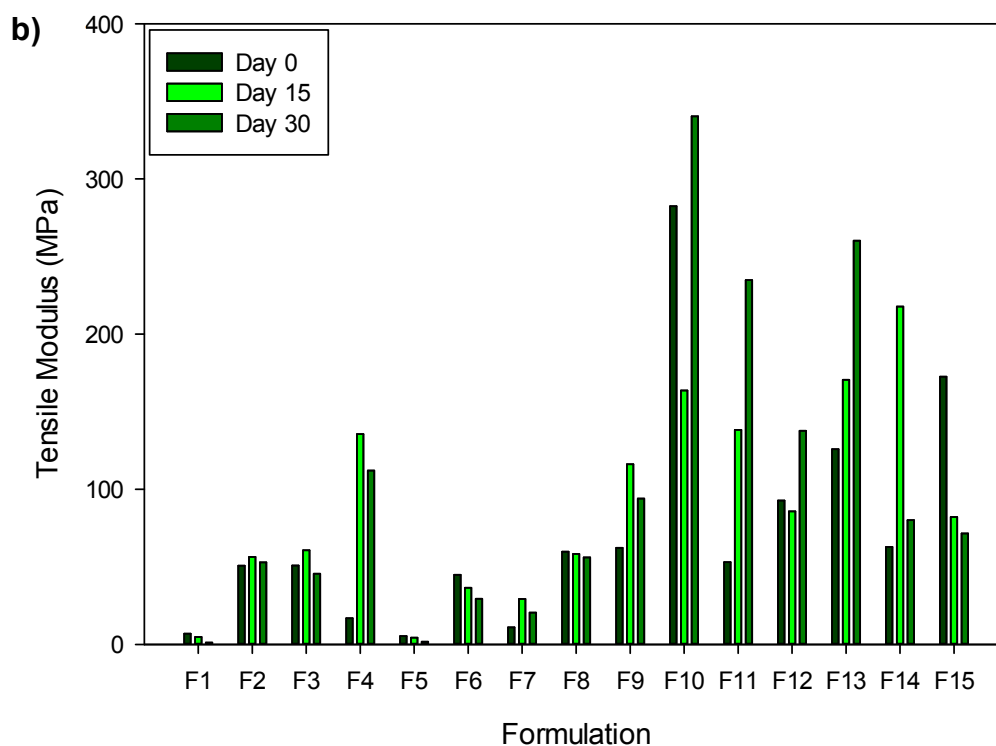


Figure 5.16: Textural analysis depicting a) tensile strength, b) tensile modulus and c) toughness of the Box-Behnken experimental design formulations before hydration (Day 0) and 15 and 30 days post-hydration in PBS pH 7.4.

A study of stress and strain, conducted by Peng *et al.* (2012) utilizing freshly harvested samples of human sciatic nerve revealed that a tensile strength of 6.71MPa was the limit for sciatic nerve physiological stress whereas as 11.63MPa resulted in rupturing of the nerve specimen. Considering the necessity for attuning of crystalline/amorphous properties, it was noted that formulations exhibiting intermediate %Crl between 50-57% seemed to possess adequate tensile strength (± 12 MPa, as established by Peng *et al.*, 2012) compared to those featuring %Crls within the lower 42-49% and higher 58-66% ranges, particularly at the end of the 30 days hence indicating the improved ability to maintain its mechanical integrity while matching the tensile properties of native nerve tissue.

5.3.7. The effect of MgOI and NAC induced crystallinity transitions on PHBV fibre hydrodynamic and erosion properties

The hydration and wettability of a biomaterial is an imperative factor affecting the type of biological response initiated by cells following its implantation into the body. PHBV, being a thermoplastic polyester polymer, is generally hydrophobic in nature and requires some modification to enhance its interaction with protein adsorption and resulting cellular adhesion. It is generally considered that a moderately hydrophilic material surface is optimal for tissue biocompatibility. However, a highly hydrophilic material may decrease cell adhesion. In addition to cellular interactions, the hydration and water-uptake properties of a drug-loaded matrix system is pertinent to elucidating its erosion and drug-release mechanisms as drug release is the process in which drug solutes dissolve in a fluid before being able to migrate out of the polymer matrix via diffusion and into the surrounding media or tissues.

Firstly, it was noted that the 4 NAC-free formulations, F15, F13, F12 and F6, demonstrated the highest water-uptake during the first 24 hours and over the 30-day study period with hydration values of 247.31%, 173.29%, 166.67% and 173.96%, respectively as shown in Figure 5.17. When compared directly to formulations equivalent in PHBV and NAC concentrations, it was found that increasing MgOI concentration from 0.1g/g to 0.6g/g decreased water-uptake of the films. This is attributed to the hydrophobicity imparted by the wax-like composition of MgOI which inhibits water penetration. NAC-loaded formulations F9 and F14 presented with the lowest water-uptake capacity. The maximum concentrations of both NAC and MgOI seemed to decrease water-uptake properties. Overall, it was noted that the less crystalline formulations F4, F6, F12, F13 and F15 with %Crls in the range of 42-52% underwent greater moisture uptake compared to formulations F7, F10 and F11 of %Crls between 64-66%. A study by Wang *et al.*, 2007, showed the effect of crystallinity on

wettability of PHBV where it was reported that decreased crystallinity of PHBV films imparted improved wettability.

Furthermore, from SEM imaging, it was noted that formulations composed of the maximum 0.6g/g MgOI formed thicker, coated fibers with a reduced number of inter-fibre spaces whereas those composed of the minimum 0.1g/g MgOI presented with thinner fibers and a greater number of open inter-fibre spaces. These interstices between the fibers during fibre deposition onto the collecting foil may be able to hold water thereby contributing a water-holding capacity to the fibrous films. Formulations high in MgOI content were observed to have occluded interstices resulting from a surface coat of excess MgOI over the fibers thus preventing water entry decreasing the hydration properties. The degree of water contact with the polymer surfaces affects its rate of erosion.

Formulations (F6, F13, F15, F12 and F4) exhibiting increased water uptake presented with low erosion rates (Figure 5.17). Similarly, formulations containing 0.6g/g MgOI underwent faster erosion compared to those containing the minimal concentration. NAC-containing formulations F7, F11, F9 and F1 which presented with the second highest change in weight after hydration in PBS when compared to NAC-free formulations, demonstrated increased erosion. In general, a higher %crystallinity index was noted to lead to increased erosion rates of the experimental formulations. The NAC-containing formulations exhibited enhanced erosion compared to NAC-free derivatives due to the loss of sample mass resulting from the dissolution of NAC particles into the surrounding media. Since PHBV undergoes enzymatic erosion via depolymerases as well as via hydrolysis (Rathbone *et al.*, 2010) and surface erosion, MgOI slows its erosion rate by inhibiting water interaction with the polymer surface. The trait of progressive surface erosion further allows maintenance of the mechanical strength of the fibers over a longer duration compared to materials which undergo rapid bulk degradation due to increased water ingress into the core of the polymeric structure. In the case of the former, the inner core of the polymeric fibers remains unaffected by erosion processes occurring on the outermost surface.

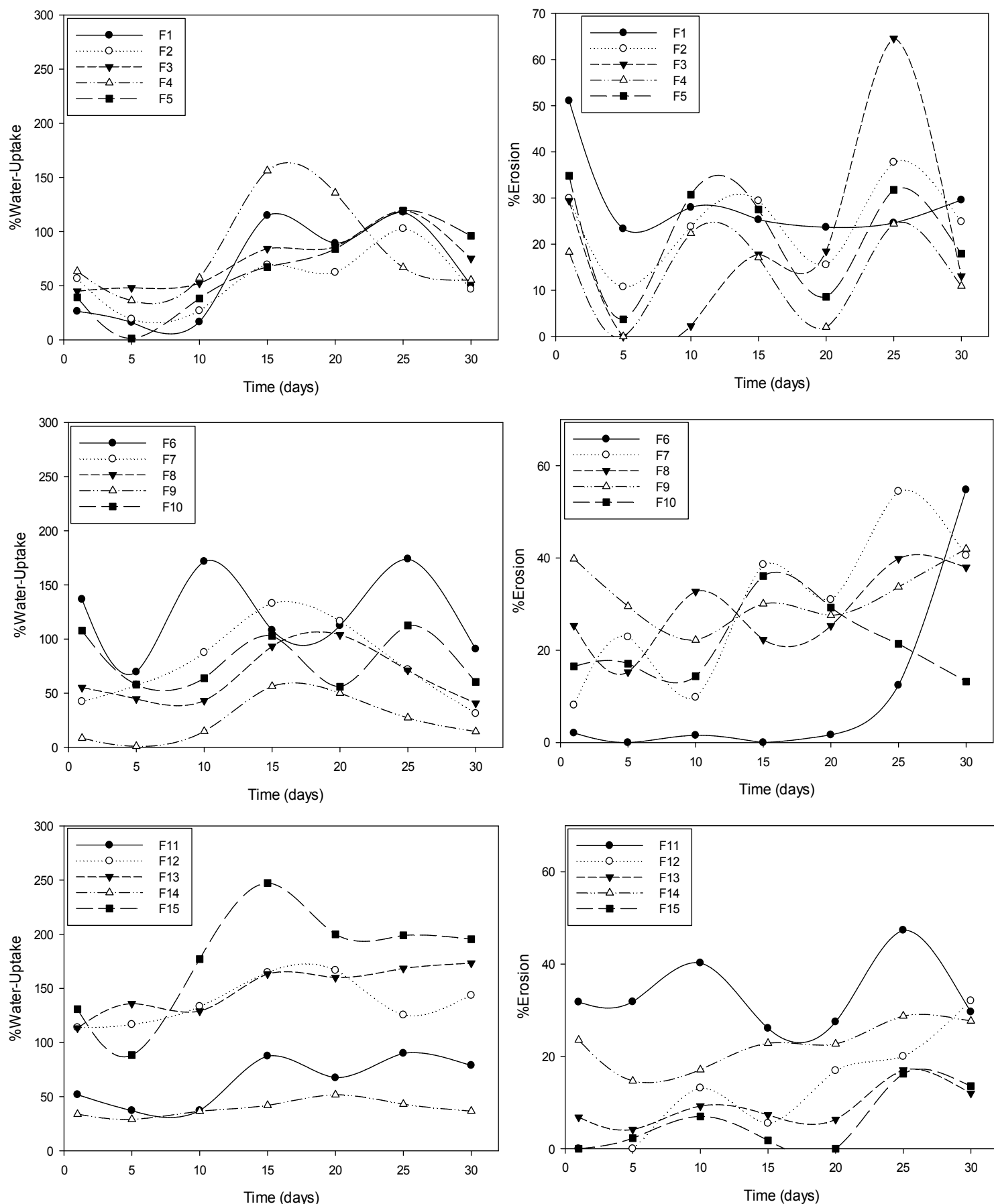


Figure 5.17: Dissolution studies showing %water-uptake (left panel) and %erosion (right panel) over a study period of 30 days in PBS pH 7.4.

5.3.8. The effect of MgOI and NAC induced crystallinity transitions on NAC release and kinetic modelling

From the drug release profiles in Figure 5.18, it is noted that F1, F4, F7, F10, F11 and F14 present with increased release rates of NAC whereas F3, F5, F8 and F9 exhibit a slower release profile. Formulations F10 and F11 demonstrated particularly increased bioactive release rates among the 11 NAC-loaded experimental formulations. Increased MgOI content resulted in slower rates whereas increased PHBV polymer content was noted to increase NAC release rates. Understandably, formulations containing 4%^{w/w} NAC released more bioactive compared to those containing 2%^{w/w} concentration as a result of an increased quantity of NAC particles available, per area unit, for dissolution into the surrounding media.

The rate of drug release is affected by a number of intrinsic physicochemical properties of the individual materials used and the resulting final formulation. Since PHBV is a non-swelling polymer that is unable to absorb water, the predominant drug release mechanisms governing NAC release are most likely diffusion-controlled and chemically-controlled. A chemically-controlled drug release mechanism is thought to encompass surface erosion, bulk degradation and other chemical interactions or processes occurring within the polymer matrix. PHBV is classed a surface eroding polymer since its water-uptake is slower than its erosion and hydrolysis is confined to the outer surface of the polymeric device whereas the interior of the matrix remains unaffected (Alexis, 2005). The bioactive release is therefore primarily erosion-controlled however, factors such as chemical composition, hydrophilic/hydrophobic properties and degree of crystallinity affect water diffusivity and hence the ultimate degradation pattern of the polymeric formulation.

Although, it is commonly thought that amorphous regions of a polymer are more susceptible to water penetration and hydrolytic attack contributing to faster bioactive release rates, the contrary was observed in the present study. The formulations demonstrating faster NAC release profiles contained a lower concentration of MgOI and consequentially contained more crystalline regions than those with an increased MgOI content. Such formulations also exhibited greater water-uptake during the first 24 hours and thereafter and presented faster erosion rates compared to those composed from higher MgOI quantities. The results reveal that erosion of the fibrous films and water penetration is directly affected by MgOI incorporation and its resulting crystallinity transitions of the formulations. It is proposed that increasing crystallinity imparts a hydrophilic property to the formulation resulting in increased water interaction at the fibre surface. Increased water contact initiates hydrolytic chain scission leading to surface erosion where escape of NAC molecules can easily occur via diffusion into the dissolution medium. In addition, accelerated erosion of the fibre surface

predisposes the entrapped NAC particles to dissolution and diffusion. Furthermore, increases water interaction in the crystalline formulations resulting in dissolution of the water-soluble oligomers contribute to the likely formation of microporous channels via erosion and hence further allows water penetration and release of NAC molecules via diffusion.

In addition to physicochemical factors, the morphological properties and general topography of the formulations play a role in modulating bioactive release. As shown in SEM imaging, formulations featuring high %crystallinities contained thinner fibers with open interstices and a rough surface morphology. The highly irregular surface of the fibers increases the total surface area of the films thereby creating a greater number of contact points for water interaction and hydrolytic degradation hence promoting rapid NAC release. Although MgOI decreased %crystallinity, excessive amounts resulted in the coating of the fibre surface, as seen in the SEM imaging. MgOI, of a wax-like consistency, is hydrophobic thereby imparting a water-resistant property to the fibrous films. Since drug release is dependent on the ability of water penetration into the matrix, reduced water entry and hydrolysis decreases NAC diffusion and its erosion-controlled release thereby imparting a slower release rate to the formulation. With regards, to the fibre topography, MgOI incorporation produces the formation of thicker but smoother fibers which decreases surface area available for water interaction contributing to a slower release rate.

Mathematical modelling, shown in Table 5.6, of the release data using the n -value derived from the Korsmeyer-Peppas model revealed a series of Fickian-diffusion, non-Fickian/anomalous transport and super case II transport where the R/F value derived from the Peppas-Sahlin model indicates the contribution ratio of relaxational release over Fickian diffusion of each formulation. The drug release of formulations of low %crystallinity were ascribed a Fickian-diffusion based release and presented lower R/F values suggesting diffusion to be the dominant contributing mechanism rather than case-II relaxational release. Formulations F4 and F5 presented with non-Fickian/anomalous transport featuring a coupled diffusion and polymer relaxational mechanism hence, presenting with higher R/F ratios of 0.035 and 0.015, respectively, thus indicating the predominant role of relaxational release. The drug release mechanism of F10, F11 and F14, of high %crystallinity were established to occur via combined polymer relaxation ensuing erosion and diffusion.

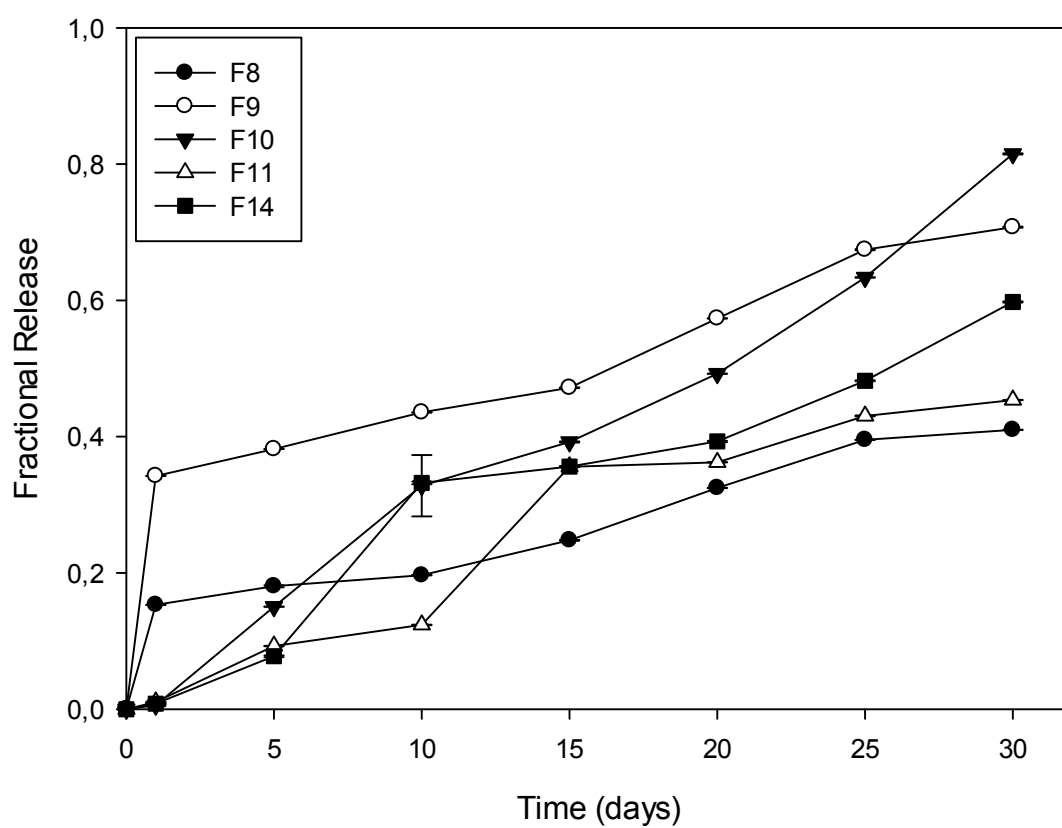
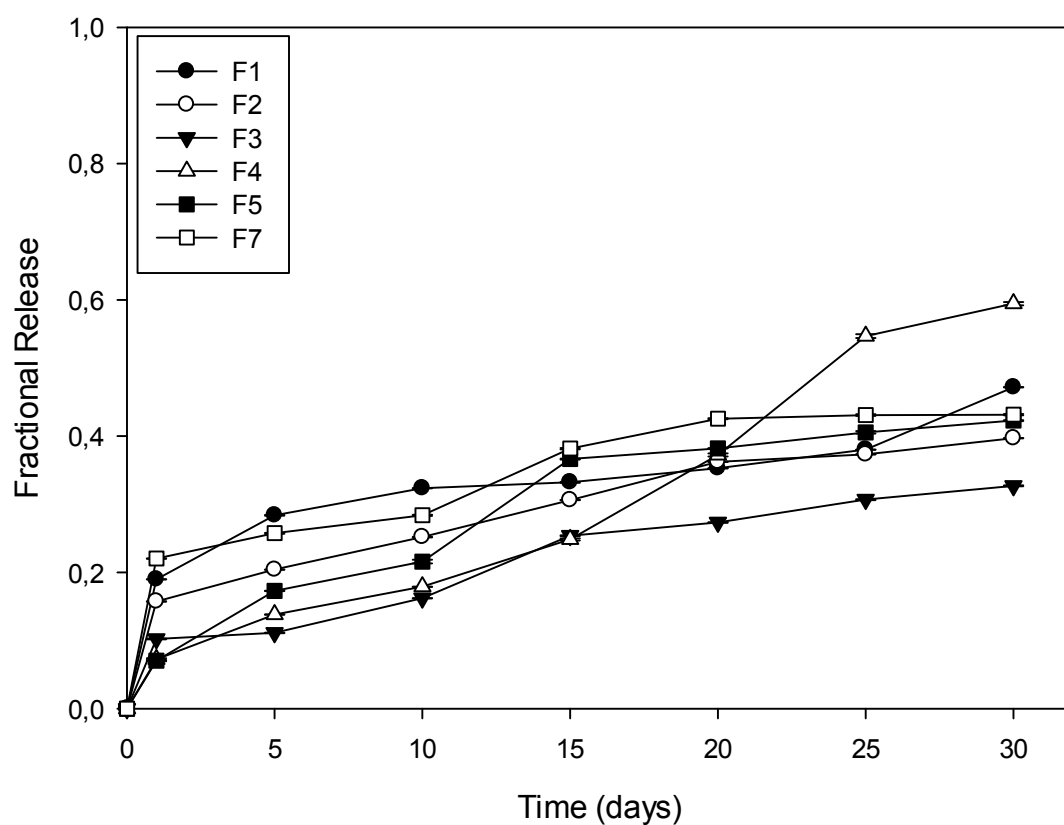


Figure 5.18: *In vitro* release profiles of NAC conducted in PBS pH 7.4.

Table 5.6: Kinetic modelling results of NAC release from the respective PHBV-MgOI electrospun fibrous films.

Formulation	Zero-Order	First-Order	Higuchi	Korsmeyer-Peppas		Peppas-Sahlin				Hopfenberg	Best-fit model
	R ²	R ²	R ²	R ²	n	R ²	K ₁	K ₂	R/F Ratio	R ²	
1	0.897	0.896	0.913	0.941	0.231	0.897	0.009	0.00003	0.003	0.897	Higuchi
2	0.965	0.975	0.981	0.948	0.284	0.993	0.015	0.00020	0.014	0.965	Higuchi
3	0.953	0.960	0.935	0.851	0.378	0.966	0.013	0.00010	0.008	0.953	First-Order
4	0.963	0.929	0.876	0.911	0.615	0.980	0.009	0.00030	0.035	0.963	Zero-Order
5	0.894	0.913	0.953	0.979	0.547	0.972	0.027	0.00040	0.015	0.894	Higuchi
7	0.904	0.908	0.925	0.875	0.221	0.953	0.016	0.00020	0.012	0.904	Higuchi
8	0.964	0.955	0.884	0.794	0.294	0.971	0.006	0.00010	0.016	0.964	Zero-Order
9	0.976	0.950	0.904	0.828	0.151	0.983	0.009	0.00010	0.011	0.976	Zero-Order
10	0.984	0.910	0.961	0.952	1.440	0.984	0.026	0.00001	0.0004	0.984	Zero-Order
11	0.919	0.934	0.927	0.973	1.133	0.946	0.028	0.00030	0.011	0.919	First-Order
14	0.929	0.950	0.952	0.964	1.272	0.949	0.031	0.00030	0.010	0.929	Higuchi

5.3.9. The effect of MgOI and NAC as neuronal cell proliferation promoters and neuroprotective agents

The level of proliferation of PC12 cells were used to confirm the neuro-protectant and growth factor-like properties of MgOI and NAC, as shown in Figure 5.19. Peripheral nerves such as the sciatic nerve, commonly used as an injury model for *in vivo* studies, are known to contain up to 45% oleic acid. It has been established that the synthesis and release of oleic acid promotes neuronal differentiation via its specific incorporation into the growth cones thereby acting as a potential neurotrophic factor (Velasco *et al.*, 2003). Oleic acid may further assist in promoting axonal growth and neuronal clustering via upregulation of GAP-43 in addition to accelerating tissue repair mechanisms (Tabernero *et al.*, 2001; Velasco *et al.*, 2003; Bonferoni *et al.*, 2014 and Chen *et al.*, 2014). Magnesium has been studied as a supplement for improved peripheral nerve regeneration. Vennemeyer *et al.*, 2015, successfully utilized magnesium metal filaments as physical guidance cues to assist the regeneration potential of transected nerve injuries. NAC is known to protect cells from oxidative damage, promote neuronal cell survival as well as possess anti-inflammatory effects (West *et al.*, 2007 and Saleh, 2015). Previous studies have considered it of potential therapeutic benefit for the treatment of peripheral nerve injuries and neuropathies.

The MTT proliferation studies indicated that the PHBV-MgOI composite fibrous films were capable of efficiently supporting the growth of PC12 cells compared to the control (cultured directly on a TPP-treated surface) over an investigation timeframe of 72hrs thus indicating biocompatibility of the fibrous films (Figure 5.19a). Increased MgOI concentration from 0.1g/g to 0.6g/g was seen to enhance cell growth. Likewise, NAC-loaded formulations increased neuronal cell proliferation compared to NAC-free fibrous films (Figure 5.19b). It has already been established above, that increased MgOI content of the fibers inhibited bioactive release thereby providing a slowed release of NAC. The release rates of NAC from formulations containing the maximum and minimum concentration of MgOI, respectively (F5 and F7; F9 and F11; F14 and F10) was compared to assess the effect on the growth of PC12 cells. It could be that a slower release of NAC may favour cell proliferation as opposed to the release of larger quantities. A highly saturated media, consisting of NAC crystals as a solute, resulting from increased drug release rates may impede cell growth by formation of a hypertonic media thereby altering cell tonicity.

Furthermore, a synergistic or additive effect on neuronal survival and promotion of growth may be acquired with the simultaneous incorporation and dissolution of MgOI and NAC.

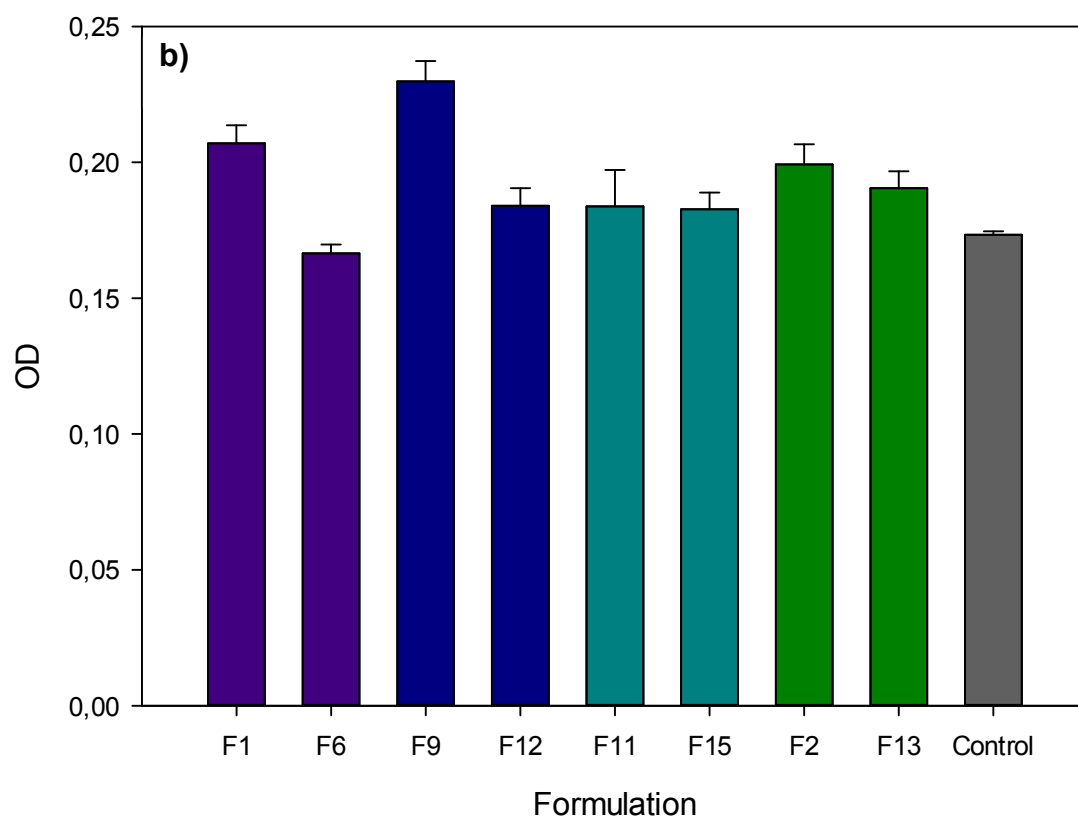
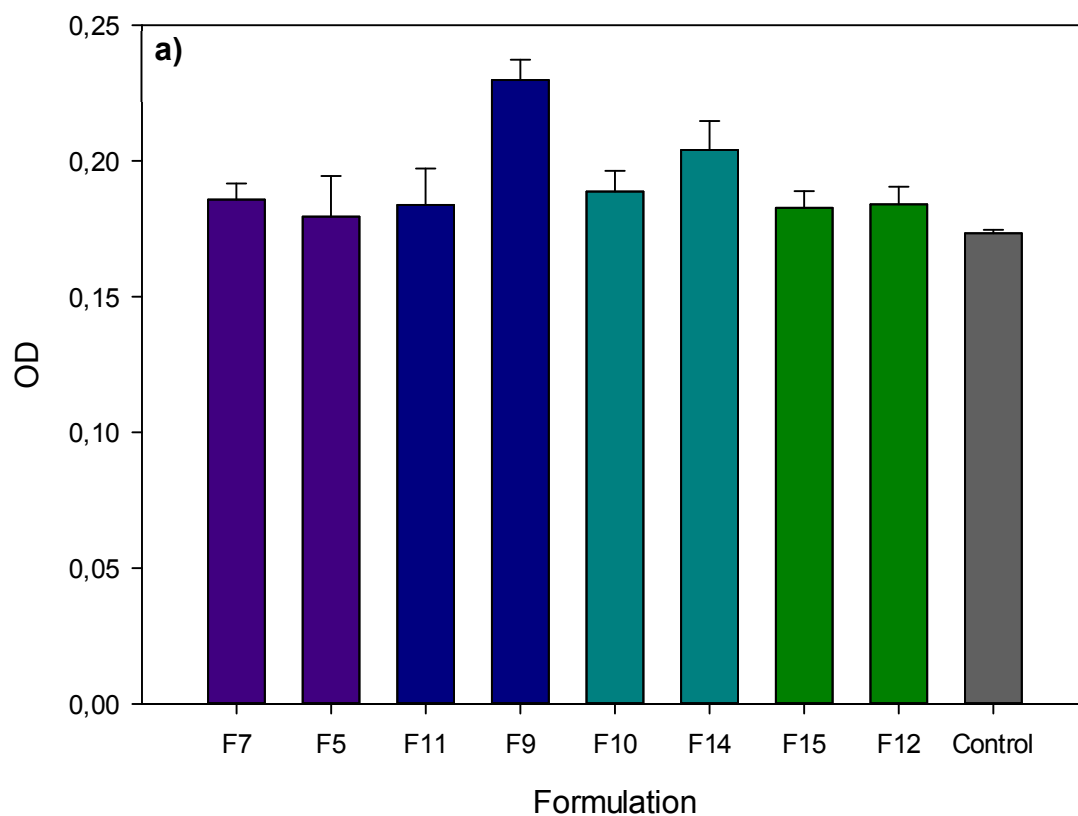


Figure 5.19: Representation of the MTT assay showing the proliferative effects of a) increased MgOI concentrations and b) NAC-loading on PC12 cells.

5.3.10. Optimization of the Box-Behnken experimental design

Minitab® V15 statistical software was employed to generate the optimal variables and response outcomes based on the results of the Box-Behnken experimental design, for the establishment of an optimized formulation. In order to fulfil the chemical and therapeutic requirements of the guidance cues, as established in Chapter 1, NAC was pertinent to the formulation for its bioactive properties in inducing neuronal proliferation. It was noted that %water-uptake conflicted with tensile strength resulting in the computation of lower tensile strength values. Tensile strength is an important factor determining the maintenance of the architectural features of the fibers *in vivo*; hence, the computation of the experimental design optimised against the responses of maximised tensile strength and minimised %erosion yielded the ideal NAC-loaded formulation. As shown in the desirability plots in Figure 5.20, the predicted ideal concentrations of 7.5%^{w/v} PHBV, 0.35g/g MgOI and 2%^{w/v} NAC could be used to obtain the optimized NAC-loaded formulation. The derived optimised NAC-loaded formulation was equivalent in composition to the experimental design centre points F3, F4 and F8. The predicted response outcomes for desirable tensile strength and %erosion were 15.67MPa and 19.06%, respectively after 15 days of *in vitro* dissolution. An optimized formulation being composed of the midpoints of the upper and lower variable limits is plausible as according to the surface response analyses and main effects plots of tensile strength (Figure 5.12) and %erosion (Figure 5.13), the midpoints represented the maxima for tensile strength (PHBV and NAC) and the minima for %erosion (PHBV and MgOI) of the acquired bell-shaped curves.

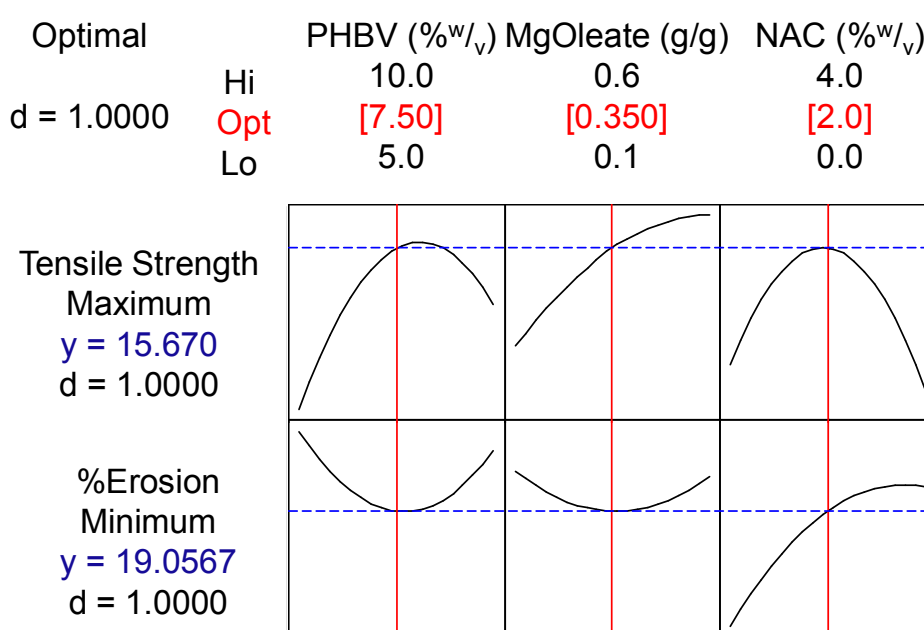


Figure 5.20: Statistical optimization yielding the desirability plots depicting the variables and predicted response outcomes for the NAC-loaded optimized formulation.

5.4. Concluding Remarks

The study pertaining to the incorporation of MgOI with PHBV demonstrated its ability to act as a nucleating agent for the modification of PHBV electrospun-fibre crystallinity and tensile strength. Through its actions of increasing crystallisation rates and decreasing spherulite size, as shown by DSC and XRD, MgOI could be used to attune the mechanical properties of tensile strength, flexibility, extensibility and toughness via the formation of amorphous regions as reflected by FTIR analysis. The addition of NAC in combination with controlled quantities of MgOI was observed to improve PC12 neuronal cell proliferation via its function as neuro-protectant agents whereas SEM imaging established the deposition of fairly linear-orientated fibers serving as a potential for enhanced cell directionality. The blending of MgOI was advantageous due to its dual and simultaneous therapeutic and non-toxic plasticising effects. Statistical optimization of the Box-Behnken experimental design generated the variable concentrations and predicted response outcomes for the attainment of the ideal formulation based on the current findings. The derived optimized formulation resulting from this investigation requires further *in vitro* and *in vivo* assessment of its capability to be employed as a guidance cue component when integrated within a hollow nerve repair conduit.

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CHAPTER 6

ASSEMBLY AND *IN VIVO* ANALYSIS OF THE BIOSIMULATED NERVE REPAIR DEVICE IN THE RAT SCIATIC NERVE MODEL

6.1. Introduction

With the gradually inclining incidences of peripheral nerve injuries, the investigation and design of artificial scaffolds for nerve repair and adequate functional recovery is indispensable. The conventional treatment procedures comprising end-to-end suturing on short gap injuries (< 10mm) and autograft repair in long gap injuries (> 10mm) have several drawbacks. Direct end-to-end repair encounters issues of increased tension across the gap defect and reduced stretching capacity of the transected nerve ends due to fibrosis, tissue adhesion and Wallerian degenerative processes (Madduri *et al.*, 2010). Although autograft repair could result in the attainment of satisfactory functional recovery and is currently considered the gold standard of treatment, it is associated with difficulties in donor tissue harvesting, donor site morbidity, multiple surgery sites and additional scar formation. The disadvantages accompanying the conventional treatment modalities for nerve repair necessitates the need for artificial, biodegradable and biocompatible nerve repair conduits with the goal of attaining functional recovery levels equivalent to or greater than that achieved with the gold standard of treatment (Belkas *et al.*, 2013 and Mukhatyar *et al.*, 2014).

Artificial nerve repair conduits are synthesized from various synthetic or natural-based polymeric materials or a blend of both. The intent of a nerve conduit is to provide a favourable internal environment permissive of neuronal survival, support and regeneration in addition to the provision of a stable neuroprotective barrier for the regenerating and newly regenerated tissues including a basic bridging function across the nerve gap defect. An environment conducive to axonal regeneration may further be obtained via the inclusion of and ability of the conduit to deliver a range of neurotrophic factors (Nerve growth factor, Glial-derived neurotrophic factor and Brain-derived neurotrophic factor) other biological actives (ECM molecular components and small-molecular weight therapeutic agents) in a sustained-release manner.

As previously outlined in Chapter 2, Table 2.1, a key characteristic of any nerve repair conduit is its mechanical properties. A nerve conduit should possess sufficient mechanical strength to withstand physical handling and surgical procedures whilst maintaining its

structural integrity from the time of implantation and throughout the duration of tissue regeneration. Simultaneously, it should be texturally compatible and appropriately flexible so as to accommodate any native tissue movements without causing additional pain or discomfort therefore enhancing biocompatibility (Ramburrun *et al.*, 2014).

Proficiency of hollow-lumen nerve conduits in the promotion of functional recovery may further be attained through the implementation of various intraluminal support scaffolds or fillers which function as physical guidance cues to assist cellular attachment, axonal elongation and direction towards the target tissues thereby reducing the occurrence of mismatched innervation. A number of strategies could be utilized for the implementation of intraluminal guidance structures consisting of gel or liquid fillers, nano-fibrous linings and fillers and arrays of hollow tubular multi-channels (Madduri and Gander, 2012).

The present study entails the *in vivo* investigation in Sprague Dawley rats on the nerve regenerative potential of the previously described optimized pristine PMMA-particle intercalated Gel-Xan hydrogel conduit (Chapter 4) in combination with the optimized electrospun PHBV-MgOI-NAC aligned nano-fibrous films (Chapter 5) as intraluminal guidance scaffolds featuring a trio guidance cue mechanism. The final assemblage of the Gel-Xan hydrogel conduit and the intraluminal PHBV-MgOI-NAC composite fibers yield the Biosimulated Nerve Repair Device (BNRD) which aims to mimic the architecture of native nerve tissue as improvements in neuro-compatibility and promotion of functional recovery outcomes. The inclusion and sustained delivery of NGF and diclofenac sodium and the presence of Mg^{2+} ions, oleic acid and NAC as bioactives function as a means of assisting the innate physiological and biological repair mechanisms ensuing nerve regeneration and healing.

The components of the BNRD system aim to improve nerve regeneration and functional recovery via provision of the following conduit features and types of guidance cues:

6.1.1. The PMMA-intercalated Gel-Xan hydrogel conduit pH-responsive in situ particulate leaching technique for porosity formation

Controlled and gradual induction of porosity allows for enhanced mechanical and resilience properties while simultaneously providing a mechanism for the dual controlled release of proteins and other small-molecular weight drugs. Furthermore, porosity induction imparts a degree of matrix permeability for the uninterrupted gaseous and nutrient exchange to the regenerating tissues.

Ionically crosslinked hydrogel matrix:

Utilization of a highly hydrophilic hydrogel matrix allows the potential absorption of excess bleeding and inflammatory fluids. The potential absorption and ionic binding of influxing calcium ions, entering the axoplasm, by the hydrogel matrix may assist with calcium-ion homeostasis and prevention of calcium-induced axonal degeneration (Schlaepfer, 1974; LoPachin and Lehning, 1997 and Coleman, 2005).

Bridging and barrier function:

Basic bridging function of the conduit serves as a macro-guidance scaffold with a protective barrier against infiltration of fibrotic tissue. In this way the conduit is capable of sustaining an enclosed suitable microenvironment for neuronal regeneration.

6.1.2. The PHBV-MgOI-NAC composite fibers

Physical cue:

The linear arrangement of the electrospun nanofibers ensure enhanced uni-directionality of axonal growth with the provision of a solid structure simulating the presence of a basal lamina-type substrate for cellular interaction. Slight roughening of the fiber surface imparted by the addition of NAC crystals (as shown in Chapter 5, Figure 5.10) provide a topographical cue for cellular attachment with an increased surface area.

Chemical cue:

The promotion of cell adhesion to the electrospun fibers and the native basal lamina may be improved by the presence of positively charge surface molecules of NGF, Mg^{2+} and the cysteine protein moiety of NAC. Oleic acid present as an oleate bound to magnesium ions has shown potential in PC12 proliferation via its function as a GAP-43 activator for neuronal growth (Velasco *et al.*, 2003). Axonal growth is believed to occur chemotactically towards the direction of such bioactives.

Therapeutic cue:

The BNRD system offers 3 types of therapeutic agents: neuro-protective, proliferative and differentiation and anti-inflammatory. The neuro-protective agents, NAC and magnesium, promotes the survival of axons upon injury and during the inflammatory degenerative processes. The provision of NGF to the axons during the early phase of injury ensures tissue viability while promoting axonal elongation and differentiation thereby assisting the body with growth factors until sufficient Schwann cells have been recruited to take over the role of growth factor production (Madduri and Gander, 2010). Diclofenac sodium was included for

its anti-inflammatory effects for the targeting of fibrotic tissue infiltration and reduction in the Wallerian degenerative effects causing potential and extensive tissue damage.

Prior to *in vivo* evaluations, the final assembled BNR device underwent characterisation of its NGF release kinetics, textural and mechanical profiling and PC12 cell compatibility to confirm its *in vitro* efficacy, safety and mechanical integrity. Procession of the study, utilizing the rat sciatic nerve injury model, involved a 4-week behavioural study consisting of weekly monitoring of walking track analysis and grid walk for assessments of functional recovery followed by muscle mass measurement as an indicator of target tissue innervation and histological analysis at termination of the study. The study aimed to elucidate the efficiency of the proposed BNRD system in promoting neuronal regeneration and functional recovery via the provision of a trio physical, chemical and therapeutic guidance cues.

6.2. Materials and Methods

6.2.1 Materials

Gellan gum (low acyl GelzanTM CM)(GG), xanthan gum (XG), poly(3-hydroxybutyric acid-co-3-hydroxyvaleric acid) (PHBV) (PHV content 12mol%) natural origin, diclofenac sodium, N-acetyl-L-cysteine (NAC) ($\geq 99\%$ TLC), 2,2,2-trifluoroethanol ($\geq 99\%$ GC) and dialysis tubing cellulose membrane (molecular weight cut-off = 14000Da) were purchased from Sigma Aldrich, Steinham, Germany. Polymethyl methacrylate (PMMA) (Eudragit S100) was purchased from Evonik, Midrand, Johannesburg, South Africa and was used without further modifications. Propylene glycol (PG), sodium hydroxide pellets, chloroform, oleic acid vegetable (extra pure) and Span 80 were purchased from Merck, Darmstadt, Germany and calcium chloride (CaCl_2) anhydrous was purchased from Rochelle Chemicals, Johannesburg, South Africa. Magnesium sulphate heptahydrate CP ($\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$), methanol, acetone and isopropyl alcohol were purchased from Associated Chemical Enterprises, Southdale, Johannesburg, South Africa. Ethanol (99% absolute) was purchased from LabChem, Edenvale, Johannesburg, South Africa. PeproTech® recombinant human β -nerve growth factor (NGF) and corresponding ELISA kits were sourced from Celtic Molecular Diagnostics, Cape Town, South Africa. Milipore water was used for washing of preparations. Dulbecco's Modified Eagle Medium (DMEM), Donor Equine Serum (DES) and Fetal Bovine Serum (FBS) were procured from Hyclone, Separations, South Africa. Pencillin/Streptomycin/Amphotericin B (P/S/AB) solution and the Roche Cell Proliferation Kit I was purchased from Lonza, USA and Sigma-Aldrich, USA, respectively. Ethilon® black nylon monofilament 9/0 sutures with needle 5.1mm 3/8C taperpoint were procured from Ethicon, Johnson and Johnson Medical (Pty) Ltd, Gauteng, South Africa. All other surgical

materials and consumables used were obtained from the Central Animal Services (CAS) of the University of the Witwatersrand.

6.2.2 Fabrication of the biosimulated nerve repair device

6.2.2.1. Synthesis of the Gel-Xan hydrogel hollow conduit

As per the predicted optimised formulation, 722.88mg gellan gum and 277.12mg xanthan gum powders were dissolved in 100mL deionised water to form a 1%^{w/v} gel solution. After solubilisation of the gum powders, propylene glycol and calcium chloride were added as the plasticiser and crosslinker in concentrations of 5%^{w/v} and 0.05%^{w/v}, respectively. Thereafter, sufficient diclofenac sodium was added to form a 0.4%^{w/v} concentration. The gel blend was then heated to and maintained at 100°C for 10 minutes. Addition of polymethyl methacrylate (PMMA) nano-sized particles was introduced into the gel blend, at a concentration of 0.25g per gram of dry polysaccharide powder blend, after cooling the solution to 60°C. Cylindrical moulds, measuring 50mm in length and 12mm in diameter, were constructed. A removable glass rod of 1.88mm diameter was inserted into the centre of the mould to form the lumen of the conduit. A volume of 3mL of the polymer gel suspension was carefully syringed into pre-lubricated cylindrical moulds and allowed to set at room temperature for 20 minutes before being removed and left to air-dry, while on the glass rods, in a fumehood for 24 hours. The dried conduits were cut into 14mm lengths for further characterisation and implantation where each piece contained approximately 4mg diclofenac sodium.

6.2.2.2. Synthesis of PHBV-MgOI-NAC electrospun fibrous film guidance cues

The polymer solutions for electrospinning were prepared by dissolving the chemical constituents in a mixture of chloroform and trifluoroethanol (3:2 ratio) under continuous magnetic stirring at 60°C. MgOleate was synthesized as described in Chapter 5.2.2.1. The optimized NAC-loaded formulation utilized 7.5%^{w/v} PHBV, 0.35g/g MgOleate and 2%^{w/v} NAC. For fabrication of the NAC-loaded and NAC-free films, 5mL of the PHBV-MgOleate solutions with or without NAC were loaded into a syringe for electrospinning (NanoSpinner24, Inovenso, Turkey) onto Span 80-coated aluminium sheets attached to a rotating drum set at 1200rpm, using a voltage of 25kV, a flowrate of 2mL/hour and a nozzle distance of 150mm.

6.2.2.3. NGF-loading into hydrogel conduit

Due to the extremely small concentration and volume of NGF used, its accurate loading into the hydrogel conduits was performed according to the technique utilized by (Madduri *et al.*, 2010). Using a micropipette, 20uL (200ng) of NGF solution in distilled water was carefully

pipetted into the centre of a 14mm length hydrogel conduit and allowed to soak in and dry in a sterilized fumehood for 8 hours.

6.2.2.4. Assembly of the final device

For the purposes of improved visualisation and mechanical stability of the conduit during surgical handling and implantation, the conduit ends were coated with a 10%^{w/v} solution of PMMA in isopropyl alcohol. The electrospun films were cut into 10mm×20mm lengths, folded in half width-ways to form a 10mm×10mm square film and rolled into a tight cylinder before being inserted into the lumen of the hollow conduits. The layers of the rolled film opened to line and form a loose spiral pattern inside the conduit thus creating an open structure (Figure 6.1).

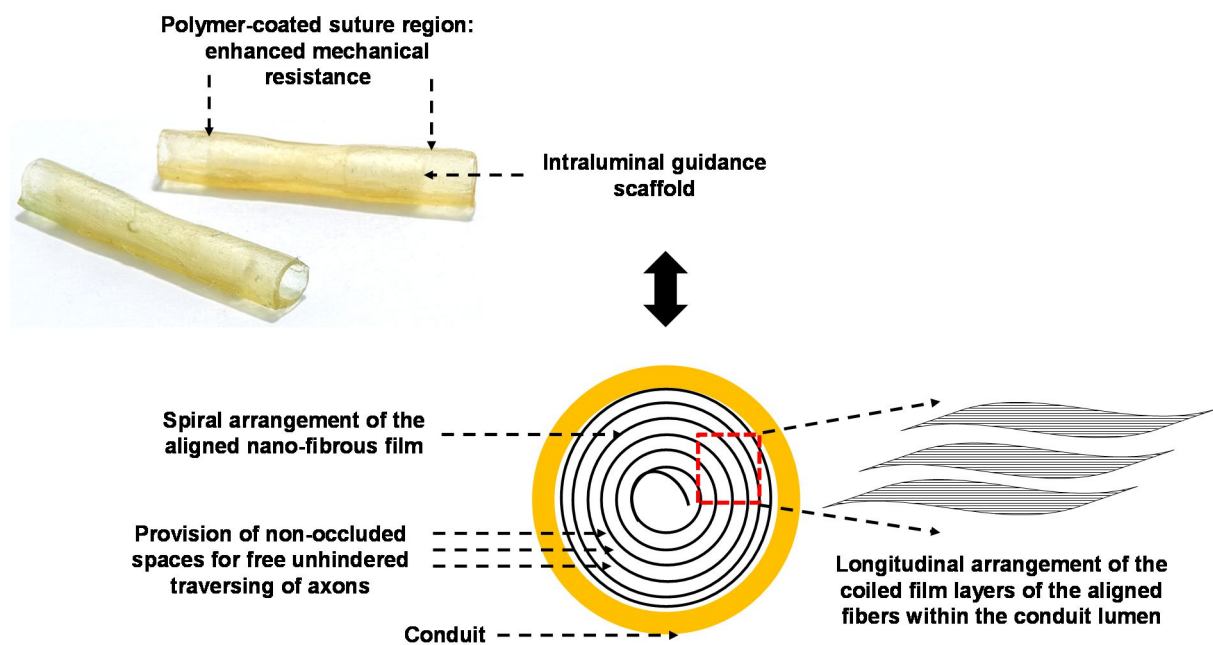


Figure 6.1: Depiction of the final assembled device and the arrangement of the intraluminal fibers.

6.2.3. Characterisation of the biosimulated nerve repair device components

6.2.3.1. Textural profiling and biaxial testing

6.2.3.1.1. Compression

Textural analysis characterization using a Texture Analyser (TAXT^{plus} Stable Microsystems, Surrey, UK) fitted with a 10mm diameter delrin probe was undertaken on hydrated conduit samples with and without the electrospun fibrous films at pre-determined sampling points. The Matrix Resilience (MR), Deformability Modulus (DF) and Fracture Energy (FE) were determined. MR (N.sec) was determined by the ratio of the areas under the compression and decompression curves generated by Force-Time textural profiles. This represented the

ability of the hydrogel conduit to recover to its original form after application of an external deforming stress. The Area under the Curve (AUC) from the Force-Distance textural profile allowed the determination of the FE (N.mm) which equates to the energy in joules required to cause rupture of or a break in the matrix of the hydrated swollen conduits. The DM (N/mm) is an indication of the hydrogel matrix flexibility and was computed from the gradient of the Force-Distance profile from zero to the point of the peak force. The textural analysis parameters utilized include a pre-test and post-test speed of 1.00mm/sec, a test speed of 0.5mm/sec, a trigger force of 0.5N and a load cell of 5kg with a target mode of 10% strain.

6.2.3.1.2. Biaxial tension

Biaxial tensile analysis of the optimized Gel-Xan hydrogel matrix and PHBV-MgOI-NAC electrospun fibrous films were performed using the BioTester5000 Biaxial Test System (CellScale, Waterloo Instruments Inc., Waterloo, Ontario, Canada) equipped with a 250mN load cell. An 8mm × 8mm sample, dusted with powdered graphite for improved visibility, was attached to the actuators via 4 0.7cm width tungsten pins on each side and stretched along a 90° and 180° axis simultaneously as shown in Figure 6.2. A strain magnitude of 10% and 100% were applied on the electrospun fibers and hydrogel matrix, respectively, for 60 seconds. The measured data points were represented as Force-Time plots. Further tracking of the measured data points in terms of displacement and strain are presented in the attached video files.

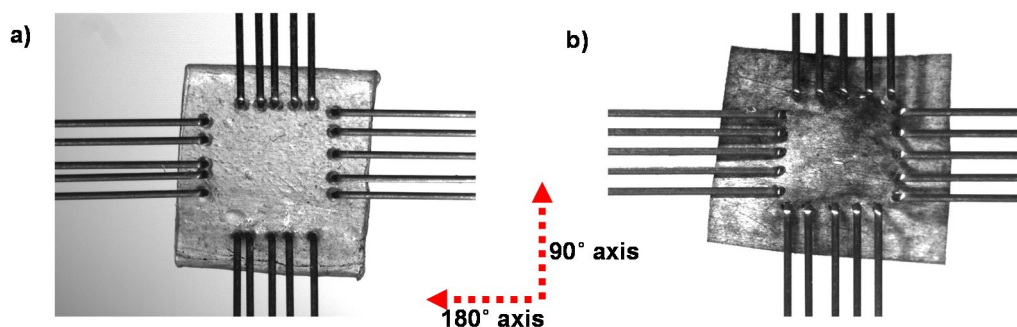


Figure 6.2: Depiction of directional strain and sample mounting for biaxial testing of a) the hydrogel matrix and b) the electrospun fibrous film.

6.2.3.2. *In vitro* release studies of NGF from the final assembled device

In vitro release studies were performed using 10mm samples of conduit each loaded with 200ng NGF. Samples were placed in glass vials containing 1mL PBS pH 7.4 and stored in an orbital shaking incubator. At pre-determined time intervals, 0.5ml of dissolution media was removed and stored at -80°C. The removed media was replaced with an equal fresh

volume to maintain sink conditions. Samples were analysed for the quantity of NGF using an NGF-ELISA kit read at 405nm with background subtraction at 650nm with an absorbance multi-plate reader (BioTek, USA). Further insight into the drug-release mechanisms was obtained via kinetic modelling of the drug-release profiles using the models listed in Table 2.

6.2.3.3. MTT proliferation assessment

Rat adrenal gland pheochromocytoma PC12 mixed adherent/suspension cell line from Cellonex (Separations, South Africa) was cultured in tissue culture treated (TPP) T-75 flasks using DMEM supplemented with 10% ν DES, 5% ν FBS and 1% ν P/S/AB solution in a humid 5% CO₂ atmosphere at 37°C. The culture medium was replaced at 75% every 2 days.

For the detection of cell proliferation in response to the exposure of the various device components, the MTT-based Roche Cell Proliferation Kit I was utilised. Samples of size 10mm×10mm were sterilized under UV light for 12 hours before overnight incubation in 400μL culture medium containing 10% ν DES, 5% ν FBS and 1% ν P/S/AB in a 48-well plate maintained at 37°C at 5% CO₂. PC12 cells were seeded onto the film samples at a density of 2×10⁴ cells/well and incubated for 72 hours. Thereafter, 40μL MTT solution was added to each well followed by a further 4-hour incubation period after which 400μL solubilising agent was added to dissolve the formazan crystals. The entire well contents were aspirated off, placed into 2mL Eppendorf tubes and centrifuged at 2000rpm for 5 minutes to separate suspended cells. The resulting supernatant was transferred into a 96-well plate and measured for absorbance at 550nm, with a background subtraction at 690nm, using a multi-plate reader (BioTek, USA).

6.2.4. In vivo analysis and experimental design

6.2.4.1. Animal procurement

The Sprague Dawely rat utilized for the sciatic injury model in this study where procured from the Central Animal Services of the University of the Witwatersrand. Ethics clearance for the study was obtained from the Animal Ethics Screening Committee of the University of the Witwatersrand (Ethics clearance number: 2014/42/C, see Appendix C1).

6.2.4.2. Experimental design

A total of 42 Sprague Dawely rats, male and female, with weights in the 250-300g range were utilized for the study. The rats were randomly assigned to 5 groups consisting of 6 rats each (n=6), as shown in the Figure 6.3, whereas 12 rats were used as cadavers for refinement of surgical skills prior to commencement of the experiment. The experimental

groups were used to assess the *in vivo* performance of the Gel-Xan hydrogel conduit and the final assembled device using drug-free and drug-loaded formulations as well as the gold standard of nerve repair as the comparative:

Group A: Comparative group

The animals in this group received the current gold standard treatment of nerve repair consisting of a reversed autograph after induction of injury.

Group B: Placebo hydrogel bioactive-free group

Animals in this group received an implantation of the bioactive-free and hollow hydrogel conduit.

Group C: Experimental hydrogel bioactive-loaded group

Animals in this group received an implantation of a hollow hydrogel conduit loaded with 100ng NGF and 4mg diclofenac sodium.

Group D: Placebo NAC-free biosimulated device

Animals in this group received an implantation of the final assembled device consisting of NAC-free fibres, 100ng NGF and 4mg diclofenac sodium.

Group E: Experimental NAC-loaded biosimulated device

Animals in this group received an implantation of the final assembled biosimulated nerve repair bioactive-loaded device consisting of 0.7mg NAC, 100ng NGF and 4mg diclofenac sodium.

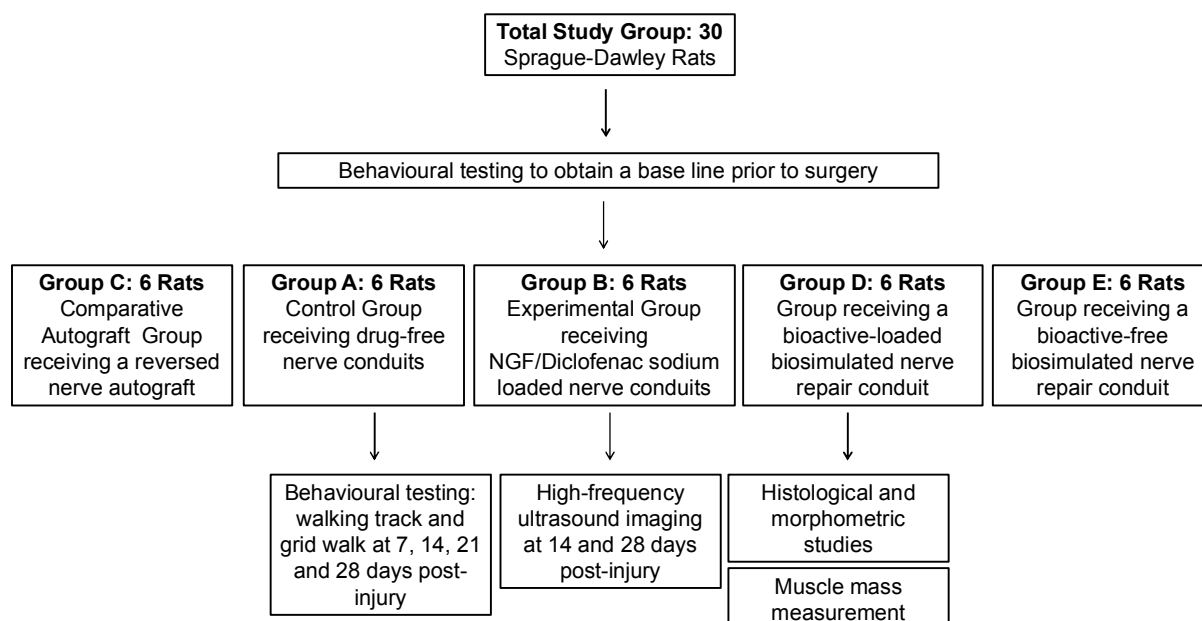


Figure 6.3: Flowchart representation of the *in vivo* experimental design and analyses.

6.2.4.3. Animal husbandry and acclimatization

Animals were individually caged at ambient temperature and kept at a 12 hour light/dark cycle. Standard laboratory rodent chow (65% carbohydrates, 15% fat and 20% protein) and water were provided *ad libitum*. Weekly monitoring of weight was used as an indication of their general state of well-being. Prior to the experimental procedures, the rats were acclimatized to the laboratory environment and trained for 5 days (twice a day for 15 minutes) on a walking track and grid walk apparatus in preparation for the behavioral analyses. Animal care and housing were executed in accordance with the standard operating procedures (SOPs) of the CAS and conforming to the South Africa Standard for the use and care of animals for scientific purposes.

6.2.4.4. Surgical procedures

The surgical procedures and techniques were first practised on rat cadavers to establish optimised surgical procedures before initiating it in live animals. This assisted in minimising potential further damage and animal suffering post-surgery while improving the surgery duration and ease. Figure 6.4 shows the practice trials of the autograft repair and conduit repair using 2 techniques. Figure 6.4b depicts the repair involving suturing of the transected nerve ends to the external edges of the conduit whereas Figure 6.4c (the optimised technique used for the study) involved insertion of the nerve ends inside the conduit using the suturing technique depicted in Figure 6.5.

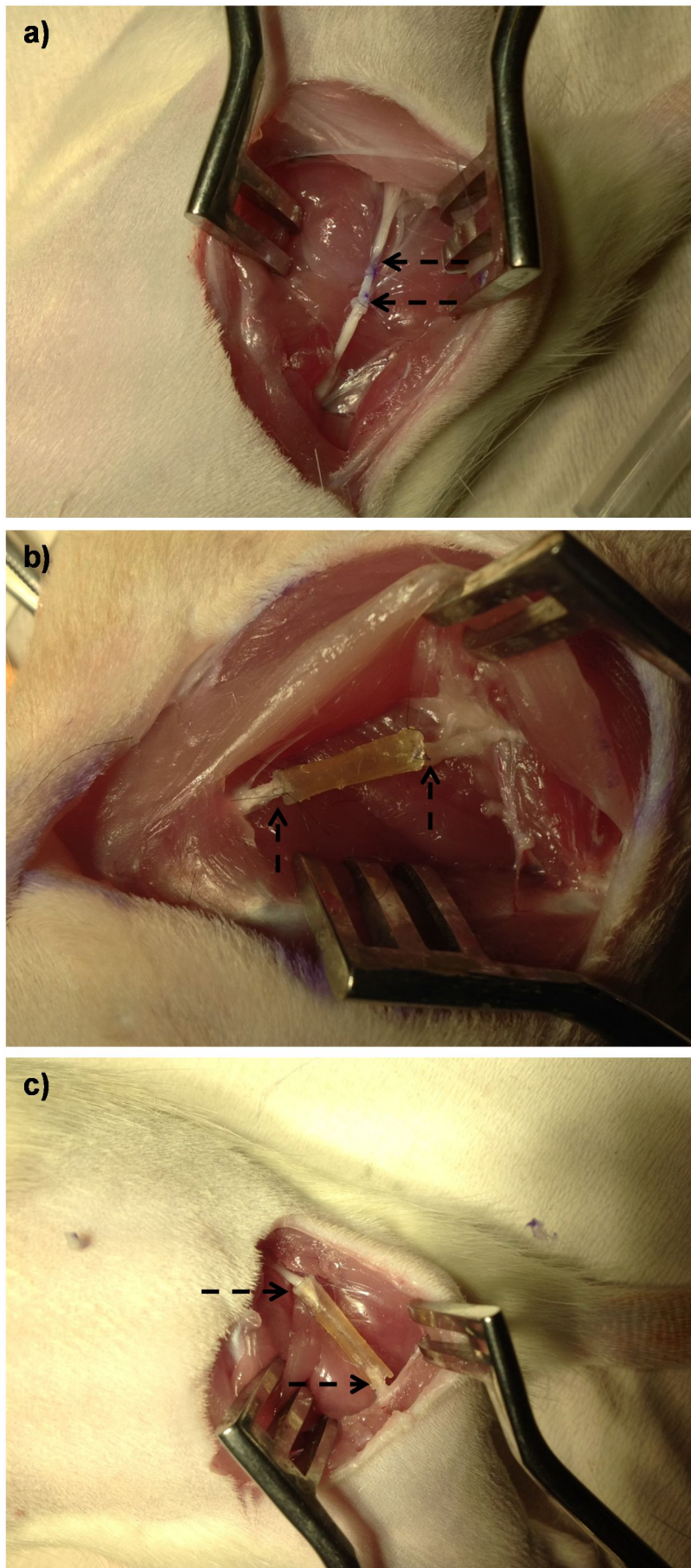


Figure 6.4: Photographs of the surgery practice trials of a) the autograft repair b) external conduit repair and c) internal conduit repair.

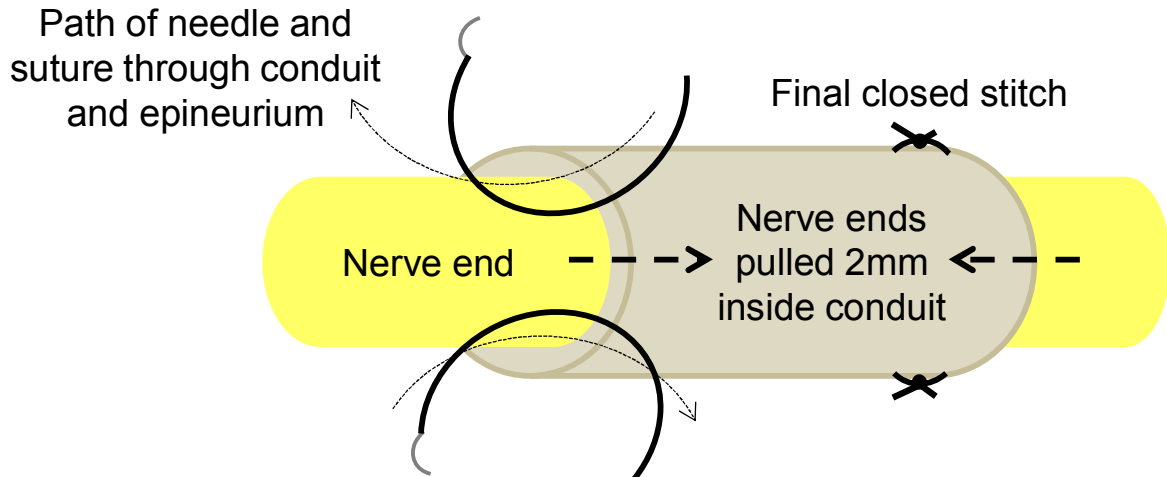


Figure 6.5: Illustration of the surgical suturing technique used for the nerve repair procedures.

6.2.4.4.1. Induction of sciatic nerve transection injury

The surgical procedures were performed according to the methods described by Xu and co-workers (2002; 2003), Madduri and co-workers (2010) and Mohammadi and co-workers (2013). Rats were anaesthetised with a mixture of ketamine (100mg/kg) and xylazine (5mg/kg) administered intraperitoneally. Anaesthesia was maintained during the surgery using a mixture of isoflurane with oxygen. Thereafter, the rats' left thigh and hind limb were shaved and the area of incision cleaned with F10 germicidal disinfectant. To expose the left sciatic nerve, a 2-3cm incision was made along the thigh and the gluteus maximus muscle was retracted. After freeing the nerve from the surrounding tissue a 5mm piece of nerve was excised, using microscissors, at the mid-thigh level proximal to the tibial and peroneal bifurcation. The retracted proximal and distal nerve stumps were pulled to create a 10mm nerve gap defect. The nerve gap defect was bridged by the implantation of a conduit or reversed nerve autograph before closure of the muscles and fascia layers with 4/0 vicryl absorbable sutures followed by closure of the skin with single 4/0 non-absorbable nylon sutures. For optimal tissue visualisation and precise implantation of conduits at the nerve transection site, all surgical procedures were performed using surgical loupes.

6.2.4.4.2. Autograft repair procedure

The autograft repair was performed as described by Yang *et al.*, 2007. After induction of sciatic nerve transection injury, the excised nerve segment was reversed 180° and re-implanted across the nerve gap defect using a single 9/0 epineurial nylon suture at the top and bottom of the proximal and distal nerve stumps as shown in Figure 6.6. The proximal

and distal transected nerve stumps were secured in place using 16mm 25 gauge needles to prevent spontaneous retraction of the nerve ends during suturing (Figure 6.6).

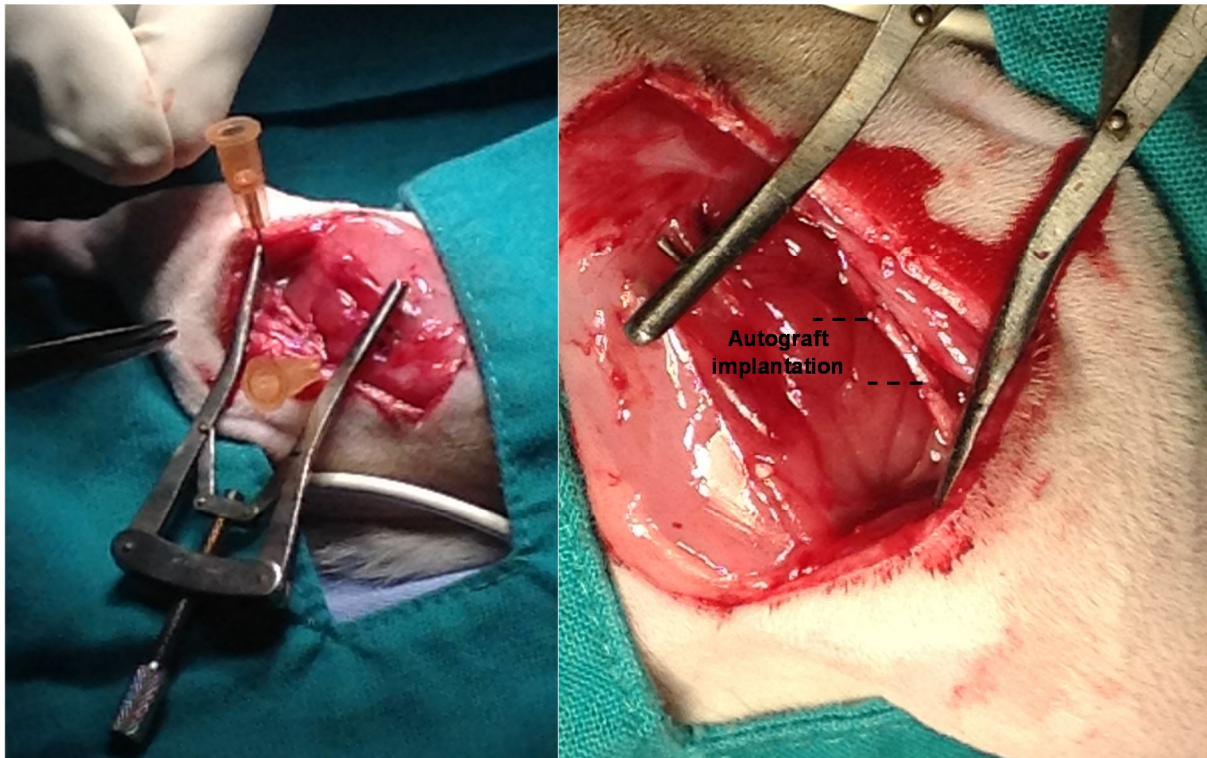
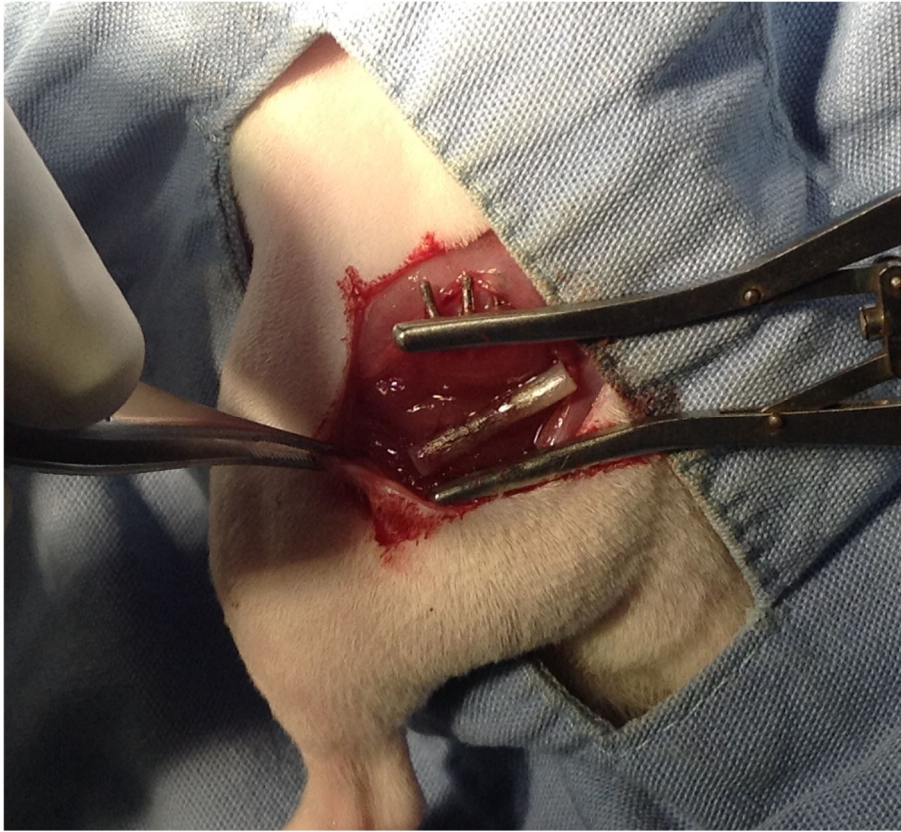


Figure 6.6: Depiction of the autograft repair procedure.

6.2.4.4.3. Conduit implantation and repair

Implantation of the nerve conduit across the nerve gap defect was performed as described by Xu and co-workers (2002; 2003) and Madduri and co-workers (2010). The conduits were sterilized via a 18-hour exposure to UV light. Immediately after induction of nerve transection injury, the proximal and distal nerve stumps were inserted a length of 2mm deep into the ends of a 14mm long hollow (Figure 6.7a) or fiber-loaded (Figure 6.7b) conduit and secured in place with two 9/0 polypropylene epineural sutures on the top and bottom of either ends of the conduit. Thereafter, the musculature was closed with single 4/0 vicryl sutures and 4/0 nylon stitches were used to close the skin.

a)



b)



Figure 6.7: Photograph of the conduit repair procedure utilizing a) the hollow conduit and b) the final assembled fiber-loaded conduit.

6.2.4.5. Post-operative care

Immediately after surgery, animals were administered naloxone for anaesthesia reversal and kept in an incubator warmed to 25°C until consciousness was regained. As post-surgery pain management, animals were administered 0.1 mL buprenorphine twice daily for 3 days. All animals were monitored twice daily for any signs of pain or distress. Auto mutilation of the affected limb was prevented by the oral administration of acetaminophen (65 mg/kg) daily for further pain management when required. In addition to this, animals were housed singly to avoid further damage or mutilation of the affected site by other animals sharing the same cage. Food and water were provided *ad libitum*.

6.2.4.6. Functional evaluation

6.2.4.6.1. Walking track analysis

Sheets of plain white paper were stained with 0.5%^{w/v} bromophenol blue/acetone solution and then air-dried. The rats' 2 hind limbs were carefully dipped in water and each rat walked a total of 3 times across a 150 cm confined wooden walking track lined with the bromophenol blue-stained paper. Contact with the rats' wet feet on the paper resulted in the immediate appearance of foot prints on the paper. After air-drying, the captured foot prints were analysed for functional recovery in terms of the sciatic function index (SFI) calculated using Equation 6.1. The following measurements were taken for the experimental left hind limb (E) and the normal right hind limb (N): the lengths between the third toe and the heel: print length (PL); the first toe and the fifth toe: toe spread (TS); the second toe and the fourth toe: intermediary toe spread (IT) (as shown in Figure 6.8) (Brown *et al.*, 1991).

$$SFI = -38.3 \times (EPL - NPL)/NPL + 109.5 \times (ETS - NTS)/NTS + 13.3 \times (EIT - NIT)/NIT - 8.8$$

Equation 6.1

A SFI value near zero represents normal nerve function whereas a SFI of -100 indicates complete nerve dysfunction. Increasing SFI values will indicate improvement in functional recovery. Walking track analysis was conducted before injury induction as a baseline recording and at days 7, 14, 21 and 28 post-injury.

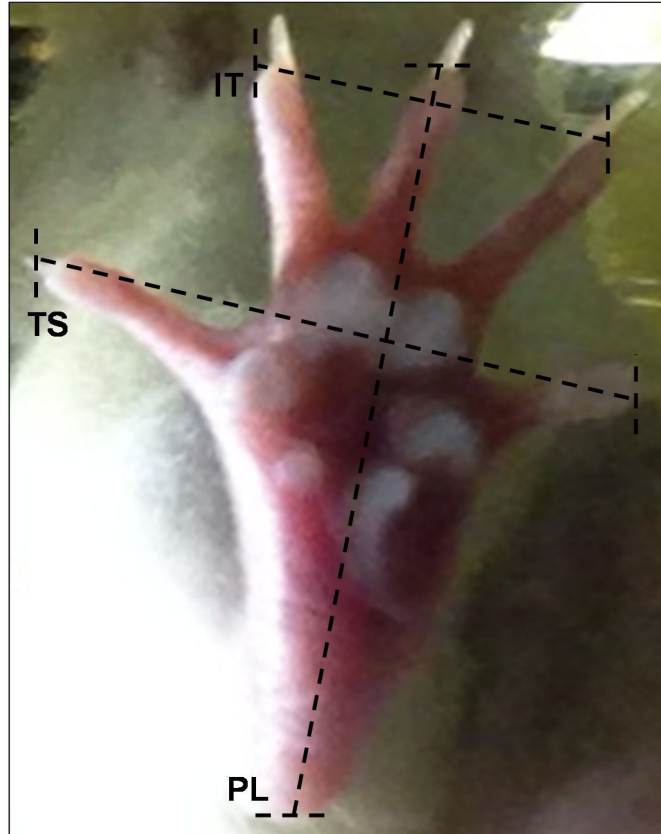


Figure 6.8: Depiction of the foot measurements required for calculation of the SFI.

6.2.4.6.2. Grid walk analysis

The grid walk test (Figure 6.9) was used as an assessment of fine motor control and gait movement. At 7, 14, 21 and 28 days post-surgery, rats were conditioned to walk across a 100cm long metal wire grid with a distance of 2cm between the wires. The rats were observed for their walking motions along the grid.

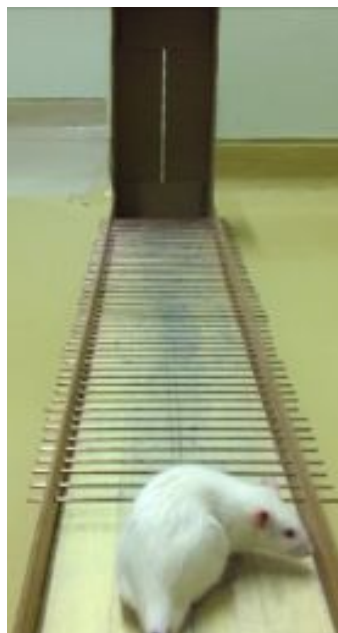


Figure 6.9: Grid walk setup for observation of motor control and gait movements.

6.2.4.7. High-frequency ultrasound imaging

The Vevo 2100 High-Frequency Ultrasound System (VisualSonics, Inc., Toronto, Ontario, Canada) equipped with an ultrasound probe of 40-MHz and a 6-mm focal depth with a spatial resolution of $40 \times 80 \times 80 \mu\text{m}^3$ at focus was utilized for imaging at 14 and 28 days post-surgery to visualise the implanted conduit. Rats undergoing ultrasound imaging were restrained on a heated stage while maintained under anaesthesia of 1.5% isoflurane in oxygen.

6.2.4.8. Muscle mass measurement

Muscle mass assessment of the gastrocnemius muscles was made at the end of the study at 28 days. The gastrocnemius muscle from both the left and right sides were dissected out immediately after euthanasia of animals with 100mg/kg pentobarbital i.p. The muscles were weighed while still wet using an electronic balance. The increase or decrease in muscle mass was used as a measure of functional recovery and target organ re-innervation.

6.2.4.9. Histology

Histological analysis of the nerve tissue for observation of axon and myelin sheath regeneration was done after euthanasia of animals with 100mg/kg pentobarbital administered intraperitoneally; the implanted nerve conduit was dissected out after 14 and 28 days post-implantation. The complete conduit containing the nerve tissue were fixed with 2.5% glutaraldehyde in a 0.1M phosphate buffered saline (PBS) solution for 2 hours before being fixed in 1% osmium tetroxide for 1.5 hours. After washing with PBS and dehydration with ethanol, the specimens were embedded in wax blocks and sectioned into 5-6 μm sections using a microtome. The cross-sections were stained with haematoxylin and eosin and viewed under a light microscope. Similarly, the dissected gastrocnemius muscle was assessed for the extent of muscle atrophy.

6.3. Results and Discussion

6.3.1. Textural profiling and SEM visualisation of the Biosimulated Nerve Repair Device

Textural profiling was conducted at weekly intervals over a 4-week study period in PBS pH 7.4 of the hollow and fiber-containing hydrogel conduits to assess the transition in mechanical properties induced by the addition of an intraluminal filler. The incorporation of pristine PMMA particles in the gellan-xanthan hydrogel for pH-responsive particulate leaching resulting in the formation of a porous matrix has previously been established (Ramburrun *et al.*, 2017) and presented in Chapter 4 of this thesis.

Figure 6.10a shows the %matrix resilience profiles obtained over 4 weeks for the hollow conduit and that containing intraluminal fibers. An improvement in matrix resilience of up to 56.90% at the end of 28 days is noted upon addition of the intraluminal filler compared to 42.01% achieved with the hollow conduit. Usually, the presence of empty voids dispersed within a polymer matrix allows the matrix to undergo a transient change in shape upon compression to a certain extent before reverting to its original form and shape after removal of the compressive force; however, singular and increasingly large unoccupied voids such as the hollow lumen would potentiate a decrease in resilience due to compromised resistance against compressive stress. The incorporation of the electrospun fibers in a spiral configuration within the conduit lumen improved resilience via partial occupation of the rather large void present in the hollow lumen of the conduit. The coiled-spiral configuration of the electrospun fibrous films enhanced the resilience of the guidance scaffolds as opposed to flat sheets of the film. Whereas induction of porosity from PMMA particulate leaching could improve permeability of the hydrogel thereby facilitating uninterrupted physiological processes, the improved resilience of the conduit imparted by the filling of the lumen resulted in enhanced ability of the system to recover upon subjection to compressive forces which may arise from contact with the surrounding tissues during *in vivo* implantation.

The improved resilience of the system corresponds to the reduced deformability moduli observed in Figure 6.10b as compared to the hollow conduit from 1.26N/mm to 0.47N/mm at the end of 28 days. Decreased deformation indicates the shift from a rigid towards a more flexible scaffold. The layers of the coiled fibrous film, of delicate thickness (10 μ m), would less likely resist deformation compared to fibers thicker in diameter. Furthermore, declined deformability moduli and increased flexibility does not imply weakened mechanical strength of the system. As noted in Figure 6.10c, the intraluminal fibers impart increasing fracture energies over the 28-day study period indicating the expenditure of higher energies required to initiate rupture or breakage of the system compared to the hollow conduit, particularly at the end of the study where the fracture of the final assembled device exceeds that of the hollow conduit at 0.19N.mm and 0.17N.mm, respectively. An easily deforming matrix has the ability to disperse energy generated from an applied force to undergo a temporary change in shape rather than resisting the energy causing immediate fracture of the scaffold. An implant should possess sufficient flexibility and toughness to withstand forces generated from body movements and conforming to the tissue shape rather than to resist deformation creating the potential for discomfort and pain resulting from textural incompatibilities with the native body tissues.

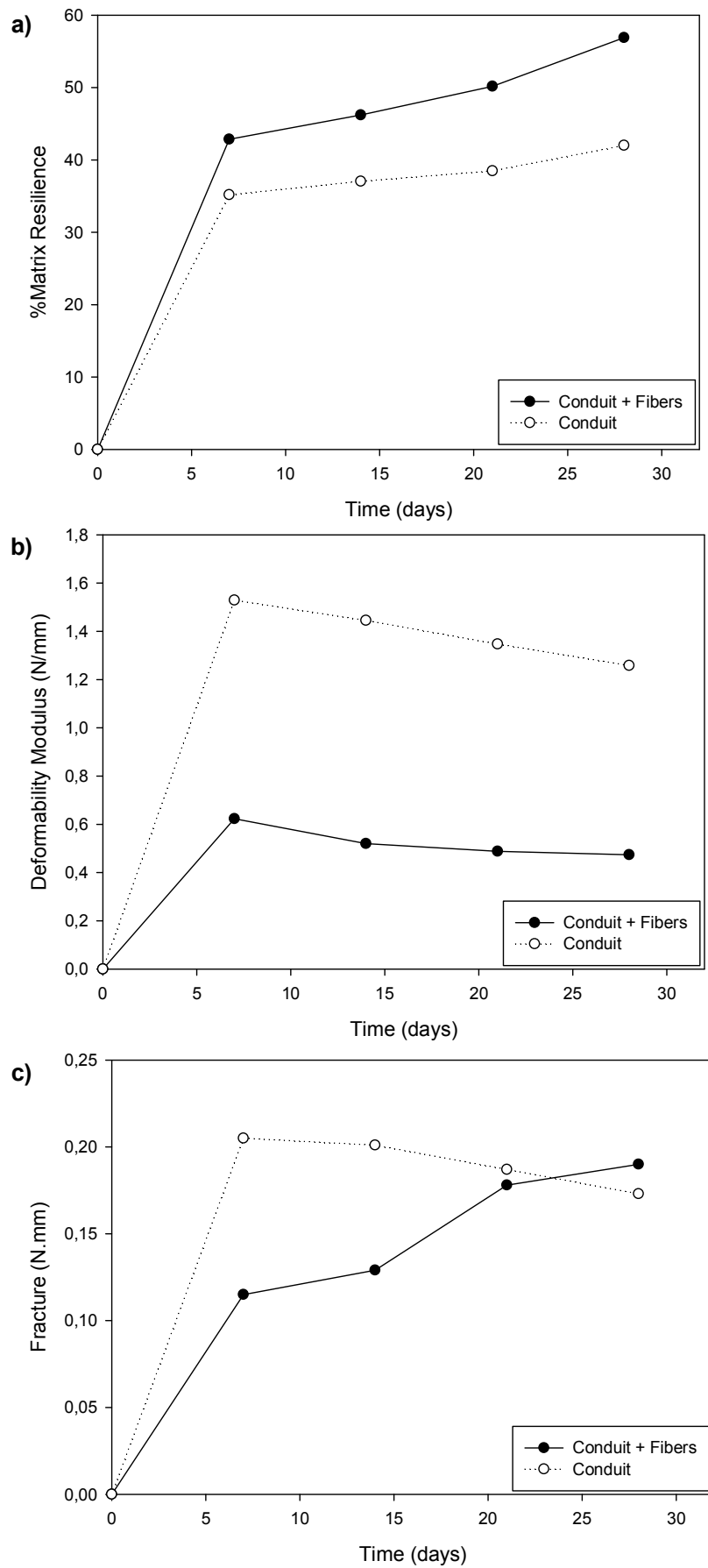


Figure 6.10: Textural analysis profiles depicting between the hollow conduit and the final assembled device a) %matrix resilience b) deformability moduli c) fracture over a 4-week study period conducted in PBS pH 7.4.

Biaxial analysis presented with varying tensile strengths in the 90° and 180° axes (Figure 6.11). This was particularly noted with the electrospun fibrous films which demonstrated a distinct pattern and enhanced tensile strength in the force-direction parallel to the fiber orientation (180°) whereas the pull in the opposing direction (90°) exhibited weaker strength (Figure 6.11a). This characteristic is imparted by weak inter-fiber adhesion points associated with the pattern of fiber deposition which further indicates the aligned orientation of the electrospun fibers as depicted in SEM imaging in Figure 6.15. As illustrated in Figure 6.12a, application of a tensile force in the direction perpendicular to the fiber alignment results in separation of the individual or grouped fiber strands whereas force applied in the direction parallel to the fiber arrangement results in slight stretching of the fibers prior to the onset of mechanical failure. Tensile failure of the fiber, in the 180° axis, is noted at 14 and 26 seconds with a break force of 88mN. Failure points at 5, 13 and 40 seconds along the 90° axis indicate separation of individual or bundles of fibers with forces of 30mN, 14mN and 17mN, respectively.

Likewise, the hydrogel sample demonstrated similar mechanical properties but varying strengths in the pull along the 90° and 180° axes (Figure 6.11b). Superior mechanical properties were noted along the 90° axis with failure occurring at 2539mN at 6 seconds whereas breakpoint along the adjacent axis was acquired at 2021mN at the same time point. This is possibly due to the crosslinking and subsequent molecular arrangement of the polymer chains around the longitudinal plane of the glass rod, during conduit synthesis, as opposed to the circumferential plane as depicted in Figure 6.12b. The superior tensile strength in the observed direction is favourable since tensile strain resulting from suturing of the conduit to the nerve ends would be subjected to the longitudinal aspect of the conduit as shown in the suturing technique in Figure 6.5.

The accompanying video files (see CD inside the back cover of this thesis), depicting computational tracking of the data points, show increased displacement in the direction where superior tensile strength is noted (Video 6.1 and Video 6.2 showing displacement tracking of the electrospun fibers and hydrogel, respectively) whereas the changing yellow and red strain colour-blocks indicate the durable and failing areas, respectively, of the sample during subjection to the applied force over the test duration (Video 6.3 and Video 6.4 showing strain tracking of the electrospun fibers and hydrogel, respectively).

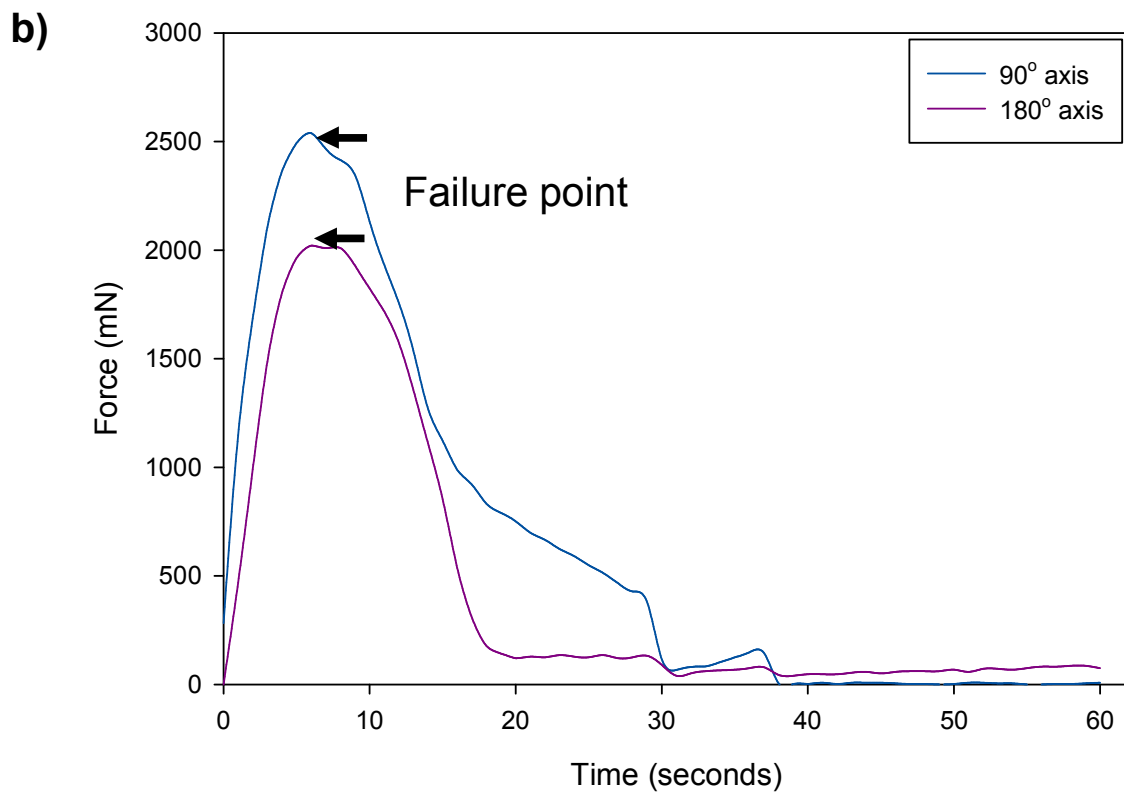
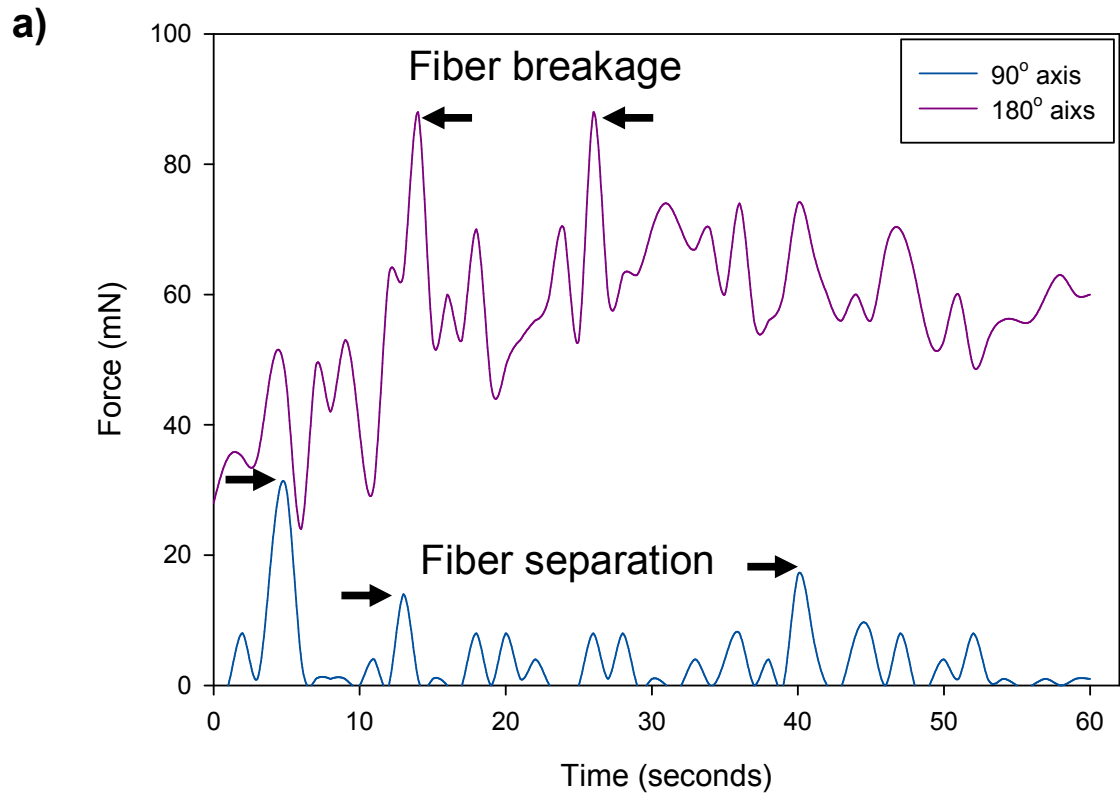


Figure 6.11: Biaxial mechanical testing of a) the electrospun PHBV-MgOI-NAC guidance fibers and b) the gellan-xanthan hydrogel conduit in its dry state.

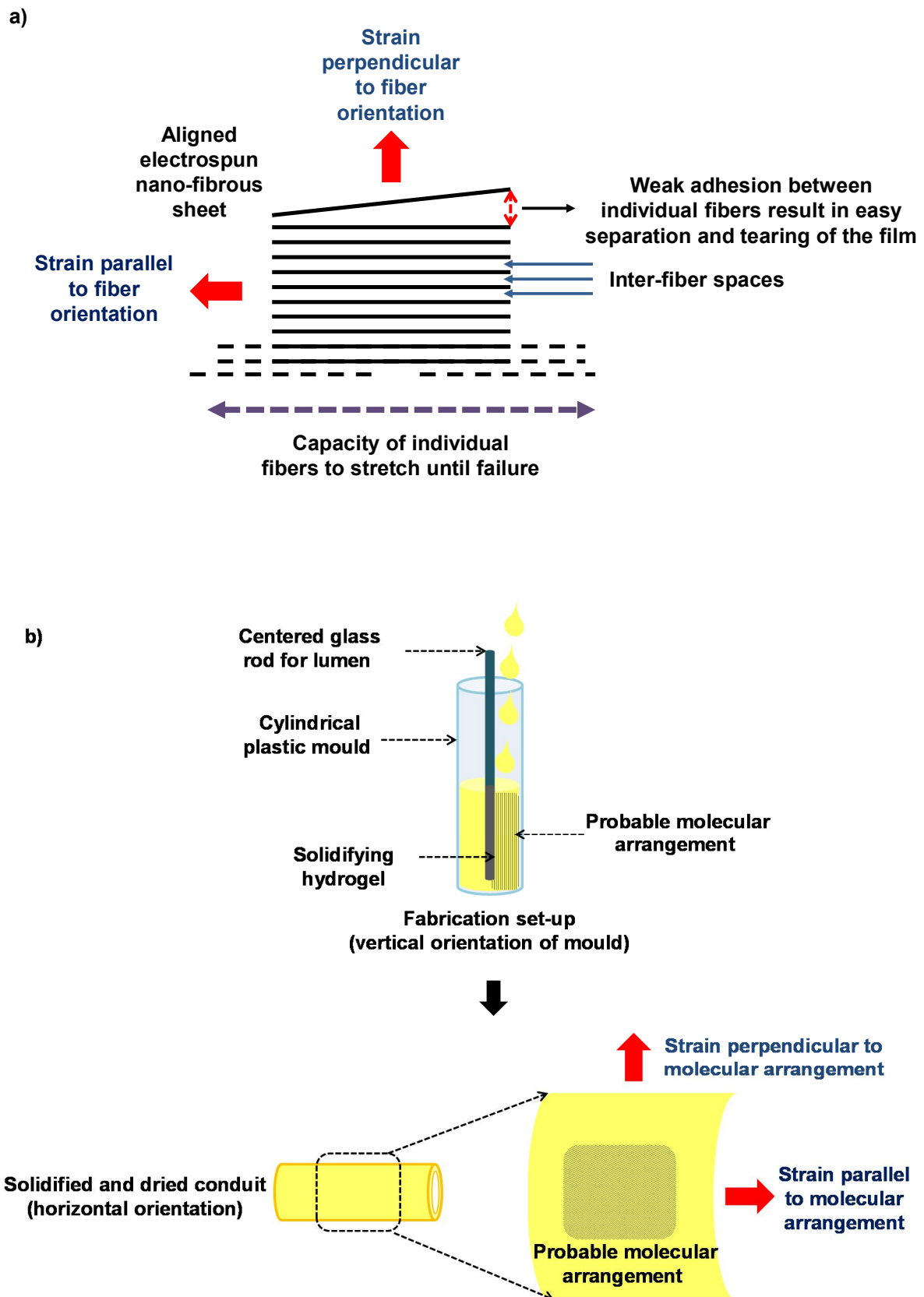


Figure 6.12: Illustration of the effect of fiber and potential molecular arrangement on biaxial tensile properties of a) the electrospun PHBV-MgOI-NAC guidance cues and b) the gellan-xanthan hydrogel conduit.

6.3.2. *In vitro* release and mathematical modelling of NGF release kinetics

The delivery of the neurotrophic growth factor, NGF, is crucial in attaining adequate neuronal survival, growth promotion and, foremost, differentiation of the regenerating axons *in vivo*. Therefore, its release profiles from the hollow and fiber-loaded conduits were evaluated over a 4-week study period. A few studies have shown that the release of high doses of NGF may hinder nerve regrowth due to down-regulation of TrkA receptors in the presence of excessive NGF molecules. Although large variations exist, lower doses of exogenous NGF are generally required as the neuronal tissue is capable (depending on the extent of the injury) of synthesizing its own NGF in the case of nerve injury after the Wallerian degeneration phase is terminated. The objective, therefore, would be to assist the body in repairing itself rather than implementing aspects to coerce repair processes.

The inclusion of the PHBV-MgOI-NAC intraluminal fibers imparted a decrease in the overall fractional release of NGF over the 4-week *in vitro* study period compared to the hollow conduit, as shown in Figure 6.13, with fiber-loading producing a 61% decrease fractional release at 28 days. Conduits containing the intraluminal fibers demonstrated a maximum release of 5.5% of the total loaded NGF whereas the hollow conduit presented a slightly rapid release reaching 14% at the end of 4 weeks. This observed decrease in release could be attributed to the further entrapment of NFG molecules upon its escape from the crosslinked gellan-xanthan hydrogel matrix. It was noted, from the previous study utilizing bovine serum albumin as a model protein, that the release of NGF was much lower. This change in drug release patterns was ascribed to the ionic charge of the polymer matrix and the bioactive release agents. Both gellan and xanthan gum are anionic polymers whereas NGF, at pH 7.4, carries a positive charge, hence its potential ionic adhesion to the dissolved polymer chain fragments. In this case, gellan is mostly crosslinked with xanthan and the released NGF molecules become entrapped within the negatively charged unreacted PHBV chains resulting in reduced escape and release of the peptide outside the conduit system. The lack of NGF release at days 1 and 3, similarly, could be attributed to such an ionic interaction between peptide and polymer molecules. Such a release pattern and entrapment of NGF acquired with implementation of the intraluminal guidance fibers could be favourable as it decreases the likelihood of NGF wastage and unnecessary leakage out of the conduit system. In this way, the released NGF molecules would be confined to the interior of the conduit lumen where it would be readily available to the regenerating axons as opposed to escape outside the conduit extremities. Furthermore, any attached NGF molecules onto the PHBV fiber surface could enhance neuronal cell interaction and adhesion along the fibers

thereby improving the physical guidance ability of the fibers towards innervation of the target muscles and functioning as a chemical cue in addition to its action as a therapeutic cue.

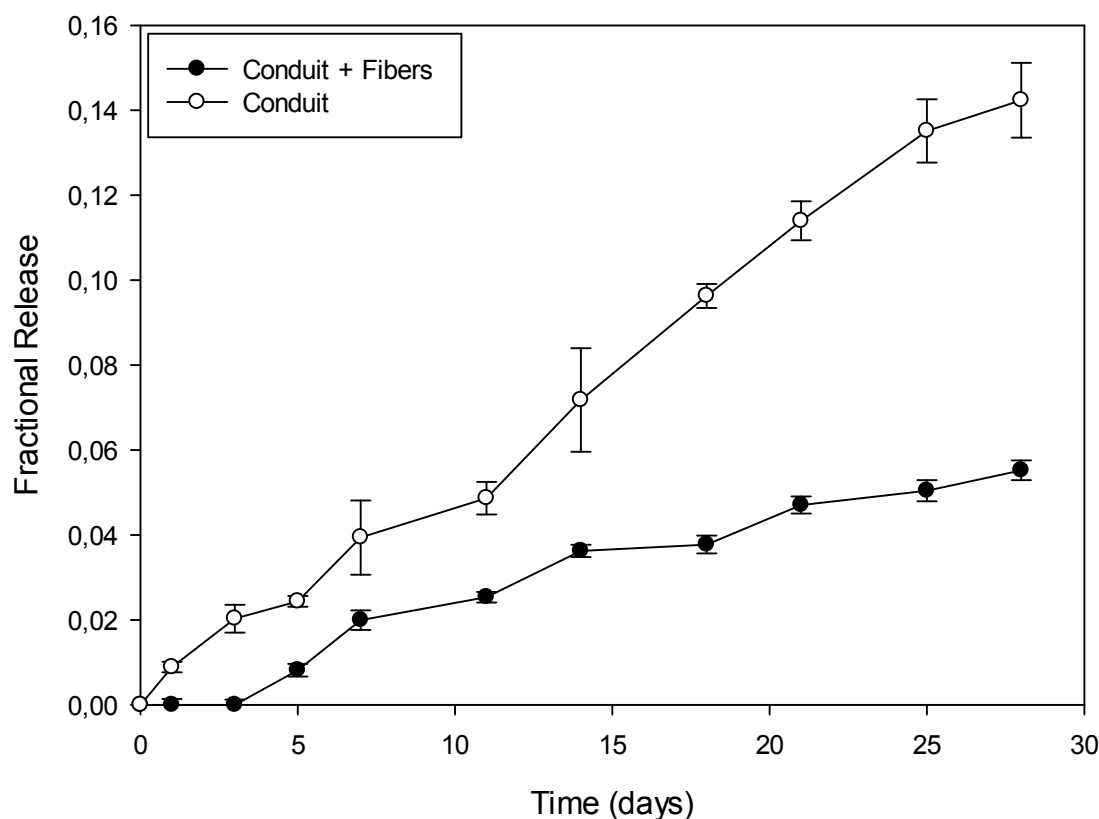


Figure 6.13: *In vitro* NGF release profiles from the hollow and fiber-containing conduits over 28 days in PBS pH 7.4.

Mathematical modelling was performed to determine the drug release mechanisms governing NGF release from the conduit systems. Mathematical modelling of the release data, shown in Table 6.1, revealed that NGF release imparted by the addition of the intraluminal fibers is best described by the Higuchi model whereas release from the hollow conduit conforms to the zero-order model, indicating the central role of diffusion in the control of NGF release from the hydrogel matrix to the PHBV-fiber matrix and out the scaffold.

The diffusional exponent, derived from n of the Korsmeyer-Peppas equation, indicates that NGF release from both the fiber-containing ($n = 0.622$) and hollow conduit ($n = 0.850$) indicate a non-Fickian anomalous transport encompassing a coupled-diffusion and relaxation mechanism. The contribution of Fickian diffusion (F) and Case-II relaxational (R) release of the system, determined from the R/F values derived from the K_1 and K_2

parameters in the Peppas-Sahlin equation, suggest a predominating release via the Case-II relaxational (increased R/F of 27.121) mechanism for the fiber-loaded conduit whereas Fickian diffusion (decreased R/F of 1.978) mainly controls NGF release from the hollow conduit. In each of the formulations, the K_2 values exceed that of the K_1 values (Table 6.1) thus indicating the increased contribution of the Case-II relaxational release mechanism as opposed to the Fickian diffusion in the overall release mechanism of NGF. Since NGF could be bound to the polymeric components via an ionic interaction, polymer relaxation ensuing erosion would be required for the peptide's subsequent release; hence the Case-II relaxational mechanism determined form kinetic modelling.

Table 6.1: Kinetic modelling results of NGF release from the fiber-loaded and hollow hydrogel conduits

Mathematical Model	Conduit + Fibers	Conduit
Zero-Order		
R^2	0.960	0.907
First-Order		
R^2	0.962	0.904
Higuchi		
R^2	0.975	0.884
Hopfenberg		
R^2	0.961	0.905
Hixson-Crowell		
R^2	0.961	0.905
Korsmeyer-Peppas		
R^2	0.821	0.958
N	0.622	0.850
Peppas-Sahlin		
R^2	0.828	0.907
K_1	185.630	262.240
K_2	5034.400	518.640
R/F Ratio	27.121	1.978
Best-fit model	Higuchi	Zero-Order

6.3.3. PC12 proliferation in response to the nerve repair device components

MTT assay for the growth of PC12 cells on the various scaffolds components and the final assembled device were determined over a 72-hour incubation period as shown in Figure 6.14. The DS-loaded hydrogels and the NAC-loaded fibers showed improved cell growth compared to the corresponding bioactive-free derivatives (Figure 6.14). The NAC-loaded fibers were shown to better support cell growth than the DS-loaded Gel-Xan hydrogels

alone. Of interesting note, the NAC-loaded fibers and the final assembled BNRD (integrating the individual bioactive-loaded hydrogel and fiber components) exceeded the growth of the control. Pristine electrospun PHBV did not efficiently enhance PC12 proliferation compared to the PHBV-MgOI and PHBV-MgOI-NAC composite fibers (Figure 6.15a-b). The observed enhanced growth reflects the favourable bioactive effects of MgOI and NAC incorporation with PHBV as the base polymer. It is noted that pristine PHBV fibers and the bioactive-free Gel-Xan hydrogel (denoted by DS-F in Figure 6.14) showed reduced cell viability in comparison to the control which could be due to the degradation of the carboxylic acid moieties present in these polymers (PHBV: butyric and valeric acids; Gellan and xanthan gum: glucuronic and pyruvate acidic moieties) thus creating a sudden decrease in pH thereby inducing a form of cellular stress resulting from the change in environmental pH. Furthermore, the uneven textured and bead-like topography of the pure PHBV fibers (Figure 6.15c) could further reduce cell compatibility and interaction

Similarly, DS exhibited potential as a neuronal-growth promoter as noted in the PC12 growth response in Figure 6.14. Combinations of DS, MgOI and NAC could function synergistically for improved PC12 cell growth as presented by the significantly improved proliferation level achieved with the BNRD (Figure 6.14). The enhancement of PC12 cell growth on the BNRD system could be mainly attributed to the combined neuro-protective actions (Hart *et al.*, 2008) of DS, Mg^{2++} ions and NAC where the presence of elemental magnesium is indeed noted in the EDS analysis in Figure 6.16a-b. Increased neuronal survival suggests a greater number of viable cells available for further proliferation via prevention of apoptotic cell death resulting from exposure to stress and sudden cellular environmental change. Furthermore, the presence of oleic acid, despite its presence in an oleate form, could enhance cell proliferation via action as a neurotrophic growth factor (as discussed in Chapter 5 Section 5.3.9). In addition, NAC could prevent cellular oxidative damage whilst simultaneously enhancing the cell adhesion ability of the cells on the scaffold surface. As per the MTT assay, the final BNRD system shows adequate PC12 cell viability and biocompatibility, thereby allowing its further investigation in an *in vivo* model.

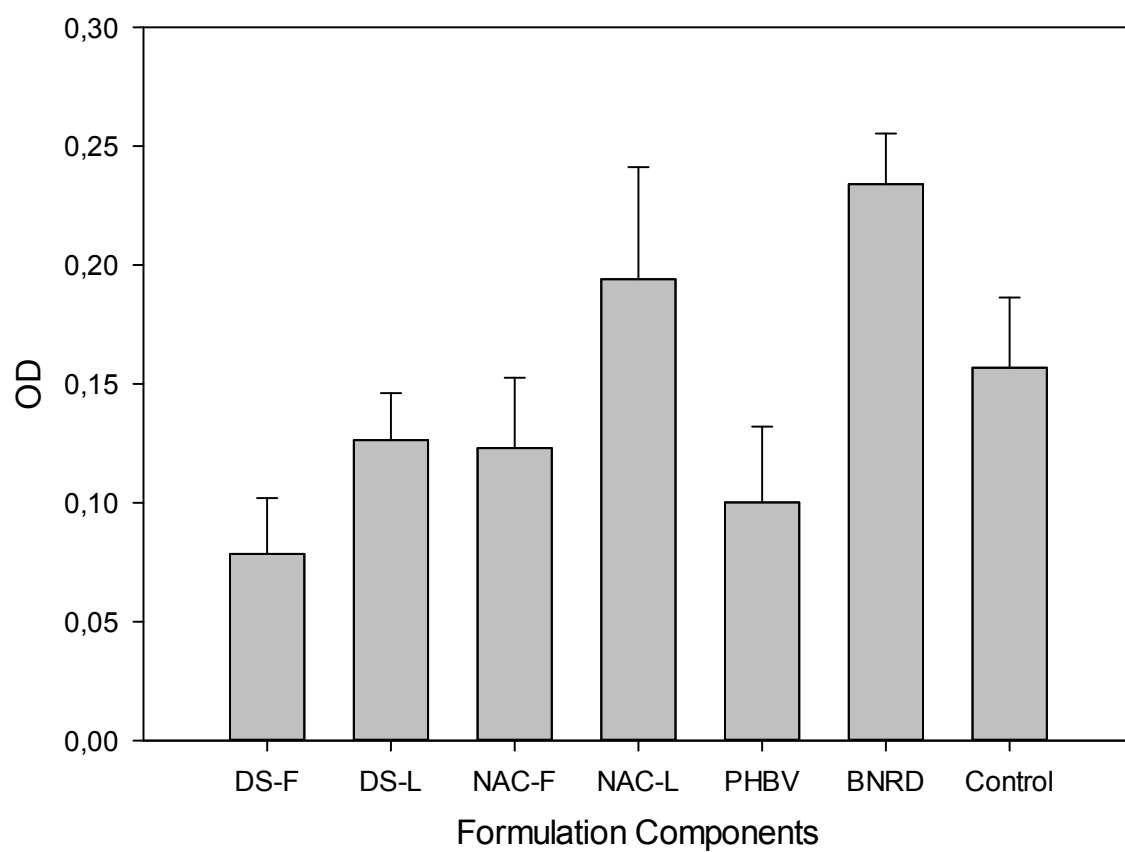


Figure 6.14: MTT PC12 viability studies in response to the drug-free (DS-F) and drug-loaded (DS-L) PMMA-intercalated Gel-Xan hydrogel conduits; the NAC-free (NAC-F) and NAC-loaded (NAC-L) electrospun guidance fibers; 100% electrospun PHBV and the final assembled biosimulated nerve repair device (BNRD) (n=3).

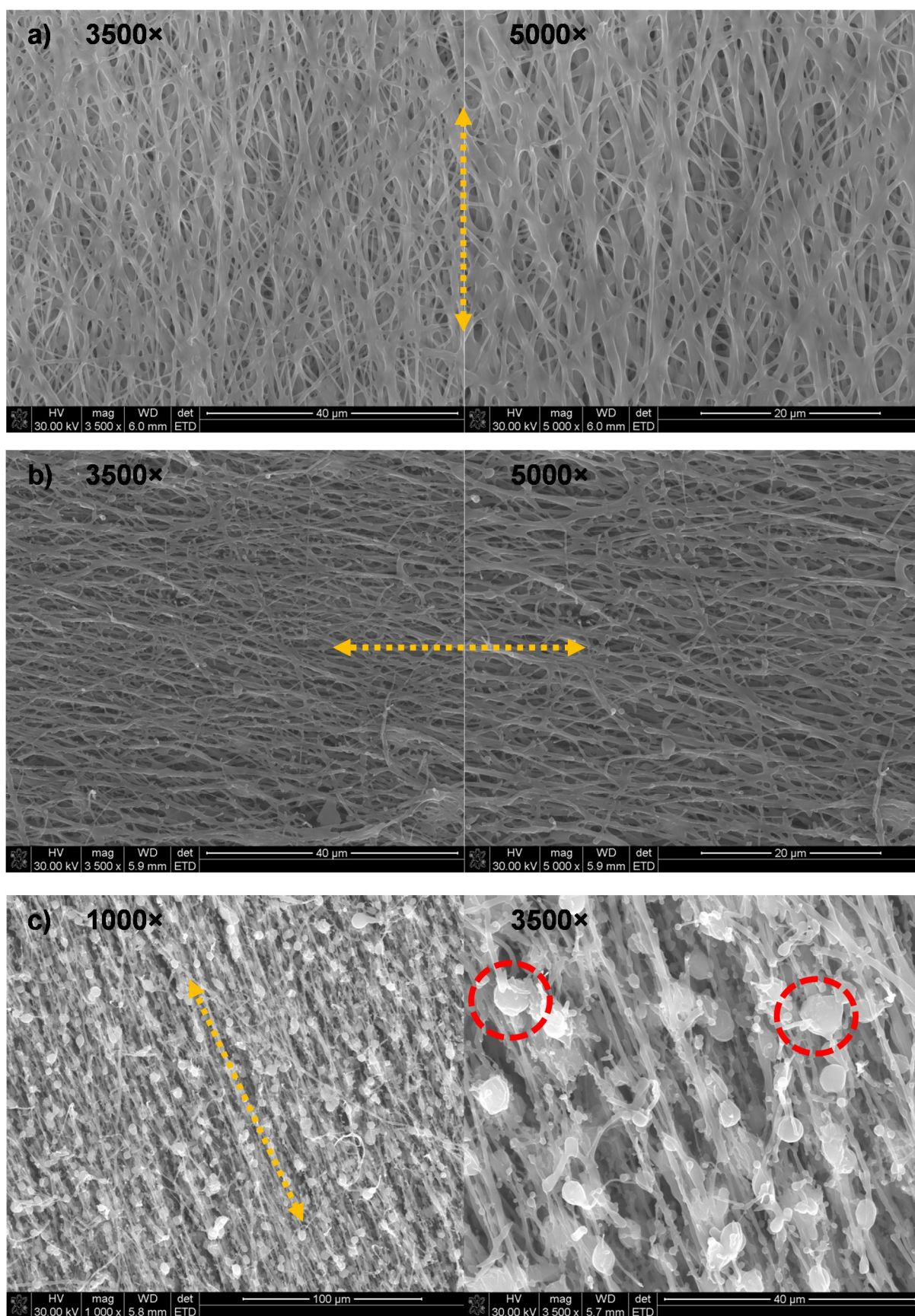


Figure 6.15: SEM imaging of a) the optimized NAC-loaded formulation b) the optimized NAC-free derivative c) pure electrospun PHBV.

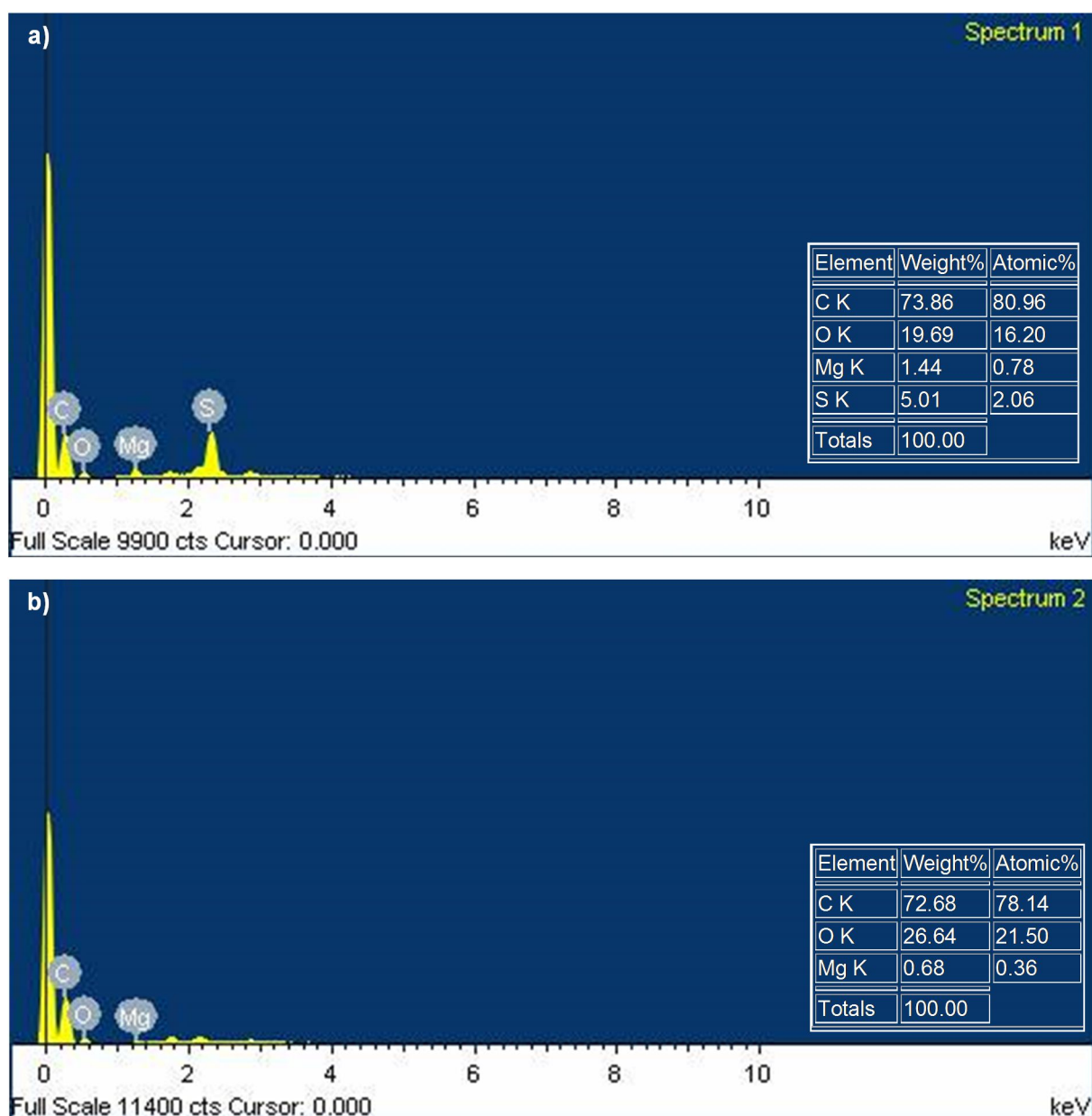


Figure 6.16: EDS analysis depicting the elemental composition of a) the NAC-loaded optimized fibers and b) the NAC-free optimized fibers.

6.3.4. Assessment of nerve regeneration by implantation of various conduits across the rat sciatic nerve defect

6.3.4.1. Walking track analysis

Pre-operatively, the mean SFI determined from baseline walking track experiments, was $-8.17 (\pm -4.15)$ lying closer to zero, thus indicating normal nerve function of the animals employed (Iohom *et al.*, 2005). After the injury and surgical procedures, 0-48 hours, the foot of the injured hind limb was completely flaccid in the plantar flexion position, as shown in Figure 6.17a, indicating total paralysis. Within 3-5 days, the rats started gaining control of the

affected limb thus assuming the original neutral orientation of the foot, as shown in Figure 6.17b of a rat in Group E. At 7 days post-injury, SFI values decreased to -75.05, -102.70, -83.52, -84.95 and -84.54 for Groups A (autograft repair), B (DS-NGF-free hollow conduit), C (DS-NGF-loaded hollow conduit), D (DS-NGF-loaded, NAC-free device) and E (DS-NGF-loaded, NAC-loaded device) respectively, indicating substantial loss of function whereas an SFI value of -100 or less (as noted with Group B) implied complete nerve dysfunction. Over the 28-day study period, the SFI values for Groups A-C were seen to progressively decrease, indicating deteriorating nerve function whereas SFI values of Group D remained fairly constant between -82.53 and -84.95 and that of Group E showed slight improvements from -84.54 to -80.17 from 7 to 28 days (Figure 6.18). The noted increases in the SFI values of Group E over the 4-week study denote the effective functioning of the final assembled BNRD system. Group B, receiving the DS-NGF-free hollow conduit, performed poorly over the 28-day study and obtaining the lowest SFI of -106.12 after 4 weeks. Group C, containing an identical but DS-NGF-loaded conduit, exhibited improved function compared to Group B with an SFI of -97.32 at 28 days therefore suggesting the importance of the reparative role of the anti-inflammatory DS and neurotrophic NGF in the treatment of peripheral nerve injuries. Introduction of the aligned intraluminal support scaffolds within the hollow conduit lumen further improved functional recovery outcomes, as depicted by the SFI values in Figure 6.18, where the inclusion of NAC in the electrospun fibers (Group E) enhanced functional recovery. These results suggest effective action of MgOI and NAC in further protecting and promoting neuronal growth (as evidenced in the MTT assays in Figure 6.14) while the provision of aligned physical guidance fibers could improve target muscle innervation, hence the improving walking ability noted. In addition, the obtained NGF release kinetics in Figure 6.13 could be deemed adequate for nerve injury treatment and regeneration.

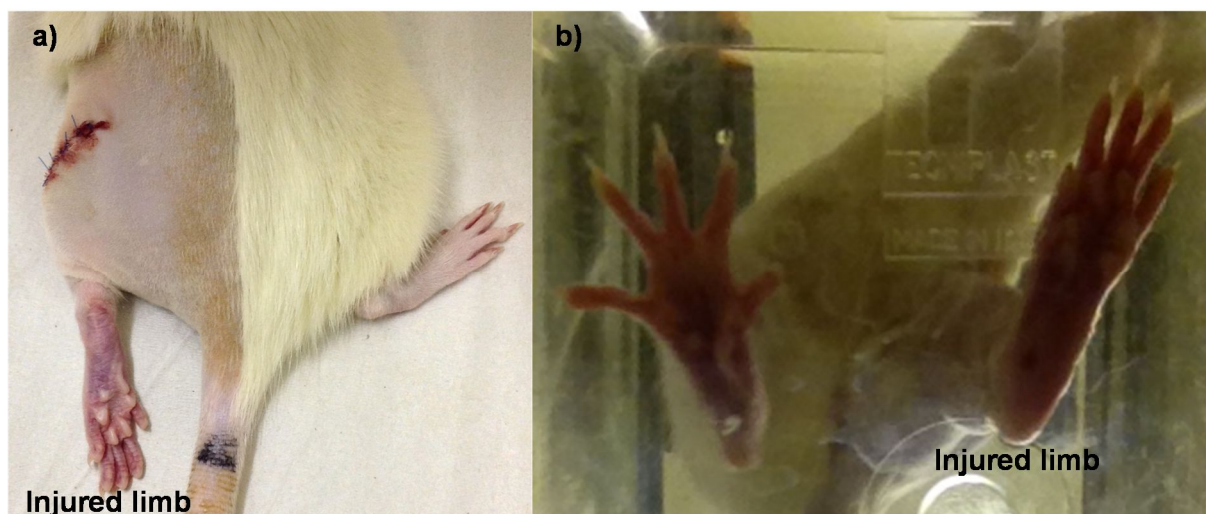


Figure 6.17: Photograph of rat in group E at a) day of surgery and b) 3 days post-surgery.

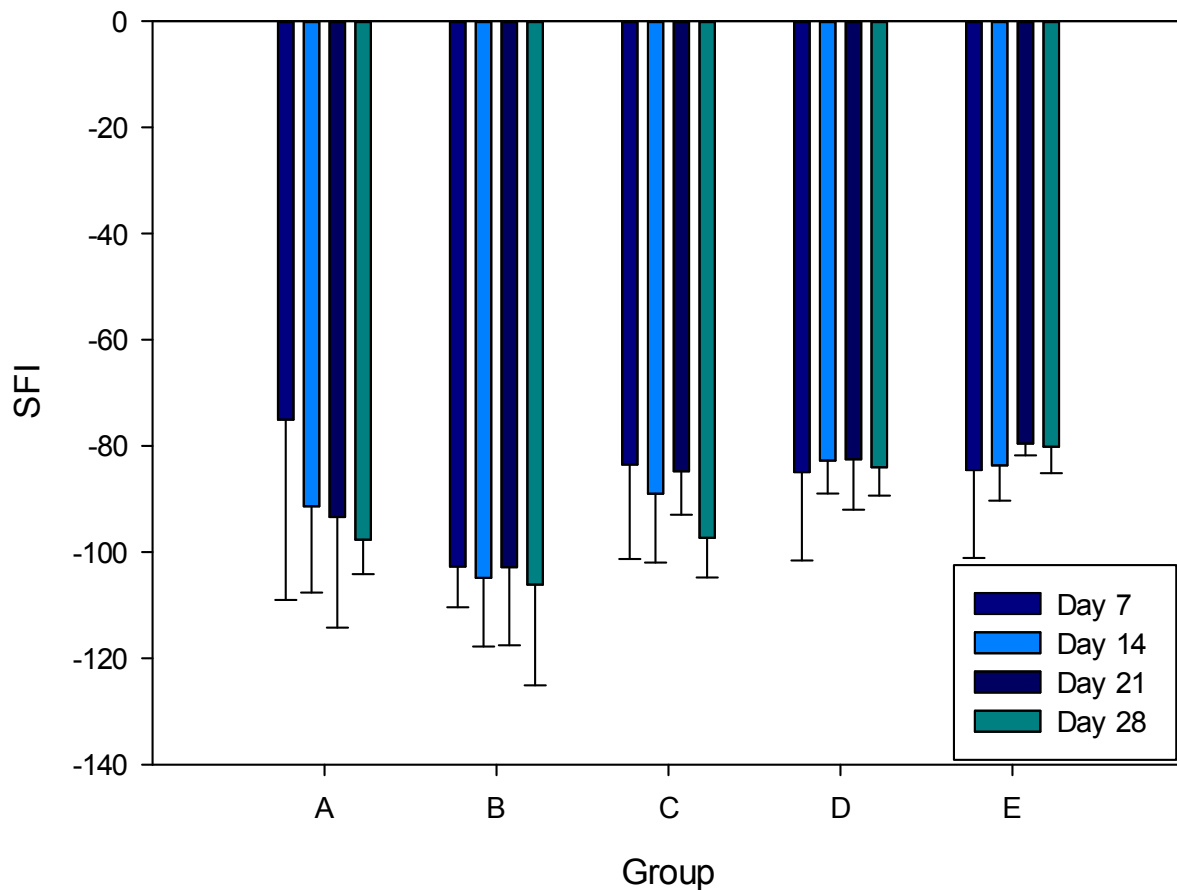


Figure 6.18: Sciatic function indices determined from walking track analysis of the various *in vivo* experimental groups over a 28-day test period.

6.3.4.2. Grid walk observations

Grid walk observations and recordings showed that all groups, at 4 weeks, showed slow recovery of fine motor control of the injured hind limb compared to the recordings taken before induction of injury. Curling and contraction of the toes on the foot of the injured limb prevented toe-spreading and the ability of the animal to grip onto the wire grids while walking. Although fine motor recovery return was gradual, minimal to no slippage of the affected hind limb was noted as the rats were able to manoeuvre across the grid via an exaggerated motion of the hip producing a hopping-like movement. Groups D and E, containing the intraluminal fibers, were observed to attain slightly improved motor function as animals in this group were able to consciously place the foot of the injured limb onto the wire grid as they walked, thus indicating the return of improved functional recovery as additionally noted with SFIs values acquired.

6.3.4.3. Ultrasound imaging and macroscopic examination of the implanted conduits

Ultrasound imaging of the injured left hind limb at 2 weeks post injury and implantation revealed the presence of an intact cylindrical-shaped conduit as noted in Figure 6.19a and b. At 4 weeks the architecture of the implanted conduit could no longer be identified and was probably due to obscuring resulting from the formation of inflammation, scar tissue or potential vascularisation of the conduit surface. Imaging of the animals receiving the autograft repair (Group A) exhibited a continuous thin linear-traversing structure depicting the intact repair of the transected nerve at both 2 and 4 weeks as depicted in Figure 6.19c. However, no abnormalities, implant rejection or detachment from the nerve ends were observed during ultrasound imaging.

At the end of the 4-week study, dissections, as shown in Figure 6.20, revealed the maintenance of an intact and hydrated conduit with minimal erosion and adequate mechanical strength as the conduits were still secured to the nerve ends at the suture points and no structural deformations were noted. A maximal swelling of the conduits up to 1.5mm was noted. A decline in swelling compared to that obtained in previous *in vitro* dissolution studies (Ramburrun *et al.*, 2017) could be attributed to the exposure of a lower fluid volume in the thigh-sciatic nerve region and the potential binding and subsequent crosslinking of plasma Ca^{2+} ions with the gellan-xanthan hydrogel, thereby strengthening polymer network and limiting its swelling and erosion capacity. Partial concealment of the implanted conduit by a thin network of vascular and connective tissue or inflammation could result in the poor conduit visibility noted at 4 weeks of the ultrasound scanning.

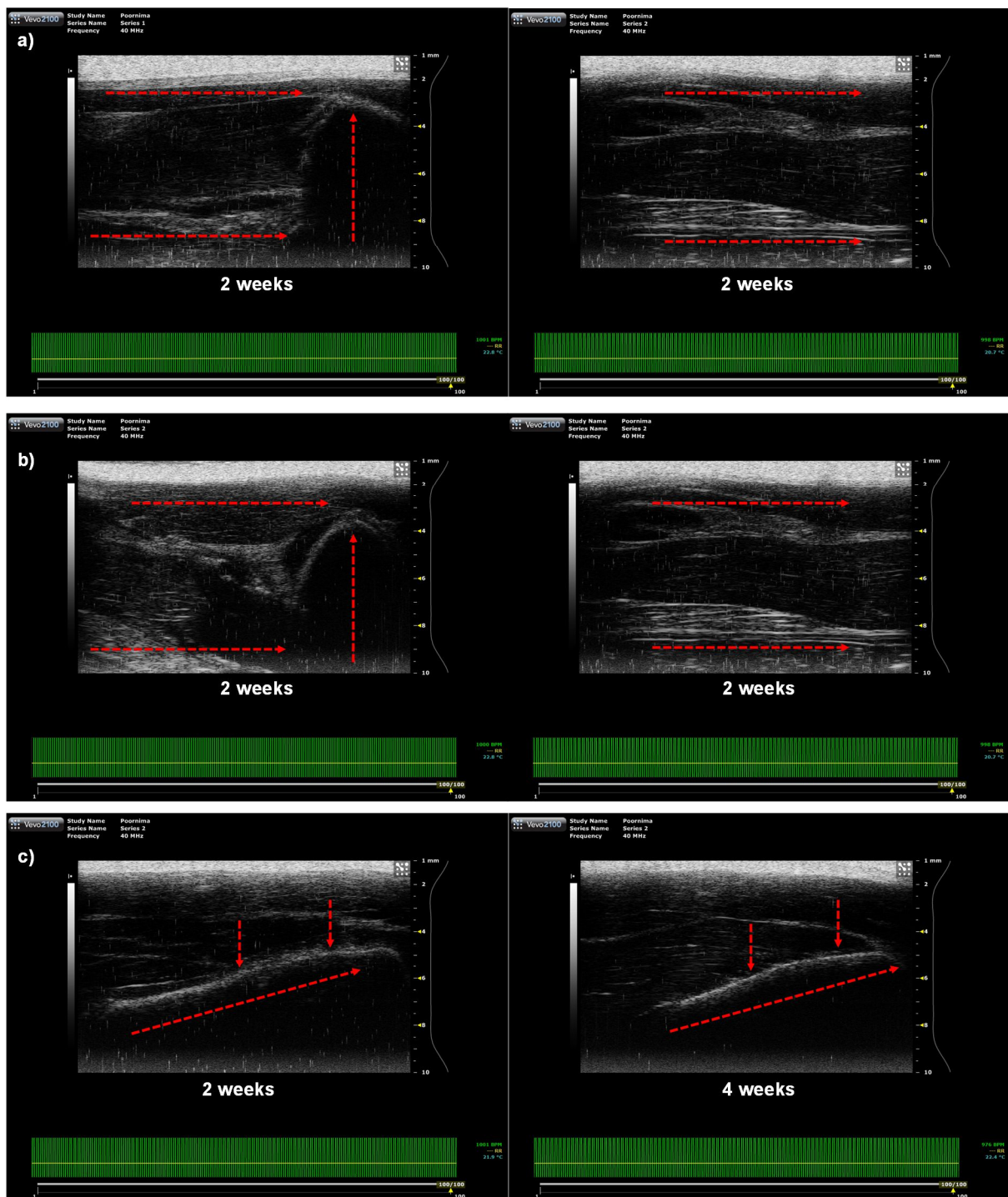


Figure 6.19: Ultrasound imaging conducted at 2 weeks post-implantation a) Group C: hollow conduit b) Group E: final assembled device and c) Group A: autograft repair at 2 and 4 weeks post-surgery.

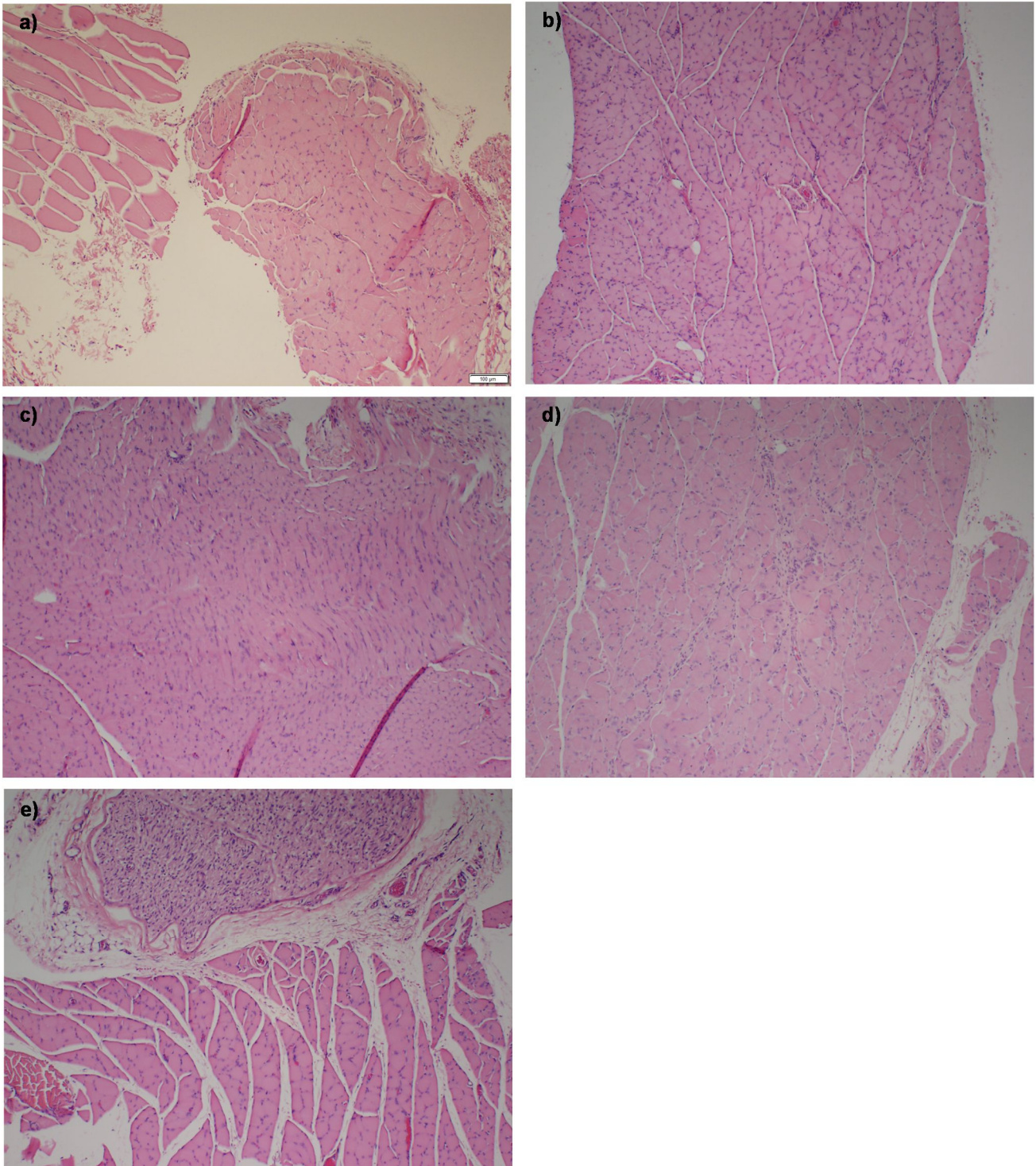


Figure 6.20: Photographic representation of the 4-week dissections showing the intact presence of the implanted conduits a) Group B b) Group C c) Group D d) Group E and e) Group A autograft repair.

6.3.4.4. Muscle mass and histological analyses

The incorporation of DS, MgOI and NAC elicited positive effects in the *in vitro* growth of PC12 cells via its collective neuro-protectant and growth-promoting actions. A similar effect was noted on the tissue appearance of the gastrocnemius muscle shown in Figure 6.21 where treatment with the aforementioned bioactives promoted the muscle fiber health as indicated by the presence of numerous interstitial cell nuclei. After 28 days of implantation of the various conduits, the gastrocnemius muscles were excised to assess the muscle mass preserved after injury-induction and subsequent surgical and conduit repair. Muscle mass has been utilized as an effective indicator of recovery outcomes from peripheral nerve injuries. Increased muscle mass suggests reduced occurrence of muscular atrophy from lack of mobilization and improved muscle re-innervation by the regenerating axons. From the %muscle mass remaining values depicted in Figure 6.22, introduction of the PHBV-MgOI-NAC aligned (as depicted in the SEM imaging in Figure 6.15a) guidance fibers via placement into the hollow Gel-Xan hydrogel conduit (Group E) improved recovery compared to the hollow hydrogel conduit (Groups B and C) alone and with slightly improved function than the comparative autograft repair (Group A) where %muscle mass remaining 40.96% for Group A and 43.05% for Group E (Figure 6.22). Although the NAC-free guidance fibers (Group D) was outperformed by the autograft Group A, it achieved higher preservation of muscle mass (34.74%) compared Groups B and C. Groups B and C comprising of the bioactive-free and DS-NGF-loaded hollow hydrogel conduits, respectively, exhibited increased muscle atrophy with only 22.44% and 21.71% muscle mass remaining at the end of 28 days. Despite Group C (receiving DS-NGF treatment) exhibiting a slightly decreased muscle mass compared to Group B (receiving no DS-NGF treatment), improved SFIs of Group B from walking track analysis suggest the return of improved muscle functionality. In addition, histological imaging in Figure 6.21b and c depicts similar muscle tissue appearance in Groups B and C. Improved muscle mass in Groups D and E indicate enhanced axonal guidance resulting in improved target muscle tissue re-innervation and functional recovery upon implementation of the trio-functionalized composite guidance scaffolds into the hollow hydrogel conduits. The appearance of improved muscle tissue regeneration, indicated by the presence of increased muscle interstitial cell nuclei (Tedesco *et al.*, 2010; Melacova and Puri, 2012 and Ceafalan *et al.*, 2014) and reduced muscle cell shrinkage, is noted in Group E of the BNRD (Figure 6.21f) compared to Group C (Figure 6.21g) who received a hollow DS-NGF-loaded conduit thereby proving the success of the intraluminal guidance cues in achieving improved levels of muscle re-innervation via formation of neuromuscular junctions between the newly-regenerated axons and the existing muscle fibers. The indication of

improved muscle health in Group E is further supported by the acquired improving SFI values and observations of fine motor control during the grid walk analysis.



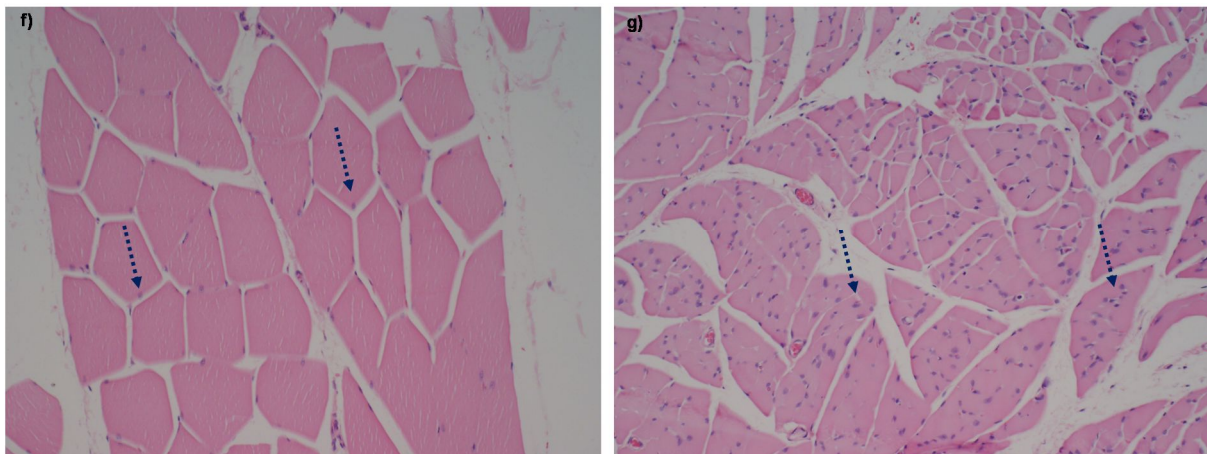


Figure 6.21: Histological imaging of the excised gastrocnemius muscle featuring interstitial cells (arrows) a) Group A b) Group B c) Group C d) Group D and e) Group E at 10× magnification and f) Group C and g) Group E at 20× magnification at the end of the 4-week study period.

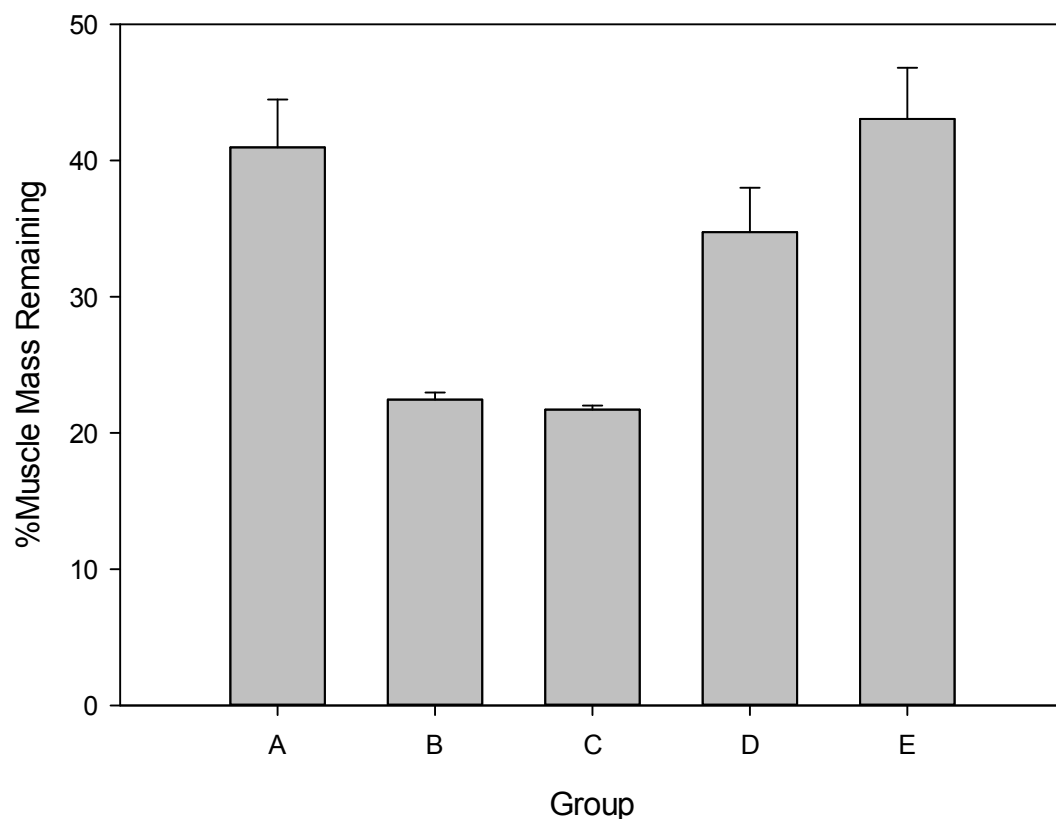


Figure 6.22: Representation of the %muscle mass remaining after 28 days following surgical nerve repair of the respective *in vivo* groups (n=3).

Histological analysis, depicted in Figure 6.23, on the regenerated nerve samples at the mid-portion of the conduit after 2 and 4 weeks were performed to assess the degree of nerve regeneration achieved and the cellular components involved in the process. All specimens collected from the 5 *in vivo* groups demonstrated a mild inflammatory response mainly attributed to the presence of foreign material such as the sutures used, the conduit matrix and the guidance scaffolds.

The autograft repair Group A presented with normal peripheral nerve tissue at 2 weeks progressing to increased cellularity from spindle and Schwann cell proliferation at 4 weeks. Certain areas however, revealed karyopyknosis: an event leading to nuclear apoptosis. The specimens collected from Group B (receiving the bioactive-free hollow conduit) presented with normal nerve tissue with prominent interstitial spindle and Schwann cell proliferation accompanied by a few lymphocytic cells. The presence of spheroids was indicative of axonal damage. At 4 weeks, the increased presence of spindle cells and granulomatous inflammation obscured most of the underlying peripheral nerve tissue, however, an interlacing formation of the spindle cells were representative of reactive Schwann cells. Inflammation responses consisting of lymphocytes, plasma cells and mast cells were present along the interstitium. After 2 weeks of the study, Group C receiving the DS-NGF-loaded hollow hydrogel conduit presented with a notable proliferation of spindle-shaped Schwann cells which appeared to form interlacing bundles with a neural-type arrangement pattern. This, however, progressed to extensive proliferation of interstitial cells and granulomatous inflammation consisting of epitheloid macrophages at 4 weeks. Group D, receiving the DS-NGF-loaded conduit with NAC-free intraluminal guidance scaffolds, presented with prominent macrophages, lymphocytes and plasma cells in the surrounding connective tissue. Spindle cells, numerous capillaries, red blood cells and fibrin deposition were noted. Granulomatous inflammation was mostly present at the ends of 4 weeks. Group E, receiving the final BNRD system, exhibited moderate interstitial and Schwann cell proliferation accompanied by inflammation consisting of lymphocytes, plasma cells, epitheloid macrophages and neutrophils. Evaluation of the collected specimens after 4 weeks, showed similar inflammatory reactions but with the prominent appearance of neovascularization as seen in Figure 6.23 (which was not evident in Groups A, B and C).

Although granulomatous inflammation was evident, in response to the conduit components as foreign material, the absence of excessive fibrotic tissue may suggest the effective role of DS as an anti-inflammatory agent thereby reducing the quantity of functionless scar tissue. Peripheral nerve regeneration requires the Schwann cell proliferation, which was evident in

the histology sections collected from the current study. Schwann cell proliferation, particularly noted in Group C and E, is pertinent for the production of intrinsic neurotrophic factors and myelin basic protein which are crucial for effective growth promotion and regeneration of damaged nerve tissue (Chung *et al.*, 2011).

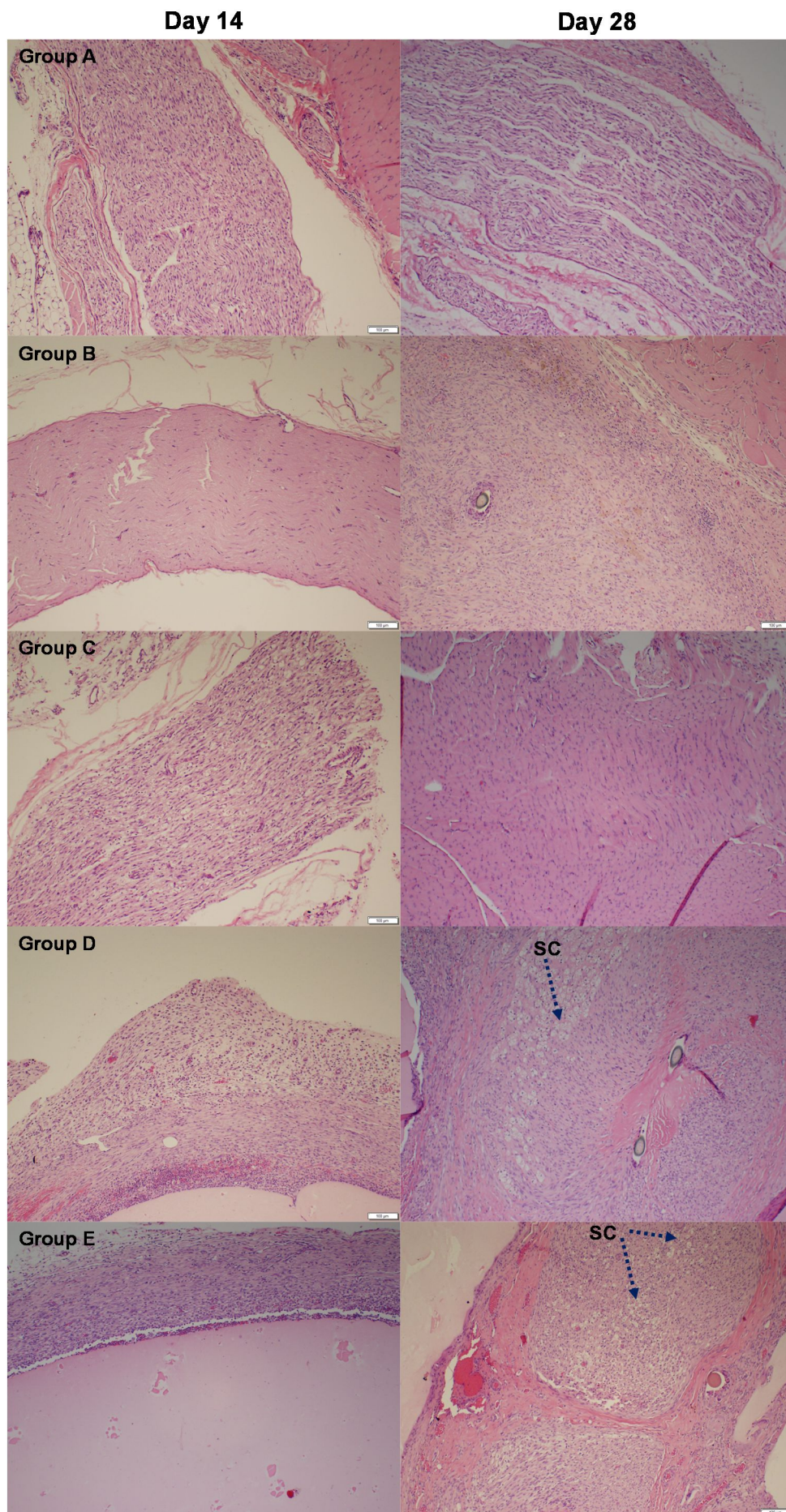


Figure 6.23: Pictographs showing histological examination of the sciatic nerve regeneration across the various treatment groups where SC represents Schwann cell proliferation.

6.4. Concluding Remarks

Analyses of textural profiling, PC12 MTT assay and NGF release kinetics showed adequate *in vitro* biocompatibility, durability and performance of the biosimulated nerve repair device comprising a polysaccharide hydrogel conduit and intraluminal aligned nano-fibrous guidance cues hence its further assessment in an *in vivo* model: the rat sciatic nerve gap model. A thermal ionic crosslinking mechanism using the divalent calcium ion resulted in sufficient holding and biodegradability of the gellan-xanthan hydrogel network over a 4-week implantation period (Ramburrun *et al.*, 2017). *In vivo* behavioural evaluations, for the determination of functional recovery outcomes post-injury, comprising walking track-derived SFI and grid walk analysis suggest improved functional recovery and return of fine motor control in animals receiving implantation of the fully assembled BNRD system where SFI values were noted to progressively increase over the 4-week study period. Further morphometric analysis of gastrocnemius muscle mass remaining revealed reduced muscular atrophy in animals treated with the PHBV-MgOI (34.74%) and PHBV-MgOI-NAC (43.05%) composite intraluminal guidance cues compared to those treated with the bioactive-free (22.44%) and bioactive-loaded (21.71%) hollow hydrogel conduit alone. Histological analysis of the regenerated nerve segments at 2 and 4 weeks post-implantation indicated the presence of prominent Schwann cell proliferation, neural-type cellular arrangement and neovascularization in Groups D and E as compared to Groups A, B and C. These observations suggest successful implementation of the intraluminal guidance scaffolds in simulating a neuronal microenvironment conducive of axonal repair. The degree of Schwann cell proliferation, neovascularization, muscle mass remaining and SFI values collectively present outperformance of the autograft repair in Group A by the BNRD system in Group E thus demonstrating the effectiveness of aligned nano-constructs within a hydrogel conduit in emulating the intrinsic architecture of peripheral nerve tissue via provision of long-term and sustained physical, chemical and therapeutic guidance cues.

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CHAPTER 7

CONCLUSIONS, RECOMMENDATIONS AND FUTURE OUTLOOK

7.1. Conclusions

The increased frequency of road and motor vehicle accidents is one of the leading causes for the inclining numbers of peripheral nerve injury sufferers globally, estimated at 2 million incidents annually. Traumatic injuries of peripheral nerves, particularly transection injuries, often culminate in permanent and lifelong debilitation causing further decline in a patient's quality of life initiated by the onset of chronic neuropathic pain, severe muscle atrophy, loss of functional recovery and diminishing social and psychological wellbeing.

Nerve tissue has an innate, but limited, capacity of regeneration post-injury depending on the severity of injury, the resulting inflammatory mechanisms and the time between injury detection and treatment. The gold standard of nerve repair currently remains the nerve autograft, however, several drawbacks and resultant morbidities associated with this treatment modality has necessitated the search for an effective yet uncomplicated strategy for promoting the innate regenerative abilities of peripheral nerves. Ongoing research has established that successful nerve regeneration and restoration of functional recovery requires an advancement of the simple bridging conduits, commercially available, towards sophisticated repair constructs capable of emulating, or preferably exceeding, the characteristics and outcomes of the autograft.

The research presented in this thesis involved facets of advanced drug delivery, polymer science and tissue engineering for the design and formulation of the BNRD system for enhanced peripheral nerve regeneration via provision of a mechanically stable, biomimetic and bioactive-releasing repair conduit. An extensive analysis comprising physicochemical, physicomechanical and *in vitro* studies of a Box-Behnken experimental design confirmed the theory of pristine-polymer-particle intercalation for extended bioactive release via a pH-responsive *in situ* porogen leaching effect in both cationic and anionic crosslinked polysaccharide matrices. Further cell cyto-compatibility studies and subsequent *in vivo* analysis in the rat sciatic nerve gap model validated efficacy of the repair device compared to the gold standard autograft repair.

The core aim of the research study was to develop a unique strategy for the controlled release of bioactives in treating peripheral nerve injuries which was achieved via the

successful implementation of pH-responsive pristine-polymer-particle intercalation within a hydrophilic swellable polymer matrix. Elucidation of the mechanisms behind the concept of particle-intercalation for extended bioactive release and improved matrix resilience properties were established utilizing indomethacin-entrapped poly-ionically crosslinked chitosan spheres as model components prior to application of the technique in a hydrogel-based scaffold suitable for nerve tissue repair.

The subsequent implementation of pristine-PMMA-particle intercalation within a novel gellan-xanthan coupled thermo-ionic crosslinked IPN hydrogel conduit offered the concurrent release of 2 bioactive types: a protein-based molecule (BSA and NGF) and a small molecular-weight non-steroidal anti-inflammatory drug (diclofenac sodium). The PMMA-intercalated hydrogel system was effective in extending NGF and diclofenac sodium release over 30 days while maintaining bioactivity and molecular integrity of the growth factor as detected by ELISA assays. The formulation of a gellan-based IPN hydrogel system via ionic and thermal crosslinking with the graded incorporation of xanthan gum yielded an effective strategy for the modulation of deformation and fracture properties of the resulting scaffold whereas PMMA-intercalation imparted improved resilience attributed to the formation of a porous network upon dissolution and leaching.

Advancement of the hydrogel conduit was further implemented via the design and integration of aligned electrospun nano-fibrous guidance cues for the provision of 3 key cue types: physical, chemical and therapeutic. The technique of high-speed rotational electrospinning yielded the formation of aligned PHBV nano-fibers blended with a metal-oleate salt, MgOI, functioning as a nucleating agent for modulation of PHBV crystallinity and resulting mechanical properties as well as simultaneous action as potential bioactives in treating peripheral nerve damage. Integration of the electrospun sheet in the form of a loose coiled intraluminal scaffold within the hollow gellan-xanthan hydrogel conduit provided the physical and topographical guidance cue via the presence of longitudinally aligned fibers whereas the amalgamation of MgOI and NAC with the PHBV base polymer, in conjunction with the bioactives loaded in the hydrogel conduit, offered the chemical and therapeutic guidance cues for enhanced cellular interaction and neuronal survival.

Cell studies, utilizing the PC12 neuronal cell line, demonstrated the proliferative effects of the various bioactive-loaded and bioactive-free components of the device. The polymeric matrices, although biocompatible and capable of supporting cell growth alone, proved to augment cell proliferation upon the loading and consequent concomitant controlled release of the various bioactives including DS, MgOI and NAC.

In vivo analysis of the final assembled optimized BNRD system in the Sprague Dawley rat sciatic nerve model served to assess the performance of the device in peripheral nerve repair and treatment. Behavioural, morphometric and histological analyses established the level of functional recovery achieved and the extent of nerve regeneration evident post injury-induction and in response to treatment with the BNRD system and its individual components in comparison to the nerve autograft. Increasing cellular activity, Schwann cell proliferation and vascularization from histological analysis corresponded to the improved muscle preservation and functional recovery in terms of SFI observed during the behavioural assessments. The release kinetics of NGF and DS, as determined from *in vitro* dissolution studies, from the BNRD system demonstrated successful execution in promoting nerve regeneration and improving functional recovery outcomes over the 4-week test period compared to that achieved with the autograft repair.

The BNRD system developed and reported in this thesis was shown to be capable of adequate peripheral nerve regeneration and restoration of functional recovery, including fine motor recovery, exceeding the outcomes achieved with the autograft repair thus indicating the efficacious design and implementation of a biomimetic construct combined with a unique strategy for the long-term and concurrent release of at least 2 bioactives in treating peripheral nerve injuries and achieving the goal in attaining an alternative repair tactic equivalent to and potentially surpassing the gold standard of nerve injury treatment.

7.2. Recommendations

The current thesis presented extensive discussions pertaining to the design, fabrication, characterization and *in vitro* analysis of the proposed BNRD system for the treatment of traumatic peripheral nerve injuries. Analyses of the device were performed up to the preclinical stage of *in vivo* evaluation in an animal model: the Sprague Dawley rat. Although the collected evidence indicated enhanced *in vivo* performance of the device compared to the autograft repair as established via muscle mass, histological and SFI walking track analysis over the 4-week test period, it may be suggested that the performance evaluation of the BNRD system be conducted over a longer test period extending 4 weeks and preferably towards 8-12 weeks or more. While studies over 4-6 weeks provide information on the early regenerative effects of the proposed treatment, long-term studies extending over several months could assist in the collection of data relating to the maximal levels of function recovery that could possibly be attained and further insight into the repair mechanisms employed by the body to bring about complete tissue healing and regeneration. The increased study time frames could be combined with an increased number of animals

employed in the study to account for the existence of natural variances and differences in the innate healing capacities among individual animals. Additional potential influencing factors affecting the outcome of peripheral nerve regeneration could be implemented in the study, such as the effects of diet and nutrition and regular physiotherapy exercises to assist with the prevention of muscle atrophy – as would typically be included in the full therapeutic regimen of a human being. Although the present study focused on the restoration of functional recovery in terms of motor control, behavioural analyses for the evaluation of sensory recovery should further be investigated.

A limitation to the present *in vivo* study was that the effects of delayed nerve repair were not investigated. The *in vivo* study conducted and described in this thesis was based on the immediate repair of the transected nerve following induction of injury. In a real world scenario, the time between acquirement of the injury, its detection and subsequent repair could vary between a few hours to days depending on the nature of the injury i.e. motor vehicle accidents and bone fractures. During that time of potential delay in nerve injury detection and treatment, the course of inflammation and fibrotic tissue infiltration has already initiated causing probable variability in the extent of tissue healing achieved. The aforementioned preclinical investigations would be essential prior to commencement of clinical trials in human subjects.

The nerve repair device presented in this thesis could further be utilized and investigated for the concurrent release of other neurotrophic growth factors and conventional therapeutics employed in the treatment strategy of peripheral nerve injuries including nutrients, analgesics and corticosteroid anti-inflammatories. A critical aspect influencing the commercialisation and ultimate clinical use of the BNRD system, in addition to mass production and requirement of specialised equipment and moulding devices, concerns effective sterilization techniques and subsequent packaging of the device to maintain sterility as well as chemical and mechanical integrity of the device with optimum preservation of bioactivity of the incorporated bioactives and fragile protein molecules from the time of manufacture till the moment it is required for implantation. Although a large component of the BNRD system utilizes affordable, naturally-sourced and renewable polymeric materials for its construction, the technique of nano-fiber synthesis for fabrication of the intraluminal guidance cues via electrospinning could be costly and time consuming regarding the large scale production of the device and therefore would require specialised instrumentation for rapid production and excellent reproducibility of the guidance scaffold component.

7.3. Future Outlook

A pH-responsive IPN hydrogel scaffold was designed and optimized of the specific repair and treatment of traumatic peripheral nerve injuries through the further implantation of an independent nano-fibrous scaffold forming an intraluminal guidance cue component for enhanced resemblance of the device to the architecture of native nerve tissue. The functional recovery results obtained from the preliminary *in vivo* studies has shown superior performance of the BNRD compared to the gold standard of nerve repair and thus holds potential for patenting, clinical trials and commercialization of the device for both human and veterinary use.

Although the polymeric components of the device presented in this thesis has been assembled specifically as a repair strategy for transected peripheral nerve injuries, the individual components and bioactive-release system could be modified and utilized in tissue regeneration applications of other organ systems which require long-term biodegradable and controlled-release scaffolds for tissue repair and healing. Simple customizable moulding and modification of the gellan-xanthan IPN hydrogel matrix through alteration of polymer and crosslinker concentration and particle-intercalation, for tailored matrix resilience, flexibility and rigidity, yields a method for adapting the system for neural (spinal cord and brain) cartilage, vascular (arterial and venous patch-up) and wound repair strategies. In addition, the approach to attaining sustained-release for the delivery of various bioactives employing the novel pH-responsive pristine-polymer-particle intercalation technique could be applied to and implemented in other hydrophilic polymer systems for either single or dual drug delivery encompassing a variety of drug classes employed in tissue-engineering and targeted drug delivery applications such as peptide-based growth factors, anti-inflammatories, antibacterial compounds and chemotherapeutics. As mentioned elsewhere in this thesis, the electrospun fibers possess alternate uses as bioactive-releasing dressings and linings or it could be utilized on its own as intraluminal guidance scaffolds in conjunction with any marketed hollow nerve conduit.

Furthermore, the use of naturally-derived biocompatible, biodegradable and renewable bacterial polysaccharides (gellan, xanthan and PHBV) in the key polymeric construct of the device implies an economical yet environmentally sustainable approach to the utilization of materials required for production of such a device thereby further supporting the trending notion of green-chemistry in the development of pharmaceuticals, as embraced by global pharmaceutical companies (Tucker and Faul, 2016).

In conclusion, the research and resulting developed device documented in this thesis holds potential not exclusively confined to nerve tissue regeneration but extends into the domain of various other tissue-engineering and regenerative medicine applications requiring the amalgamation of advanced drug delivery techniques.

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APPENDICES

APPENDIX A1

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Review Article

A Review of Bioactive Release from Nerve Conduits as a Neurotherapeutic Strategy for Neuronal Growth in Peripheral Nerve Injury

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Peripheral nerve regeneration strategies employ the use of polymeric engineered nerve conduits encompassed with components of a delivery system. This allows for the controlled and sustained release of neurotrophic growth factors for the enhancement of the innate regenerative capacity of the injured nerves. This review article focuses on the delivery of neurotrophic factors (NTFs) and the importance of the parameters that control release kinetics in the delivery of optimal quantities of NTFs for improved therapeutic effect and prevention of dose dumping. Studies utilizing various controlled-release strategies, in attempt to obtain ideal release kinetics, have been reviewed in this paper. Release strategies discussed include affinity-based models, crosslinking techniques, and layer-by-layer technologies. Currently available synthetic hollow nerve conduits, an alternative to the nerve autografts, have proven to be successful in the bridging and regeneration of primarily the short transected nerve gaps in several patient cases. However, current research emphasizes on the development of more advanced nerve conduits able to simulate the effectiveness of the autograft which includes, in particular, the ability to deliver growth factors.

1. Introduction

The persistence of peripheral nerve injuries as a challenge has over the years stimulated a significant response in the amount of research produced towards the investigation of strategies to overcome the ordeals of this debilitating clinical condition. The peripheral motor and sensory nerves form an extensive and intricate network encompassing a substantially large area of the body. This extensive placement of nerve tissue makes the peripheral nervous system susceptible to trauma inflicted by external forces to any site of the body [1]. Injuries to peripheral nerves are becoming increasingly common due to frequent incidents of trauma resulting from motor vehicle accidents, fractures, lacerations, crush injuries, and surgical complications that cause direct or indirect nerve compression from oedema and haematomas [2–6]. Peripheral nerve injuries greatly impair the ability to feel normal sensations and exercise muscle movements due to

denervation of adjacent tissues and muscles bringing about the loss of sensory and motor function [7]. This results in paralysis, chronic pain, and neuropathies leading to severe disability and a diminished quality of life in those patients who have sustained such injuries [1, 3, 8–12].

Initially, strategies focused on the design of a support structure to perform a similar neuronal function as the gold standard of treatment, a nerve autograft, thereby eradicating the need of obtaining a donor nerve via lengthy or multiple surgical procedures as well as eliminating the concerns of donor tissue availability, resulting morbidities, and additional injuries and scarring [2, 13–18]. A nerve autograft involves the transplantation of a donor nerve across a nerve gap defect. In certain cases, a cable graft is required wherein several donor nerves are attached together to create an appropriate sized graft to fit the recipient nerve. Although nerve autografts have a good success rate in the regeneration of nerve across gaps of less than 10 mm, it becomes less feasible for longer gap



Design of chitospheres loaded with pristine polymer particles for extended drug delivery via polyelectrolyte complexation and particulate leaching



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ABSTRACT

The aim of this study was to investigate the drug release from swellable chitospheres laden with pristine polymethylmethacrylate (PMMA) nanoparticles. Chitosan matrices were prepared by sodium tripolyphosphate crosslinking from a chitosan suspension containing the model BCS class II drug, indomethacin. PMMA particles were added to the chitospheres as the modulator for drug release. Swelling and erosion studies in conjunction with textual profiling provided an understanding of the dominant and underlying drug release mechanisms of the ionically crosslinked chitospheres loaded with the pristine PMMA particles. A series of drug release studies performed in PBS pH 7.4 showed that the pristine particle-loaded chitospheres released indomethacin over 144 h in a first-order manner with 50% drug release occurring over 48 h. The study also revealed that in situ pore leaching for pore creation and polyelectrolyte complex formation were the main mechanisms of release from the chitospheres. The results of this study may be utilized for the development of neuro-implants for controlled delivery of bioactives to the brain where fields of superior mechanical strength and reduced swelling properties are required.

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1. Introduction

Chitosan matrices, particularly spheres, formed from ionic or covalent crosslinking has long been used as a three-dimensional matrix for the study of release profiles of numerous drugs with the intent to modulate drug release profiles in a manner that provides drug release of kinetic order and therapeutic effectiveness (Gupta and Ravi Kumar, 2000; Mi et al., 2003; Sarkar et al., 2013). These multiparticulate spherical matrices (Paspalakis and Bouropoulos, 2006; Srinatha et al., 2008; Kotadiya et al., 2009; Yang et al., 2011; Ko et al., 2002; Lin et al., 2005; Harris et al., 2010; Jonassen et al., 2012; Rampino et al., 2013) comprise chitosan alone or blended with other property-modifying natural or synthetic polymers that have been widely used for the oral delivery of therapeutic agents and for long-term sustained delivery of proteins, peptides and other essential growth factors for tissue regeneration applications and gene delivery technologies (Anal et al., 2003; Vimal et al., 2013).

Chitosan is readily soluble in acidic pH, with the resultant protonated amine group available for ionic interaction with multivalent ions for immediate gelation (Jonassen et al., 2012; Shenoi et al., 2014). The cationic nature of chitosan also makes it simple for producing physically crosslinked gelling matrices and the ability to electrostatically interact with counter ionic polymers to form polyelectrolyte complexes (PECs) (Cerf et al., 2014; Nath et al., 2015). Previously reviewed PEC applications show that PECs have unique physicochemical and mechanical properties to that of the parent polymers and offer an additional dimension to the delivery system and enhanced properties for tailoring drug release patterns to the desired site (Hamman, 2010). Sodium tripolyphosphate (STPP) has been frequently used as an ionic crosslinking agent for chitosan matrices due to its rapid crosslinking action and safety compared to glutaraldehyde, epichlorohydrin and ethylene glycol diglycidyl ether that have proven physiological toxicity (Mi et al., 1999; Shu and Zhu, 2002; Li and Huang, 2012; Shenoi et al., 2014). Chitosan-based multi-particulates can instantly be synthesised using STPP which forms either intermolecular or intramolecular linkages that stabilize the positively charged amino groups of chitosan resulting in crosslinking (Mi et al., 1999, 2003). Previous studies, aiming to extend the release of indomethacin from crosslinked chitosan and other

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APPENDIX A3



Expert Opinion on Drug Delivery



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Design and characterization of neurodurable gellan-xanthan pH-responsive hydrogels for controlled drug delivery

Poornima Ramburrun, Pradeep Kumar, Yahya E. Choonara, Lisa C. du Toit & Viness Pillay

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APPENDIX B1

Design of Hydrogel Matrices with Intercalated Pristine-Polymer-Particles for Enhanced Porosity and Extended Bioactive Release via *In situ* pH-Responsive Particulate Leaching

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Purpose:

The release kinetics of bioactive molecules and the structural integrity of the delivery scaffold are pertinent in achieving optimal tissue healing. This study investigates the effect of pristine-polymer-particle intercalation on mechanical properties and bioactive release from chitosan matrices for extended drug release in tissue regeneration.

Methods:

Preparation: Sodium tripolyphosphate ionically crosslinked polysaccharide-based (chitosan) matrices were loaded with quantities between 50-200%^{w/w} of pristine polymethyl methacrylate (PMMA) anionic nanoparticles.

Drug release: Chitosan spheres were loaded with indomethacin as a model drug. *In vitro* release studies in phosphate buffered saline (PBS) pH 7.4 and UV spectroscopy were used to quantify drug release.

Porosity analysis: Porosity imparted by particulate leaching of PMMA particles were examined via Porositometric analysis in accordance with BET and BJH models. Surface morphology and pore visualisation was conducted on an FEI Quanta 400 FEG scanning electron microscope prior to gold-palladium sputter-coating of samples.

Textural profiling: Changes in mechanical properties of the matrices over 30 days of PBS immersion was evaluated in terms of resilience, deformation and fracture using the TAXT.plus StableMicrosystems texture analyser.

Results:

Drug release from chitosan spheres loaded with pristine PMMA particles was controlled via the pH-responsive dissolution (pH 7.4) of PMMA leading to the simultaneous formation of pore structures on the matrix surface and polyelectrolyte complexation (PEC) between the opposing charged polymers. PEC formation improved matrix fracture point and extended indomethacin release over 144 hours in a first-order manner as opposed to PMMA-free matrices providing release for only 72 hours. Increase in PMMA particle concentration led to enhanced resilience (27.27% increase) via increased number and size (10.77nm increase in diameter) of pores formed as validated by porositometric analysis and SEM imaging.

Acknowledgements:

This research was funded by the National Research Foundation (NRF) of South Africa.

APPENDIX B2

Intercalation of Hydrogel Tissue-Repair Scaffolds with Pristine-Polymer-Particles for Enhanced Porosity and Extended Bioactive Release

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Purpose: To investigate the effect of pristine-polymer-particle intercalation on mechanical properties and bioactive release from anionic and cationic hydrogel matrices for tissue regeneration.

Methods: Polysaccharide-based (chitosan and gellan/xanthan gum blend) ionically crosslinked hydrogel matrices with intercalated pristine polymethyl methacrylate (PMMA) nanoparticles were used to investigate the effects on drug release of model bioactives indomethacin (chitosan), BSA and diclofenac (gellan/xanthan). *In vitro* release studies and UV spectroscopy were used to quantify bioactive release. Porosity imparted by particulate leaching of PMMA particles was examined via Porositometric analysis in accordance with BET and BJH models. Mechanical properties in terms of resilience, deformation and fracture were evaluated at predetermined time points during erosion. Thereafter, matrix surface morphology and pore visualisation was conducted on a scanning electron microscope to assess the matrix erosion mechanisms and pore-formation.

Results: Drug release from chitosan loaded with pristine PMMA particles was controlled via the pH-responsive dissolution (pH 7.4) of PMMA leading to the simultaneous formation of pore structures and polyelectrolyte complexation. PMMA intercalation extended indomethacin release over 144 hours in a first-order manner as opposed to PMMA-free matrices (72 hours). PMMA pristine particles enhanced resilience (27.27% increase) via increased number and size (10.77nm increase in diameter) of pores formed as validated by porositometric analysis and SEM imaging. Similarly, varying degrees of crosslinked gellan/xanthan matrices containing 75%^{w/w} pristine PMMA particles exhibited improved resilience (45-56%) compared to formulations containing 25%^{w/w} (31-40% resilience). Pristine PMMA particles, with the synergistic retarding property of xanthan gum, reduced release rates for both BSA (37-75% release over 20 days) and diclofenac sodium (14-20% release over 30 days). Although PMMA particle intercalation hastens erosion rates particularly after dissolution and leaching, swelling of the matrices remained steady.

Conclusions: Intercalation of PMMA pristine nanoparticles for *in situ* pH-responsive particulate leaching resulted in pore formation and polyelectrolyte complexation which were key mechanisms for enhancing mechanical properties and extending drug release

APPENDIX B3

ABSTRACT

TITLE: Design of Hydrogel Matrices with Intercalated Pristine-Polymer-Particles for Enhanced Porosity and Extended Bioactive Release via *In situ* pH-Responsive Particulate Leaching

PURPOSE: To investigate the effect of pristine-polymer-particle intercalation on mechanical properties and bioactive release from anionic and cationic hydrogel matrices for tissue regeneration.

METHODS: Polysaccharide-based (chitosan and gellan/xanthan gum blend) ionically crosslinked hydrogel matrices were loaded with quantities between 25-200%^{w/w} of pristine polymethyl methacrylate (PMMA) anionic nanoparticles. Sodium tripolyphosphate crosslinked chitosan spheres loaded with indomethacin and calcium crosslinked gellan/xanthan tubes loaded with BSA and diclofenac sodium were used as the model matrix-bioactive systems. *In vitro* release studies and UV spectroscopy were used to quantify bioactive release. Porosity imparted by particulate leaching of PMMA particles were examined via Porositometric analysis in accordance with BET and BJH models. Changes in mechanical properties were evaluated in terms of resilience, deformation and fracture using the TAXT.plus StableMicrosystems texture analyser. Matrix surface morphology and pore visualisation was conducted on an FEI Quanta 400 FEG scanning electron microscope prior to gold-palladium sputter-coating of samples.

RESULTS: Drug release from chitosan loaded with pristine PMMA particles was controlled via the pH-responsive dissolution (pH 7.4) of PMMA leading to the simultaneous formation of pore structures on the matrix surface and polyelectrolyte complexation. The latter extended indomethacin release over 144 hours in a first-order manner as opposed to PMMA-free matrices providing release up to 72 hours. Increase in PMMA particle concentration led to enhanced resilience (27.27% increase) via increased number and size (10.77nm increase in diameter) of pores formed as validated by porositometric analysis and SEM imaging. Similarly, varying degrees of crosslinked gellan/xanthan matrices containing up to 75%^{w/w} pristine PMMA particles exhibited improved resilience (45-56%) compared to formulations containing 25%^{w/w} of PMMA particles (31-40% resilience). Pristine PMMA particles, with the synergistic retarding property of xanthan gum, assisted in reducing release rates for both BSA (37-75% release over 20 days) and diclofenac sodium (14-20% release over 30 days). Although PMMA particle intercalation hastens erosion rates particularly after dissolution and leaching, swelling of the matrices remained steady.

CONCLUSIONS: A strategy involving the intercalation of PMMA pristine nanoparticles for *in situ* pH-responsive particulate leaching resulting in pore formation and polyelectrolyte complexation as key mechanisms of enhancing matrix mechanical properties and extension of drug release was studied. The features of enhanced matrix resilience, porosity, minimal swelling and controlled drug release appeals to implanted scaffold designs for the repair of injured tissues where sustained delivery of growth factors and other bioactives are imperative.

APPENDIX B4

Title: Bioactive-releasing neurodurable porous hydrogel bioscaffolds for tissue-repair

Poomima Ramburrun, Pradeep Kumar, Yahya E. Choonara, Lisa C. du Toit and Viness Pillay*

Wits Advanced Drug Delivery Platform Research Unit, Department of Pharmacy and Pharmacology, School of Therapeutic Sciences, Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, 7 York Road, Parktown, 2193, South Africa.

Introduction: The purpose of this study was to investigate the effect of pristine-polymer-particle intercalation and crosslinking on mechanical properties and bioactive release from interpenetrating networks of microbial expo-polysaccharide hydrogel matrices for tissue regeneration.

Methods: Ionically crosslinked microbial polysaccharide-based hydrogel matrices were loaded with quantities between 25%^{w/w}-75%^{w/w} of pristine polymethyl methacrylate (PMMA) anionic particles. The model matrix-bioactives utilized were BSA and an NSAID. Changes in mechanical properties, at different dissolution time points, were evaluated in terms of resilience, deformation and fracture using the TAXT.plus StableMicrosystems texture analyser. *In vitro* release studies with UV spectroscopy was used to quantify bioactive release. Scaffold morphology imaging was conducted on an FEI Nova NanoLab 600 scanning electron microscope.

Results: The concentration-dependent intercalation of PMMA enhanced matrix resilience from 31% to 56% and provided a near zero-order release of BSA (37-75% fractional release) and NSAID (14-22% fractional release) over 20 and 30 days, respectively. Increased crosslinker concentrations from 0.05%^{w/v} to 0.1%^{w/v} results in 20% declined resilience, higher fracture energies ranging from 0.14-0.16N.mm to 0.19-0.24N.mm and low matrix flexibility of 1.6-1.9N/mm whereas low crosslinking concentrations promoted scaffold flexibility with values in the 1.6-2.9N/mm range. Fracture and flexibility gradually decreased as erosion continued over 30 days, reaching lower values of 0.11-0.15N.mm and 0.1-1.3N/mm, respectively.

Discussion: In addition to the large macro-porous architecture of the scaffold, matrix resilience was enhanced by the *in situ* pH-responsive (pH 7.4) particulate leaching effect of the pristine-polymer particles which produced porous channels throughout the hydrogel matrix and similarly, modulated the mechanism of sustained bioactive release. Crosslinking influenced the mechanical strength of the scaffolds via formation of rigid polymer chains of varying flexibility and water-uptake upon hydration. The features of enhanced matrix resilience, porosity, and controlled drug release appeals to implanted scaffold designs for the repair of injured tissues.

APPENDIX C1



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CLEARANCE CERTIFICATE NO. 2014/42/C

APPLICANT: Ms P Ramburrun

SCHOOL: Pharmacy & Pharmacology

LOCATION: Faculty of Health Sciences

PROJECT TITLE: *An evaluation of the regenerative potential of a polymer-based nerve conduit for the controlled release of bioactives in treating peripheral nerve injury in Sprague Dawley rats*

Number and Species

75 Rats (of which 18 to be used for a pilot study and 12 as cadavers for practicing and refining techniques).

Approval was given for the use of animals for the project described above at an AESC meeting held on 29 July 2014. This approval remains valid until 28 July 2016.

The use of these animals is subject to AESC guidelines for the use and care of animals, is limited to the procedures described in the application form and is subject to any additional conditions listed below:

None.

Signed: _____
(Chairperson, AESC)

Date: 7th August 2014

I am satisfied that the persons listed in this application are competent to perform the procedures therein, in terms of Section 23 (1) (c) of the Veterinary and Para-Veterinary Professions Act (19 of 1982)

Signed: _____
(Registered Veterinarian)

Date: 7th August 2014

cc: Supervisor: Prof V Pillay
Director: CAS

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APPENDIX D1

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