

OPTIMISATION OF LIGAND-BOUND DRUG-LOADED NANOSPHERES FOR INTRACELLULAR DRUG DELIVERY IN MOTOR NEURON DISEASE

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DECLARATION

I, Zamanzima Mazibuko declare this dissertation as my own work. It is being submitted for the degree of Master of Science in Medicine (Pharmaceutics) in the Faculty of Health Sciences in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at this or any other University.

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This 4th day of February 2016

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SUMMARY

This dissertation discusses Amyotrophic Lateral Sclerosis (ALS) and the progress, or lack thereof, in its treatment thus far. ALS is a neurodegenerative disorder characterised by the degeneration of both upper motor neurons in the motor cortex and lower motor neurons connecting the spinal cord and brain stem to muscle fibers. The prognosis is poor with death occurring approximately 3 years after the detection of symptoms. Numerous therapeutic agents have been tested in clinical trials but only one drug, riluzole, has received FDA approval over the years.

A review examining various ALS treatment options previously investigated explains how most of these have failed possibly due to, amongst many other factors, poor clinical trial designs as well as the difficulty in delivering therapeutic agents across the blood-brain barrier. Therefore, extensive exploration into the disorder and the barriers encountered when designing treatment for it is necessary in order to unearth a solution to this problem. This dissertation proposes that nanotechnology increases the chances of improving the efficiency of therapeutic agents previously tested with minimal or no positive results. Nanotechnology has been investigated in other neurodegenerative disorders such as Alzheimer's disease and Parkinson's disease and although most of these studies are at a foundation level, they are showing a lot of promise.

In an attempt to tackle the difficulties presented by the treatment of ALS, a nano-enabled drug delivery system was formulated to deliver amantadine in the brain. This delivery system comprises of poly(D,L-lactide)-Eudragit® L100 nanospheres prepared by the double solvent evaporation technique and conjugated to the chelating ligand diethylenetriamine penta-acetic acid (DTPA). A design of experiments approach was used to formulate an optimal nanosphere formulation subsequent to preformulation studies. Fifteen formulations were prepared using the Box-Behnken design and were tested for particle size, drug entrapment efficiency, mean dissolution time and zeta potential. The optimal formulation was determined according to the suitable responses and was followed by insertion into an N-Vinylcaprolactam ϵ -caprolactone injectable hydrogel. The hydrogel prepared responded to temperature changes and was developed to form a solid at body temperature.

In vitro and *ex vivo* tests were performed on the drug delivery system. Motor neuron cells were used to investigate the cytotoxicity and the cellular uptake of the delivery system.

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“Those we love don't go away, They walk besides us every day unseen, unheard, but always near, still loved, still missed and very dear, for death leaves a heartache no one can heal but love leaves a memory no one can steal”

Continue resting in peace Muna. You will never be forgotten.

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where V_s is the hydration energy

$$\text{Equation 3.8} \quad M_n = \frac{\sum N_i M_i}{\sum N_i} \quad 52$$

where M_i is the molecular weight of a chain and N_i is the number of chains of that molecular weight

$$\text{Equation 3.9} \quad M_w = \frac{\sum N_i M_i^2}{\sum N_i M_i} \quad 52$$

M_w considers the molecular weight of a chain to confirm the contributions to the molecular weight average

$$\text{Equation 3.10} \quad M = \frac{\sum N_i M_i^{n+1}}{\sum N_i M_i^n} \quad 52$$

where: $n = 1$ gives $M = M_w$
 $n = 2$ gives $M = M_z$
 $n = 3$ gives $M = M_z + 1$

$$\text{Equation 3.11} \quad \text{Polydispersity index} = \frac{M_w}{M_n} \quad 53$$

$$\text{Equation 3.12} \quad BS = \frac{1}{\sqrt{\lambda^*}} \quad 53$$

$$\text{Equation 3.13} \quad \lambda^*(\phi, d) = \frac{1}{n (\pi d^2/4) Q_e} = \frac{2d}{3 \phi Q_e} \quad 53$$

$$\text{Equation 3.14} \quad \phi = n \frac{\pi d^3}{6} \quad 53$$

Where λ^* is the photon transport mean free path, ϕ the particle volume fraction, n is the particle density, d the particle mean diameter and Q_e the extinction efficiency factor for scattering and absorption phenomena

Equation 4.1. 71

$$\% \text{ DEE} = \frac{D_a}{D_t} \times 100,$$

where % DEE is the percentage of drug entrapped, D_a is the actual drug quantity (mg) measured by UV spectrophotometry and D_t is the theoretical drug (mg) added to the formulation.

Equation 4.2. 71

$$\text{MDT} = \frac{\sum_{i=0}^n t_j \Delta M_j}{\sum_{i=0}^n \Delta M_j}$$

Equation 4.3. 72

$$\text{DE} = \frac{\int_0^t y \times dt}{y_{100} \times t} \times 100\%$$

where y is the drug percentage dissolved at time

Equation 4.4 $\text{DE} (\%) = \frac{SA}{R} \times 100$ 72

where SA is the shaded area and R is the rectangle area ($y_{100} \times t$)

Equation 4.5 $\text{Conjugation efficiency} (\%) = \frac{\text{Actual quantity of DTPA on nanospheres}}{\text{Theoretical quantity of DTPA employed}}$ 73

Equation 5.1 $\tau = G\gamma$ 94

Equation 5.2 $\tau = \eta \frac{\Delta\gamma}{\Delta t}$ 94

Equation 5.3 $G = G' + iG'' = (G'^2 + G''^2)^{1/2}$ 96

Equation 5.4 $\tan \delta = \frac{G'}{G''}$ 96

Equation 5.5

$$\eta' = \frac{G}{\omega}$$

96

where δ is the phase angle, η' is the complex viscosity and ω is the angular frequency

CHAPTER ONE

BACKGROUND AND MOTIVATION FOR THIS STUDY

1.1. Introduction to Amyotrophic Lateral Sclerosis

Motor neuron disease (MND) refers to a group of progressive neurodegenerative disorders which are distinguished by the deterioration of upper motor neurons and/or lower motor neurons (Leigh and Ray-Chaudhuri, 1994). In upper motor neuron degeneration, the condition is known as Primary Lateral Sclerosis and presents as Babinski signs, Hoffmann signs and excessive tendon reflexes. Progressive Spinal Muscular Atrophy is the term used to describe lower motor neuron degeneration. Symptoms for this condition are muscle atrophy, fasciculation and weakness (Rowland and Shneider, 2001). Additionally, a disorder known as Amyotrophic Lateral Sclerosis (ALS) results in the degeneration of both upper and lower motor neurons and has the most cases reported on it. The frequency of this disease is approximately 2 in 100 000 with an average age of onset of 58 years (Talbot, 2002). The expected survival from the initiation of the symptoms is 3 years. In approximately 90% of the cases, the disease occurs sporadically (Sporadic ALS) whereas the remaining 10% is inherited or familial (Familial ALS) (Shaw, 1999).

There is currently uncertainty regarding the pathogenesis of motor neuron degeneration. Mechanisms which appear to contribute to the neurodegenerative progression comprise of protein aggregation, glutamatergic toxicity, mitochondrial dysfunction, oxidative stress, neuroinflammation, cytoskeletal derangements, growth factor dysregulation, apoptosis as well as high expression of copper/zinc superoxide dismutase (Cu/Zn SOD1) (Shaw, 1999; Bedlack et al., 2007).

The only drug which is approved by the USA Food and Drug Administration (FDA) for the treatment of MND is riluzole. It is a glutaminergic transmission modulator which inhibits the release of glutamate at the presynapsis. It also non-competitively inhibits the action of excitatory amino acids postsynaptically. Riluzole has additional mechanisms of action including the inactivation of voltage-gated sodium channels and activation of G-protein-dependent processes (Iłżecka, 2003).

In a study by Bensimon (1994), survival rate, being the primary end point, proved to be considerably improved with the use of riluzole. This study showed that riluzole had greater effects in patients with ALS of bulbar onset than those of limb onset. Therefore, according to this study, there is a dependence on site of onset of disease for a larger and substantial

effect of riluzole. Treatment with riluzole showed major therapeutic effects over the first 12 months of use and a decline thereafter ensued. An enhanced and infinite way of treating neurodegenerative disorders is therefore needed in order to achieve improved results. The blood-brain barrier (BBB) is one of the factors that require attention when developing new therapies as it contributes to the minimal success achieved in finding an effective treatment.

1.2. The physiology of the BBB

The BBB is a monolayer of cells that regulates the passage of molecules between the CNS and the blood. The restrictive properties of the BBB are dire and emulate that of a continuous cell membrane. The tight junction (also referred to as a zonula occludens) is a site where the membranes of two cells come so close together that the membranes of the contacting cells appear to be fused (Figure 1.1). Tight junctions act as a barrier, preventing materials from passing between two interacting cells. These junctions present as an uninterrupted stretch of sheets completely encircling the cell. Molecules therefore, cannot pass from one side of the sheet to the other by squeezing between cells. Alternatively, these molecules must go through a cell, and hence the cell can regulate their passage. The endothelial cells that create the barrier at the capillaries and arterioles as well as at the epithelial cells that make up the barrier at the choroid plexus direct the homeostatic environment of the CNS, coordinate the route of peptides and regulatory proteins, and manage the access of metabolic fuels, neurotransmitter precursors, and essential nutrients into the CNS. Additionally, the cells that mould the BBB have active enzymes, supply cytokines and nitric oxide, and are able to secrete toxic molecules. Consequently, the BBB goes beyond its role as a physical barrier and is aptly observed as a regulatory boundary and progressively established to be a target for therapeutic interventions. (Banks, 1999; Francis et al., 2003)

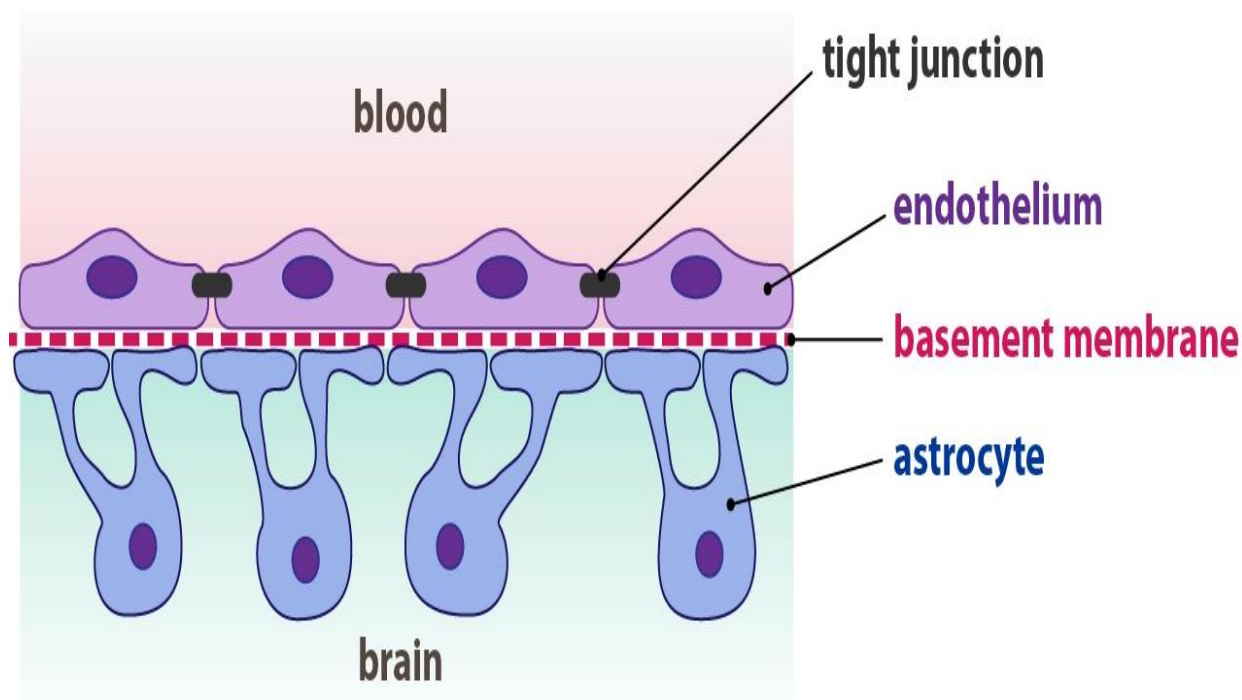


Figure 1.1. The blood–brain barrier (BBB). The BBB is created by the tight junctions made up of endothelial cells lining blood vessels in the brain, constructing a barrier between the circulation and the brain parenchyma (Francis et al., 2003)

1.3. Rationale and Motivation for the Study

The penetration of the BBB is essential in obtaining effective treatment of any CNS disorders. Currently, the conventional methods are not designed to specifically target the affected sites nor are they able to adequately bypass the BBB. Consequently, the prescribed doses of these particular agents have to be increased to levels that have a likelihood of reaching therapeutic concentrations at the intended site. This therapeutic dose, however, becomes toxic to the rest of the body and in due course insignificant therapeutic effects are achieved.

Polymers have been used extensively in the preparation of drug delivery systems and are an enhanced alternative to the commonly used routes of drug administration. Nanospheres can be used to create a system that will deliver therapeutic agents in a controlled process. This can be achieved by distributing the therapeutic agent into the nanocapsule's matrix. The nanocapsule is able to control the release of the drug because of its biodegradation features that allow a more predictable control of drug release kinetics (Peracchia et al., 1997; Modi et al., 2010). Greater therapeutic efficiency is accomplished when using nanospheres as they

provide a protective environment for the drug. This results in the drug remaining in its active conformation until it reaches its target site (Peracchia et al., 1997). Furthermore, in order to produce nanospheres which will be endocytosed by neuron cells, chelating ligands can be strategically conjugated onto these nanospheres resulting in a targeted complex. Drug-loaded ligand-bound nanospheres (DLLBN) can be injected at the site of action; however, the incorporation of these nanospheres into a biodegradable polymeric biomaterial (hydrogel) is beneficial for targeted drug delivery and is synergistic in accomplishing controlled release of the drug. Furthermore, the integration of biodegradable polymers in the formulation, evades the need for surgical procedures in order to remove the material once its drug-load has been exhausted. Injectable scaffolds, which are cross-linked forms of hydrophilic polymers, have shown to be biomaterials with a prospective for use in the biological and medical fields (Peppas et al., 2006). The use of a thermosensitive hydrogel as a carrier of the drug-loaded nanospheres is proposed to be an effective drug delivery system. The approach would be to intracranially inject the DLLBN-carrying implantable hydrogel as opposed to the conventional systemic therapy.

Since there is currently only one (inadequate) FDA approved drug for the treatment of ALS, looking elsewhere for an alternative therapeutic agent to incorporate in this system, an agent that has perhaps been overlooked in the past, brings about the possibility of much needed progress in the treatment of ALS.

Amantadine, an antiviral drug, is amongst a series of drugs indicated for the treatment of influenza A. However, its mechanism of action with regards to the treatment of Parkinson's disease appears to be isolated from that of the treatment of influenza. Amantadine induces dopamine synthesis and release, and blocks the reuptake of dopamine to increase availability for dopaminergic receptor activation. It is also a potent antagonist at the N-methyl D-aspartate (NMDA) receptor. In a study by Parkes et al. (1970), amantadine significantly improved the patients' tremor, hypokinesia as well as their appearance showing its worth in patients with Parkinson's disease. It also reduced dyskinesia in patients with Huntington's disease (Calon et al., 2003). Amantadine shows partial neuroprotective properties in the MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) model of Parkinson's disease demonstrating valuable effects on disease progression (Bonuccelli and Del Dotto, 2006). According to Guttman et al. (2003), patients requiring minimal treatment should consider using amantadine. It should, however, be used with caution in elderly patients as they are much more susceptible to CNS effects such as confusion (Guttman et al., 2003). Amantadine is better tolerated in younger patients and is beneficial as a first-line therapy. It is also suitable for wearing-off phenomenon which occurs initially in Parkinson's disease patients, thus reducing motor oscillations.

Oral amantadine is well absorbed by the gastrointestinal tract (GIT) and according to the excretion rate has a comparatively long half-life. The rate of excretion is dependent on the dose administered (Bleidner et al., 1965; Parkes et al., 1970). Blood levels of amantadine reach a peak 4 hours following oral administration of 2.5mg/kg. Up to 92% of amantadine is excreted unchanged showing minimal metabolism. Side-effects caused by amantadine include insomnia, depression, hallucinations, nausea, constipation, orthostatic hypotension and dizziness (Millet et al., 1981). This extensive list of undesired side-effects can lead to non-compliance from patients and therefore the design of a system that would accomplish minimal adverse events would be exceedingly advantageous. This would necessitate an intracellular delivery system that would have negligible contact with the GIT, for instance. There is currently insufficient literature on the use of amantadine in ALS. This creates an opportunity to develop a novel, targeted intracellular drug delivery that will be successful in ALS clinical trials and will eventually become a useful treatment in clinical practice. Moreover, one of the proposed pathogenesis of sporadic ALS, according to Rowland and Schneider (2001), is persistent viral infection. This is therefore an additional reason to explore the use of amantadine as an alternative treatment of ALS because of its foremost antiviral property.

1.4. Proposed Drug Delivery System for ALS

The polymer based delivery system was to be prepared using amantadine-loaded nanospheres homogeneously incorporated within an injectable thermosensitive hydrogel which would be directly as depicted in Figure 2.1. The nanospheres were proposed to be formulated employing a blend of poly-DL-lactide and a polymethacrylate. The thermosensitive hydrogel would be prepared using ϵ -caprolactone and N-vinylcaprolactam. The nanosphere-hydrogel composite would be injected into the target site, where the thermosensitive gel would react to the increase in temperature and transition from a liquid to a sol implant that would gradually disintegrate, releasing the nanospheres to be taken up by motor neuron cells, mediated by DTPA (chelating ligand). The nanospheres would then release amantadine which would increase dopamine levels while the DTPA removes ions hypothesised to play a role in the oxidative stress theory of ALS.

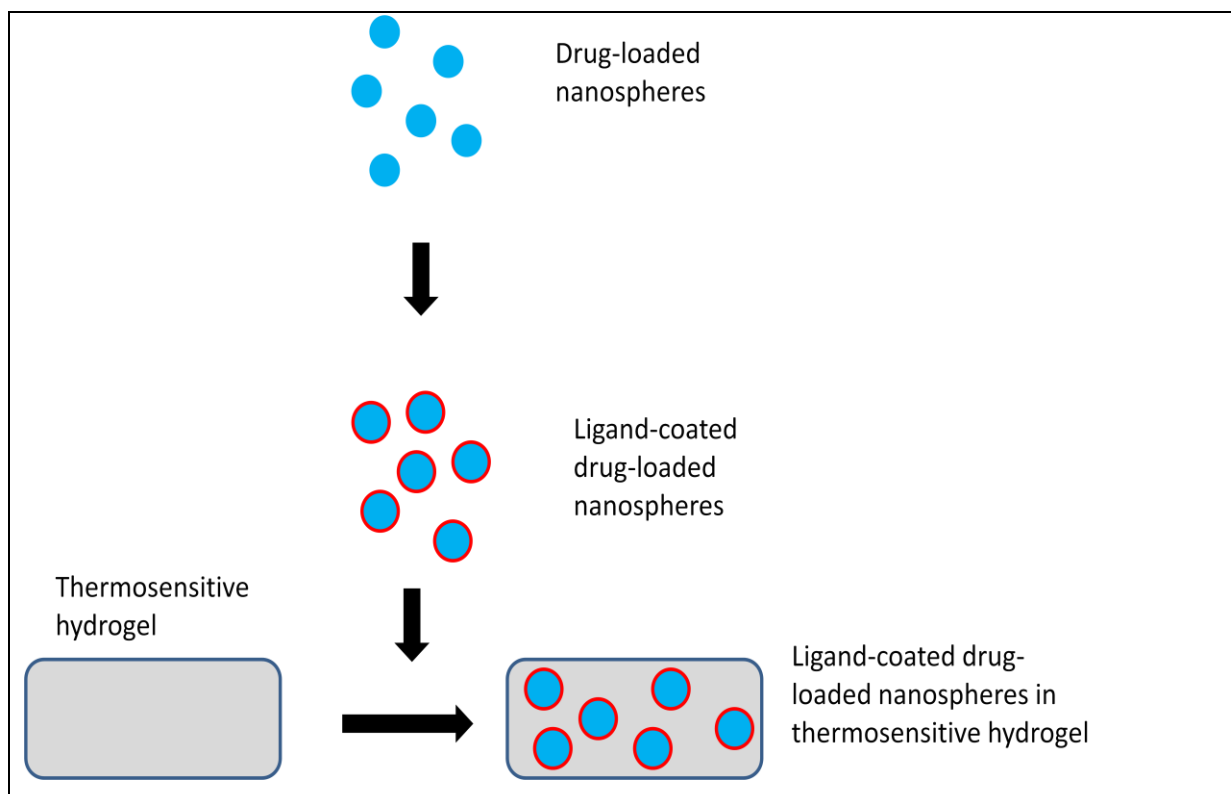


Figure 1.2: A schematic of the proposed drug-loaded ligand-bound nanosphere system

1.5. Aims and Objectives

The aim of this study was to design a ligand-bound nanostructure for optimal intracellular drug delivery, carried by a hydrogel, and incorporating the antiviral, amantadine, as the model drug for the treatment of ALS.

This aim was achieved by meeting the following objectives:

1. Synthesis of drug-free and drug-loaded nanospheres using poly (DL-lactide) as well as a polymethacrylate (Eudragit® L100) and conjugation of chelating ligand on the surface of nanospheres
2. Morphology observation and characterisation of the nanospheres
3. *In vitro* drug entrapment and release studies for nanospheres
4. Preparation of polymeric injectable scaffold and integration of nanospheres within the polymeric injectable scaffold
5. Morphology and characterisation of intact drug delivery system
6. *In vitro* drug release studies of intact delivery system
7. Investigation of the extent to which the NSC-34 cell-line was able to absorb amantadine-nanospheres and the toxicity thereof.

1.6. Overview of the Dissertation

Chapter one begins with an introduction to Amyotrophic lateral sclerosis (ALS), its prevalence, as well as the various theories surrounding the pathophysiology of the disease. It outlines the rationale for this study, looking at the blood-brain barrier as one of the obstacles to developing an effective treatment and introduces nanotechnology as a possible solution. The chapter also delineates the aims and objectives of this research and the potential benefits that may arise from this research.

Chapter two is a literature review delving deeper into the pathophysiology of ALS and the various clinical trials that have investigated a number of potential therapeutic agents. It looks at a range of approaches that have been investigated throughout a number of years including stem cell therapy and suggests more innovative ways in tackling the treatment of ALS emphasizing on nanotechnology.

Chapter three details the characterisation of the drug amantadine, the biodegradable and biocompatible polymers; poly-(D,L)-lactide, three anionic polymethacrylates and two cationic polymethacrylates. The aim of the preformulation studies was to determine a suitable method to detect amantadine as it lacks a chromophore and conventional methods are inefficient. The aim was also to investigate which polymethacrylates in combination with poly-D,L-lactide would result in the most stable nanospheres. The techniques used for the preformulation studies included; attenuated total reflection Fourier transform infrared spectroscopy (ATR-FTIR), thermal and thermodynamic analysis using differential scanning calorimetry (DSC), ultraviolet spectroscopy for amantadine detection and Turbiscan measurements of the nanoparticles in suspension. The most pertinent formulation was observed and further investigations on the particular formulation were carried out in chapter four.

Chapter four presents the details of the formulation of amantadine-loaded ligand-bound polymethacrylate-poly-DL-lactide nanospheres using the double emulsion solvent evaporation method. A 3 factor Box-Behnken statistical design was employed in the optimisation of the preparation of the nanospheres. In the Box-Behnken design; amount of polymer, ultrasonication time and total volume of solvent were regarded as the independent parameters whereas; particle size, zeta potential, drug entrapment and mean dissolution time were considered as dependent responses. Furthermore the optimised nanospheres were conjugated to a chelating ligand, diethylenetriamine penta-acetic acid (DTPA) for increased cell uptake. Techniques used to verify the distribution and characteristic of the

amantadine-loaded DTPA-bound poly-DL-lactide-polymethacrylate (PDLLA-PMA) nanospheres include; ATR-FTIR, DSC, Turbiscan[®] Lab system and X-ray diffraction.

Chapter five describes the formulation of an N-Vinylcaprolactam ϵ -caprolactone thermosensitive injectable hydrogel. Physicomechanical and physiochemical properties were analysed using ATR-FTIR and rheometer. This chapter also looked at the incorporation of the amantadine-loaded DTPA-bound nanospheres into the hydrogel and drug release of amantadine from the nanosphere-hydrogel composite.

Chapter six describes the *in vitro* testing of the thermosensitive injectable hydrogel and the nanospheres in all of their different forms (i.e. drug-loaded; ligand-bound; placebo) on a motor neuron cell line. The uptake of the nanosystems is investigated using fluorescence microscopy as well as Cellvizio[®] imaging system. Toxicity of the different components of the system is investigated using MTT assays.

Chapter seven is the conclusions and recommendations component of this dissertation. The synopsis of various aspects discussed in the dissertation on ALS treatment and the amantadine-loaded DTPA-bound nanospheres embedded in a thermosensitive injectable hydrogel is provided. Recommendations for further research on ALS treatment and the shortcomings of this study are presented in this chapter.

CHAPTER TWO

A REVIEW OF THERAPEUTIC INTERVENTIONS FOR THE TREATMENT OF AMYOTROPHIC LATERAL SCLEROSIS

2.1. Introduction

An unrelenting predicament that has faced physicians and scientist alike is the failure to conquer the on-going complexity of treating central nervous system (CNS) disorders. Most detrimental of these, are the neurodegenerative disorders that gradually lead to the loss of bodily functions and eventually death. Neurodegenerative disorders include, but are not limited to, Alzheimer's disease, Parkinson's disease, Huntington's disease and Amyotrophic Lateral Sclerosis (ALS). Extensive research has been done in the development of diagnostic tools for early detection as well as successful treatment of these disorders. However, very modest advancement has been achieved. To date, the blood-brain barrier (BBB) remains one of the reasons for the lack of success in the development of treatments as it averts the penetration of therapeutic agents and diagnostic tools. There are a few approaches imminent in tackling the treatment of neurodegenerative disorders including the use of stem cells and antitoxins against mutant forms of the copper and zinc superoxide dismutase (SOD1) (Gros-Louis et al., 2010) as well as nanotechnology which require widespread innovation.

In this review, we look at the various theories for the pathophysiology of ALS and some of the ways these hypotheses led to the various clinical trials. Numerous ALS clinical trials have failed and for the past decade only one drug (riluzole) has been approved by the Food and Drug administration (FDA). The approval of only one drug over the years bears testimony to the very minimal progress that has been achieved in the treatment of ALS. We theorize that some of the unsuccessful therapeutic agents could have prospectively produced better results if firstly clinical trials had suitable, effective designs and secondly, if innovative drug delivery systems were employed to enhance the bioavailability of potential

agents. We then look at studies and lessons from similar disorders that incorporate delivery systems to try overcoming the various barriers presented by neurological disorders.

2.2. The hypotheses surrounding the cause of ALS

Motor neuron disease (MND) refers to a group of progressive neurodegenerative disorders which are distinguished by the deterioration of upper motor neurons and/or lower motor neurons (Leigh and Ray-Chaudhuri, 1994). Upper motor neurons have cell bodies located in the motor area of the cerebral cortex and have processes connecting with motor nuclei in the brainstem or the anterior horn of the spinal cord while lower motor neurons have cell bodies located in the brainstem or the spinal cord and have axons innervating skeletal muscle fibers. However, MND is commonly used to refer specifically to ALS and is the preferred term for this paper. ALS is characterised by the deterioration of both upper and lower motor neurons and presents as spasticity, hyperreflexia, muscle atrophy, fasciculation and weakness (Rowland, 1998; Rowland and Shneider, 2001; Nishimura et al., 2004).

The frequency of ALS ranges between 1.5 and 2.5 in 100 000 (Rojas-Garcia et al., 2012) with an average age of onset being between ages 55 and 75 years (Zaldivar et al., 2009). The expected survival from the initiation of the symptoms is 3 years (Vance et al., 2009). In approximately 90% of the cases, the disease occurs sporadically (Sporadic ALS) whereas the remaining 10% are inherited or familial (Familial ALS) (Shaw, 2009). The events underlying the disease triggers are completely unknown in ALS which makes developing an efficient therapy complicated. Therapies that have been tested for the treatment of ALS range from glutamate antagonists, antioxidants, neurotrophic factors, immunomodulatory agents, and antiviral agents (Rowland and Shneider, 2001). However, the majority of ALS clinical trials have produced unfavourable outcomes, classifying a large number of agents as non-beneficial. Many of these clinical trials were discontinued because of the severe side-effects caused by these particular therapeutic agents, without any improvement in survival. Various reasons have been suggested for the failure of these clinical trials. The complexity of designing ALS clinical trials and the resulting inconsistent administration of the trials need

the most attention. It has been reported that some clinical trials lacked efficient sample sizes, while others had contentious duration times as well as ambiguous endpoints (Orrell et al., 2007), ultimately causing a decrease in statistical significance.

Mechanisms that are reported to potentially contribute to the neurodegenerative progression in ALS include cytoskeletal derangements, oxidative stress and mitochondrial dysfunction, protein aggregation, glutamate and excitotoxicity, gene defects, immune dysregulation, and growth factor dysregulation amongst others. A few of these mechanisms are discussed below:

2.2.1. *Oxidative stress and mitochondrial dysfunction*

Jones (2006) described oxidative stress as “a disruption of redox signalling and control” whereas Sies (1997) had previously expressed it to be “an imbalance between the oxidants and antioxidants in favour of the oxidants, potentially leading to damage”. Figure 2.1 summarises the reactions that generate reactive oxygen species (ROS) and the consequences thereof. The presence of ROS in cells as a result of aerobic metabolism can lead to the escape of some electrons from the mitochondrial respiratory chain which in turn may result in partial reduction of molecular oxygen during oxidative phosphorylation to finally generate hydrogen peroxide (H_2O_2) and the superoxide radical ion (O_2^-) (Barber and Shaw, 2010). By definition, the reduction of a portion of oxygen forms superoxide. Characterisation of calcium-dependent isoforms of nitric oxide synthase has given evidence that suggests that ROS can also be produced by the inflammatory activation of neurons (Heneka and Feinstein, 2001). Peroxynitrite (ONOO^-), a potent oxidant, is produced when superoxide reacts with nitric oxide radicals (Squadrito and Pryor, 1998). This highly potent oxidant can cause damage to macromolecules, including DNA, thus possibly leading to DNA mutations (Marnett, 2000). Enzymes in the body, such as superoxide dismutase, are able to eradicate harmful reactive agents and thus neutralise such reactions (Ischiropoulos et al., 1992).

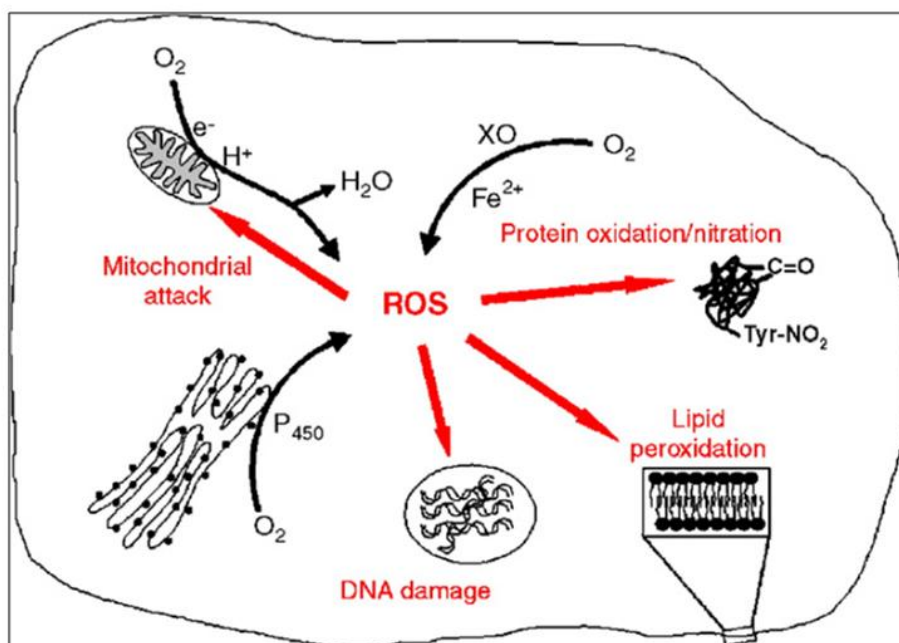


Figure 2.1: Depiction of the sources of reactive oxygen species (ROS) and their targets. ROS in cells can lead to the escape of electrons from the mitochondrial respiratory chain which may cause partial reduction of molecular oxygen during oxidative phosphorylation leading to the production of hydrogen peroxide (H₂O₂) and the superoxide radical ion (O₂⁻). Oxidative enzymes such as cytochrome P450 in the endoplasmic reticulum, xanthine oxidase (XO) and nitric oxide synthase (not depicted) are responsible for the production of ROS. Cellular targets attacked by ROS include DNA, proteins, membrane lipids, and mitochondria and this attack leads to DNA damage, protein oxidation/ nitration, lipid peroxidation and mitochondrial dysfunction respectively (Barber and Shaw, 2010).

Several studies have linked oxidative stress in neurons to familial ALS because protein carbonyl groups, which are markers of oxidative stress, were detected in ALS post mortem tissue (Bowling et al., 1993). In the brain, the inducible form of nitric oxide synthase (NOS) has been characterised in microglial cells and its expression described after injury or trauma which results in excess nitric oxide (Murphy et al., 1993; Heneka and Feinstein, 2001). Almer and co-workers showed the up-regulation of inducible nitric oxide in a transgenic mouse model of ALS (Almer et al., 1999). Furthermore, increased levels of 8-hydroxy-2'-deoxyguanosine (8-OHdG), an indication of oxidative damage to DNA (Alam et al., 1997), have been located in motor cortex of sporadic ALS patients (Ferrante et al., 1997).

Additionally, 4-hydroxynonenal (HNE), a neurotoxic marker of lipid peroxidation, was found in increased quantities in lumbar spinal cord as well as CSF of ALS patients (Pedersen et al., 1998; Smith et al., 1998).

2.2.2. Gene defects

A number of recognized disease-causing mutations have been recorded in FALS families (Bedlack et al., 2007). These mutations can be inserted into animals to produce an ALS phenotype. Decreasing the assembly of FALS gene products through RNA interference (RNAi) technology or antisense oligonucleotides can delay disease progression in animals (Bedlack et al., 2007). The ALS-causing mutations that occur in the *SOD1* gene are the most widespread and best understood. Over 100 various *SOD1* point mutations can cause an ALS phenotype. These mutations do not appear to result in disease by a loss in function; however a toxic gain in function for the mutant *SOD1* protein is alleged. More recently, the gene encoding a DNA/RNA binding protein FUS/TLS, has been reported to be repeatedly mutated in ALS. This mutation results in the cytoplasmic accumulation of mutant FUS protein, which is characteristic to ALS pathophysiology (Zhou et al., 2013). It has also been reported that a large hexanucleotide repeat expansion positioned within the non-coding segment of *C9orf72* is the cause of chromosome 9-linked ALS and accounts for 40% of FALS (DeJesus-Hernandez et al., 2011; Majounie et al., 2012; Mori et al., 2013).

2.2.3. Protein aggregation

Superoxide dismutases (SOD) are a group of ubiquitous enzymes capable of catalysing the conversion of superoxide anions (Zelko et al., 2002). *SOD1* is a copper and zinc homodimer (CuZn-SOD) that acts as an antioxidant and is present primarily in the cytoplasm (Green et al., 2002; Zelko et al., 2002). One of the hypotheses of ALS is that a mutation in this enzyme results in neurotoxicity, and is considered to be as a result of a gain in function (Watanabe et al., 2001).

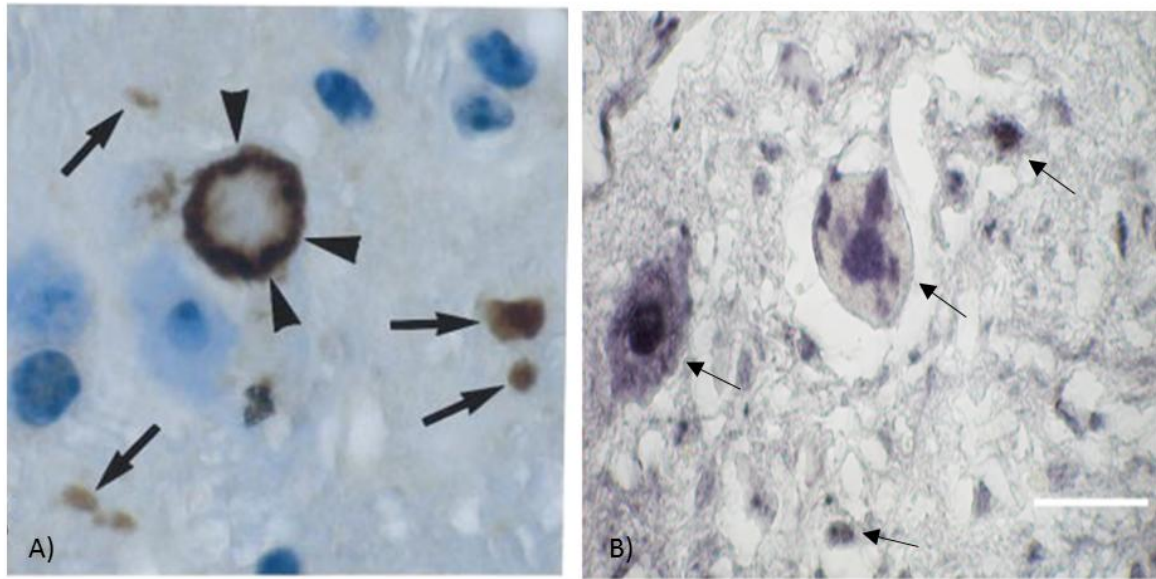


Figure 2.2: SOD1-immunoreactive aggregates that have formed in: A) transgenic mice expressing FALS-linked mutations in the SOD1 protein. Hemotoxylin and eosin (H&E) staining was used to detect aggregates in the ventral portion of a spinal cord from an end-stage mouse expressing mutant SOD1. The figure shows an inclusion that reveals a halo of intense immunoreactivity to SOD1 antibodies (Bruijn et al., 1998). B) anterior horn cells in FALS patients observed on H&E also showing immunoreactivity to SOD1 antibodies (Okamoto et al, 2011).

There are evidently 100 single point mutations of SOD1 that result in the familial type of ALS (Banci et al., 2008). Familial ALS promotes the formation of cytoplasmic mutant SOD1 aggregates found in motor neurons (Vance et al., 2009), (Figure 2.2) but the factors leading to this aggregation are still elusive. However, metallation of SOD1 proteins is suggested to play an important role in reducing the propensity of these proteins to aggregate (Banci et al., 2008). Conversely, the 43kDa TAR-DNA-binding protein (TDP-43) has been identified as the key component of ubiquitinated protein aggregates found in many patients with sporadic ALS and (SOD1)-negative FALS (Yokoseki et al., 2008). The irregular molecular weight fragments of TDP-43 have been found in affected neurons suggesting that this protein is involved in the pathophysiology of SALS, although the pathogenesis is uncertain (Yokoseki et al., 2008). Furthermore, there have been demonstrations showing that the pathological forms of TDP-43 are ubiquitinated and hyperphosphorylated and show a loss of the normal nuclear TDP-43 staining in cells harbouring neuronal cytoplasmic inclusions (Mackenzie et

al., 2007). This evidence shows that TDP-43 is the major pathological protein in sporadic ALS.

2.2.4. Glutamate and excitotoxicity

The irregular stimulation of glutamate receptors (GluRs) results in excitotoxic injuries leading to highly increased levels of calcium entering into neurons (Foran and Trotti, 2009). This process is proposed to be one of the theories of the pathophysiological mechanisms of both types of ALS. It has been found that motor neurons have a high concentration of GluRs, and are the most susceptible neurons to excitotoxicity caused by influx of Ca^{2+} through GluRs (Le Verche et al., 2011). GluR2 subunit expression and editing results in Ca^{2+} impermeability to the AMPA GluRs which mediate fast excitatory transmission. In ALS, however, there is reduced GluR2 RNA editing at the glutamine/ arginine (Q/R) site which causes Ca^{2+} permeability at the GluR2 (Q)-containing AMPA receptors (Heath and Shaw, 2002; Le Verche et al., 2011). *In vitro* studies have shown that an increase in glutamate levels and the over-stimulation of NMDA receptors lead to motor neuron death (Urushitani et al., 2001). Additionally, an increase in calcium influx in motor neurons through activation of AMPA receptors containing an unedited GluR2 subunit demonstrated an analogous effect (Vandenberghe et al., 2000). Furthermore, cerebrospinal fluid retrieved from ALS patients caused excitotoxicity in neuronal cultures, which was not evident in healthy controls (Cid et al., 2003). A decrease of glutamate transport in ALS has also been observed, which is evidently caused by the loss of EAAT2 protein (a high affinity glutamate or excitatory amino acid transporter) in the spinal cord and motor cortex of ALS patients (Guo et al., 2003; Le Verche et al., 2011). EAAT2's main function is to keep extracellular glutamate at levels that are suitable for both neurotransmission and neuronal survival. EAAT2 has been described as not having a primary role in the degeneration of motor neurons but rather a contributive role. This contributive role was shown in a study designed to increase EAAT2 through over-expression in ALS SOD1 mice, where a 2-fold expression of EAAT2 amplified glutamate

uptake by 2-fold, delayed symptom onset (by 17%) and motor neuron death, with negligible effects on the inception of paralysis, weight loss or life span (Le Verche et al., 2011).

2.2.5. Growth factor dysregulation

Defective neurotrophic factor coordination has been proposed as a primary or secondary cause for the loss of motor neurons seen in ALS (Ekester, 2004). The theory is based on the fact that the administration of neurotrophic factors to rat ALS models presented with neuroprotective effects (Corse et al., 1999). In a study by Corse and co-workers (1999) on spinal cord cultures from postnatal rats, insulin-like growth factor-I (IGF-I), glial cell-line derived neurotrophic factor (GDNF), and NT-4/5 were shown to have highly neuroprotective properties. Comparably, brain-derived neurotrophic factor (BDNF), ciliary neurotrophic factor (CNTF), and NT-3 exhibited no notable outcome. An additional neurotrophic factor that has been investigated is vascular endothelial growth factor (VEGF), which has shown to encourage motor neuron survival in animals. ALS patients have been reported to have considerably decreased plasma levels of VEGF, which is distinct from previously mentioned growth factors (Ekester, 2004). In an interesting *in vivo* study by Storkebaum and co-workers (2005), VEGF delayed onset of paralysis by 17 days, improved motor performance and prolonged survival by 22 days in a rat model of ALS. Overall, ALS patients have, however, not experienced significant benefits from treatment with neurotrophic factors. The lack of positive results may be due to the fact that *in vivo* studies have been performed mostly on rat models of FALS while most ALS patients have the sporadic version.

2.3. Drug Formulations in ALS Clinical Trials

These various aforementioned theories formed the premises of the many clinical trials that ensued. The oxidative stress theory led to clinical trials that investigated anti-oxidants such as acetylcysteine, selegiline, creatine, selenium as well as coenzyme Q10. In a representative study to establish whether vitamin E (α -tocopherol), with well-known anti-oxidant properties, would be efficient in treating ALS, it was concluded that vitamin E had no effect on the survival and motor function of ALS patients (Desnuelle et al., 2001). A phase II study testing coenzyme Q10 (CoQ10) was completed in 2008 and it was concluded that 2 700mg of CoQ10 daily for 9 months produced results that were not satisfactory enough to continue to phase III studies (Kaufmann et al., 2009). Similar conclusions were reached with all the other anti-oxidant therapeutic agents.

As mentioned earlier, neurotrophic factors have also been tested to tackle the growth factor dysregulation theory. The brain-derived neurotrophic factor (Ochs et al., 2000), insulin-like growth factor 1 (Sorenson et al., 2008), ciliary neurotrophic factor (Miller et al., 1996), and thyrotropin-releasing hormone (Brooke et al., 1986) have all produced negative outcomes in ALS clinical trials. In two randomized, double-blind, placebo-controlled, multi-centre, multi-national clinical trials investigating Xaliproden (a small non-peptide composite that has the potential of promoting the release of neurotrophic factors), minimal success was observed (Meininger et al., 2004). Vascular endothelial growth factor (VEGF) is a protein found in the body that plays a fundamental role in angiogenesis and the survival of motor neurons (Zinman and Cudkowicz, 2011). Patients with FALS and SALS have been reported to have considerably decreased levels of this protein leading to cause motor neuron death (Van Den Bosch et al., 2004). Phase I and II studies are still in progress to further investigate intracerebroventricular administration of VEGF in patients with ALS using an implanted catheter and SynchroMed[®] II Pump (An open label, safety and tolerability continuation study

of intracerebroventricular administration of sNN0029 to patients with amyotrophic lateral sclerosis, 2013).

Minocycline is an antibiotic that inhibits apoptosis and also has anti-inflammatory effects (Bernardino et al., 2009). Moreover, minocycline has shown to have neuroprotective properties in neurodegenerative disorders (Tikka et al., 2001). This knowledge prompted a study to assess the efficacy of minocycline in ALS patients as a way to tackle the apoptosis theory in the pathophysiology of ALS. Although results from animal models were positive, studies did not translate into positive results in human ALS (Gordon et al., 2007).

Clinical trials employing the anti-glutamatergic approach i.e lamotrigine, dextromethorphan, gabapentin and branched amino acids were all found to be non-effective. However, riluzole, also a glutamate antagonist, became the only drug that received approval by the Food and Drug Administration for the treatment of ALS (Cheah et al., 2010; Bellingham, 2011). It is a glutamatergic transmission modulator which inhibits the release of glutamate at the presynapsis (Lamanauskas and Nistri, 2008). It also non-competitively inhibits the action of excitatory amino acids postsynaptically (Samuel et al., 1992). Riluzole has further mechanisms of action including the inactivation of voltage-gated sodium channels and activation of G-protein-dependent processes (Iłzecka et al., 2003).

In a study by Bensimon and co-workers (1994), survival rate was the primary end point and it proved to be considerably improved with the use of riluzole in ALS patients. The study showed that riluzole had greater effects in patients with ALS of bulbar onset than those of limb onset and that there is a therapeutic dependence on site of onset of disease for a larger and substantial effect of riluzole. Treatment with riluzole showed major therapeutic effects over the first 12 months of use and a decline thereafter ensued. An enhanced and infinite way of treating ALS and similar neurodegenerative disorders is therefore needed in order to achieve improved results.

More than 30 therapeutic agents have been tested for their efficacy and effectiveness in ALS. However, countless trials have been negative and researchers are now focusing on ways to improve clinical trials and identify new medications for ALS. A major emphasis of research is now to discover biomarkers which would enable earlier diagnosis of ALS (which currently takes more than a year on average) and optimistically result in future therapies being more effective early in the course of the disease (Wagner, 2009). Trialists also understand that there is a need to uncover a method to measure the effect of a drug (akin to viral count in trials of HIV). Previously, trialists detected a large size effect to save costs when testing a new agent and therefore aimed for at least a 30% difference from placebo (Mitsumoto et al., 2004). Currently trial designs aim to lower the effect size and look for effects of perhaps only 10% to 20% which includes drugs that have a modest impact on the disorder (Mitsumoto et al., 2004). Moreover, a few methods have been suggested to minimise missing data in clinical trials and there has been emphasis on early trial phase trials to select dose (Mitsumoto et al., 2004; Fleming, 2011; Dziura et al., 2013). Many phase III trials may have failed because of inadequate definition of dose and pharmacodynamics markers in animal and early phase human trials (Levy et al., 2006). Selecting dose is done in sequence where pharmacokinetics and safety of different doses are tested in phase I following investigations in the laboratory. Phase II follows with in-depth safety, biological effect and dose selection and lastly phase III with efficacy (Mitsumoto et al., 2004). Trialists are also looking to address the issue of using animal models as predictors of therapeutic agents' effects in human studies. The accuracy of using the SOD1 transgenic mouse model to extrapolate data for human sporadic ALS is questioned as trialists have limited experience in ALS trials and there is uncertainty whether human ALS and ALS in mouse models are truly representations of each other (Suzuki et al., 2012). Attending to these issues will surely lead to better therapies following the discovery of causes.

The failure of these therapeutic agents can perhaps furthermore be attributed to the challenges that these agents encounter in reaching their target site. The BBB has brought forth major obstacles for the development of therapeutic agents targeting the central nervous system. It obstructs the passage of xenobiotic molecules to the brain thus making the penetration of these agents to their target sites difficult. It is highly expected for this, together with the issues experienced in clinical trials to be amongst the factors in the lack of efficacy of these agents. As a result of the hindrance by the BBB, the bioavailability of most of these agents is very low. Furthermore, gastric absorption of some of these agents is highly reduced and the increase in dosage of therapeutic agents in an attempt to overcome this reduction is often counteractive (Amsterdam, 2003; Rais et al., 2011). Increased doses may lead to saturation of transporter uptake in the intestine. Gabapentin for example has a bioavailability of 60% but this can decrease to almost 35% with increasing dosage (Rais et al., 2011). Selegiline also has difficulty in being taken up by the intestine, with a bioavailability of only 4%. A Selegiline Transdermal System, however, has been used to increase the bioavailability up to 74% (Amsterdam, 2003). This increase in bioavailability enhances the chances of this agent to reach its target site in the brain. The application of delivery systems that will drastically alter the bioavailability of therapeutic agents, through increased gastric absorption and most crucially by manipulating the BBB is an aspect that needs extensive research.

2.4. Intricacies and Prospects for Formulation Success Across the Blood-Brain Barrier in ALS Therapy

The BBB is relatively impermeable to various molecules such as proteins, small peptides and amino acids (Gabathuler, 2010). Small lipophilic molecules are able to pass easily from blood capillaries via passive diffusion (Jolliet-Riant and Tillement, 1999), whereas larger charged molecules require some form of aid, for instance gated channels (Roney et al., 2005). This rigid control of the passage of molecules is maintained by the brain capillaries which are made up of endothelial cells that have exceedingly tight junctions with extremely high electrical resistance (Rubin et al., 1991). Molecules moving across the BBB do so in a unidirectional manner. This movement is dependent on the concentration of the molecules and occurs from the plasma to the brain or vice versa (Ma et al., 2005). The difference between the two unidirectional flow rates provides the net flux. This net flux establishes the therapeutic concentrations achieved by drugs targeting the CNS (Modi et al., 2010). To bypass the BBB, drug delivery approaches include osmotic disruption of the BBB using hyperosmolar agents, Trojan horse receptor mediated transport, nasal drug delivery, receptor-mediated transport, active efflux, carrier-mediated transport, and transcranial delivery (see Figure 2.3).

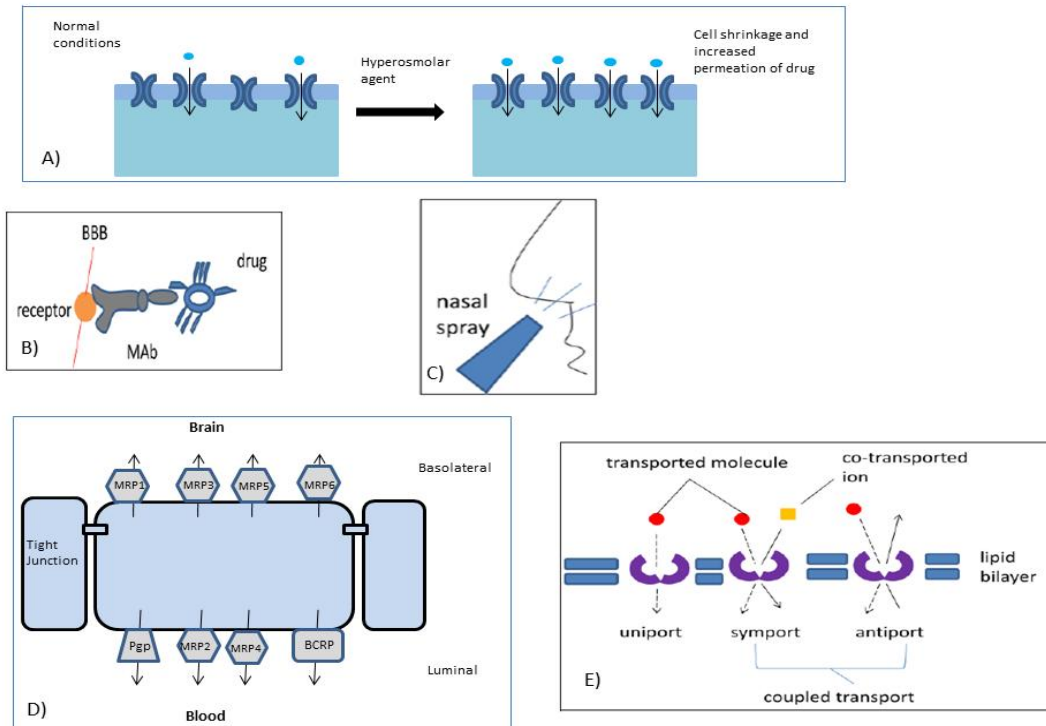


Figure 2.3: Mechanisms for drug delivery to the brain: **A) osmotic disruption of the BBB.** A hyperosmolar agent (e.g. mannitol) is introduced to the endothelial cells of the BBB which results in temporary shrinkage of the cells, allowing therapeutic agents to pass through to the brain and reach their target site. BBB disruption is able to increase drug delivery to the brain by up to 50-100 fold (Siegal et al., 2000; Angelov et al., 2009); **B) trojan horse receptor mediated transport.** A receptor-specific monoclonal antibody (mAb) referred to as a BBB molecular Trojan horse, is fused to a non-transportable drug. The Trojan horse binds a specific receptor on the BBB and this enables transport across the BBB. The drug can then bind its particular receptor, on brain cells to trigger the desired pharmaceutical effect in the brain (Pardridge, 2006); **C) nasal drug delivery.** Drugs are able to reach the CNS from the nasal cavity by a direct transport across the olfactory region located at the roof of the nasal cavity. It is the only site in the human body where the nervous system is in direct contact with the surrounding environment. The drug can cross the olfactory epithelium by a transcellular route through the cells and/ or a paracellular route between the cells. The drug can also be transported through the olfactory neuron cells by intracellular axonal transport mainly to the olfactory bulbs (Illum, 2003); **D) active efflux.** ATP-binding cassette (ABC) transporters such as P-glycoprotein (Pgp), multidrug resistance protein (MRP1-6), and breast cancer resistance protein (BCRP) are membrane proteins situated at the luminal site of brain capillary endothelial cells that form the BBB. These transporters use the energy of ATP hydrolysis to translocate solutes across cellular membranes. They are effective as efflux pumps and drug transporters and are a target for alteration of the BBB for entry of therapeutic drugs into the brain (Loscher and Potschka, 2005); **E) carrier-mediated transport.** Therapeutic agents that have low lipid solubility are able to pass through the BBB via transport proteins. Drugs are designed so they can be transported into the brain via various transport systems such as the hexose transport systems, amino acid transport systems, monocarboxylic acid transport systems and amine transport systems (Tsuji and Tamai, 1999).

However, it has been reported that the BBB as well as the blood-spinal cord barrier (BSCB) are altered in ALS patients (Garbuzova-Davis and Sanberg, 2014; Nicaise et al., 2009). This alteration is evident in endothelial cell degeneration, capillary leakage, perivascular oedema,

down-regulation of tight junction proteins, and micro-haemorrhages (Garbuzova-Davis and Sanberg, 2014). Deterioration of endothelial and pericyte cells, which results in compromised vascular barrier integrity in the brain and spinal cord in ALS patients, may possibly be a significant impediment for successful drug delivery to the CNS. An accumulation of basement membrane collagen from the damaged barriers also modifies barrier influx and efflux transport systems which leads to a decrease in nutritional agents being transported to the brain and an increase in metabolites. In addition, augmentation of P-glycoprotein (P-gp) and the breast cancer resistance protein (BCRP) were determined in brain and spinal cord micro-vessels in both SOD1 animal models and ALS patients. It is suggested that the impairment of these P-gp and BCRP efflux transporters may encourage an obstruction of therapeutic agents in ALS (Jablonski et al., 2012). Overall, most drug delivery mechanisms rely on an intact BBB, which unsurprisingly presents a vast complication for drug discovery in ALS. It is important, therefore, to consider the implications that a damaged BBB and BSCB exhibit when developing a treatment for ALS.

2.5. Specialized Therapeutic Agent Delivery Systems: A Possible Solution

2.5.1. Delivery systems for gene therapy

There have been prospects in using gene therapy in the treatment of ALS where neurotrophic factors have shown potential in preventing the degeneration of motoneurons in culture and *in vivo*, after peripheral nerve injury. Systemic administration of neurotrophic factors, however, limits their efficacy. Several studies have reported incorporating biological components as carriers for these neurotrophic factors (Table 2.1). A combination therapy of glial cell line derived neurotrophic factor (GDNF) and human mesenchymal stem cells (hMSC) was investigated in a rat model of familial ALS by Suzuki and co-workers (2008). hMSC are mostly isolated from bone marrow tissue, although they are also found in other mesenchymal tissues (Aggarwal and Pittenger, 2005). They have shown neuroprotective effects and the ability to release cytokines as well as other growth factors which are believed to save cells from continuous neurodegeneration (Cova et al., 2012). Suzuki and co-workers

(2008) further demonstrated that the combination of the therapeutic effects of hMSC transplants and GDNF administration results in increased motor neuron survival as well as function in a rat model familial ALS model. In this way, the hMSC were used as a delivery system for the GDNF in the skeletal muscle thus evading the need for host cells to provide the growth factors while enduring neurodegeneration thereby increasing the overall lifespan of the rats by up to 28 days.

In a similar study, human neural progenitor cells (hNPC) were used as carriers for GDNF in rats (Klein et al., 2005). The hNPC differentiated into astrocytes and neurons after incorporation into the nervous system and restored the damaged neurons. New astrocytes repaired the extracellular environment of motor neurons and aided in their regular function. The hNPC survived for up to 11 weeks after implantation into the lumbar spinal cord of rat ALS model where they released GDNF (Klein et al., 2005).

Mohajeri and co-workers (1999) prepared primary myoblasts from skeletal muscles of infant mice and infected them with retroviral vectors. These retroviral vectors contained within them either GDNF or β -galactosidase. The myoblasts were then implanted into the hindlimbs of a transgenic mouse model of ALS before the onset of symptoms or motor neuron degeneration. Later, the tracer fluorogold was injected into the same muscles in order to determine the number and size of the motor neurons that were not damaged as well as the number of motor neurons that preserved axonal projections to the treated muscles. This study showed that GDNF-secreting myoblasts protected the motor neurons in these muscles from degeneration, enhanced their motor function, and delayed the onset of symptoms. In a phase I study, encapsulated genetically modified xenogeneic cells were used to transport ciliary neurotrophic factor (CNTF) in ALS patients (Aebischer et al., 1996). Systemic delivery of CNTF caused many undesirable side effects and thus clinical trials were discontinued. This delivery system was implanted in the patients' lumbar intrathecal space where CNTF was released without the drawback of side effects observed in systemic delivery (Aebischer et al., 1996). A similar delivery system was developed for human retinal degeneration and

tested in a phase I trial. Cells transfected with the human *CNTF* gene were encapsulated and surgically implanted into the vitreous of the eye (Sieving et al., 2006). In an *in vitro* study for controlled release and sustained delivery of CNTF, nanospheres and microspheres were used to optimise CNTF encapsulation and release (Nkansah et al., 2008).

Table 2.1: Outline of stem cell transportation in Motor Neuron Disease. Adapted from Gowing and Svendsen (2009)

Model	Cell-Type Grafted	Method of Administration	Effect on Pathology	Mechanism
C7-C8 ventral root avulsion rat	Spinal rat npc ^{GFP}	Intraspinal	4.1-fold ↑ MN Survival	Trophic support
SOD1 ^{G93A} rat	Rat NSC	Tail vein injection	Not investigated	Not investigated
SOD1 ^{G93A} rat	hNPC ^{GDNF}	Intraspinal	↔ Lifespan approx. 2-fold ↑ MN survival	Trophic support
SOD1 ^{G93A} mice	hNSC ^{VEGF}	Intrathecal (L5-L6)	1.1-fold ↑ Lifespan	↓ apoptosis, trophic support
SOD1 ^{G93A} rat	Spinal hNSC	Intraspinal	Not investigated	Not investigated
SOD1 ^{G93A} rat	Spinal hNSC	Intraspinal	Delayed onset by 1.08-fold 1.12-fold ↑ lifespan	Not investigated
SOD1 ^{G93A} mice	hNPC ^{BDNF} , IGF-1, GDNF or VEGF	Cisterna magna or lateral ventricles	1.27-fold ↑ MN survival -GDNF ♂ 1.08-fold ↓ survival-GDNF ♀	Not investigated
SOD1 ^{G93A} mice	hMSC	Irradiation and tail vein injection	Delayed onset 1.1-fold ↑ lifespan 1.45-fold ↑ MN survival	Not investigated
SOD1 ^{Leu26de1TT} mice	Rat MSC ^{GFP}	Fourth ventricle	No effect	N/A
SOD1 ^{G93A} rat	Rat MSC	Intrathecal	1.13-fold ↑ lifespan 1.71-fold ↑ MN survival	↓ microgliosis, ↓ inflammation
SOD1 ^{G93A} mice	hMSC	Intraspinal	1.06-fold ↑ MN survival ♀ motor behavior	↓ microgliosis ↓ astrocytosis

hMSC = human Mesenchymal stem cells; GFP = glial fibrillary protein; MSC = Mesenchymal stem cells; IGF-1 Insulin-like growth factor-1; GDNF = glial cell line-derived neurotrophic factor; BDNF = brain derived neurotrophic factor; MN = motor neuron; hNPC = human neural progenitor cells; NSCs = neural stem cells; SOD1 = superoxide dismutase 1; VEGF = Vascular endothelial growth factor

2.5.2. Nano-drug delivery systems: Structure and functional properties

According to the U.S. National Nanotechnology Initiative (NNI), nanotechnology is “the ability to understand, control and manipulate matter at the level of individual atoms and molecules...in order to create materials, devices, and systems with fundamentally new properties and functions because of their small structure” (Roco et al., 2007). Using this knowledge it is possible to control the primary elements of this technology and incorporate them into biological systems. Polymeric resources are used to formulate structures on a nanometer scale capable of interaction with biological structures at a molecular level. Continuous research of these nano-structures could be beneficial in treating CNS diseases that have presented complicated challenges such as decreased bioavailability of therapeutic agents.

There are various nano-sized particles that have been formulated for biomedical applications; each with different physicochemical properties. These nano-sized particles are able to by-pass the BBB and carry out their required therapeutic or diagnostic functions. Systemically delivered nanoparticles cross the BBB via either receptor-mediated transcytosis, which necessitates the attachment of specific ligands on the nanoparticle surface, or adsorptive-mediated transcytosis, which employs charge-based interactions (figure 2.4a). Moreover, nanoparticles coated with polysorbate T-80 have shown to mediate the endocytosis of drugs across the BBB through the interaction polysorbate T-80 has with brain micro-vessel endothelial cells (figure 2.4b) (Sun et al., 2004; Tian et al., 2011). Polysorbate 80-coated nanospheres adsorb apolipoprotein E (ApoE) from blood plasma and imitate low density lipoprotein, having particular receptors at the surface of endothelial cells of the BBB. The therapeutic agent may then be released into these cells from the nanospheres and diffuse into the brain, or the particles may be transcytosed. Other processes such as tight junction modulation or P glycoprotein inhibition may also occur. Polysorbate T-80 therefore plays a role in brain targeting, which improves the transporting properties of the nanospheres and gives them an added advantage. The appeal of

nanoparticles is further enhanced by their biodegradability and biocompatibility characteristics. Additionally, it is possible to insert nanoparticles into a biodegradable polymeric biomaterial (hydrogel) to form a dual drug delivery system (Gong et al., 2012). Hydrogels have been developed to be utilised in the treatment of CNS disorder (Lee et al., 2006), however, in combination with nanoparticles the functions of both the nanoparticles and the hydrogel could be enhanced.

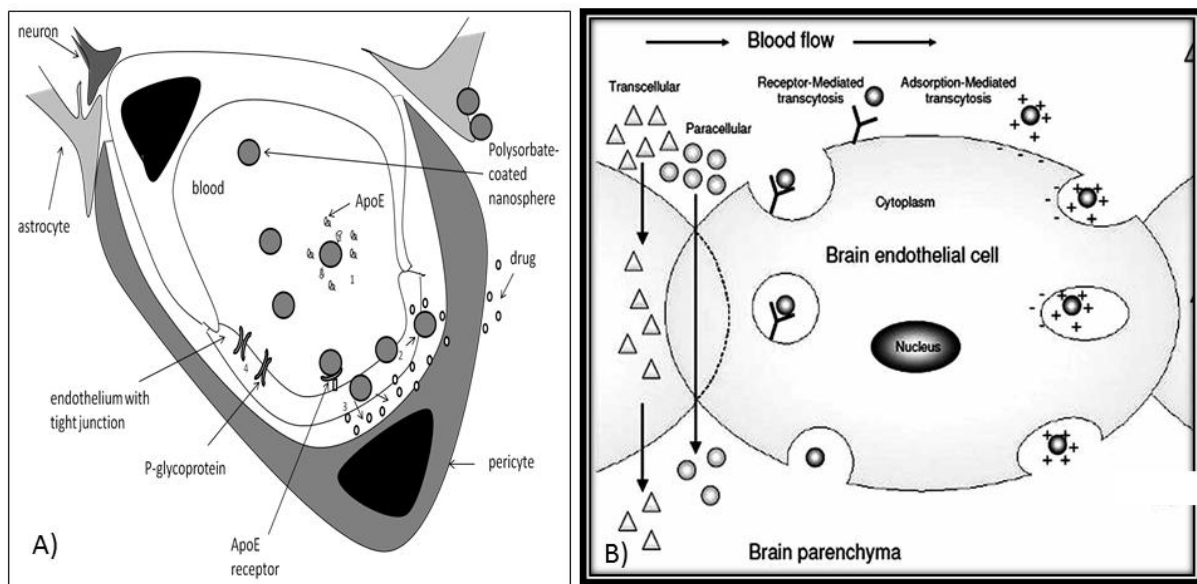
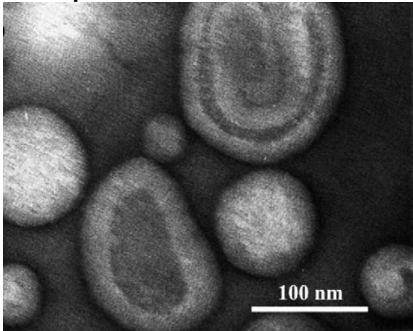
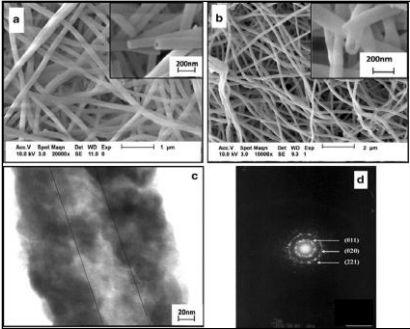
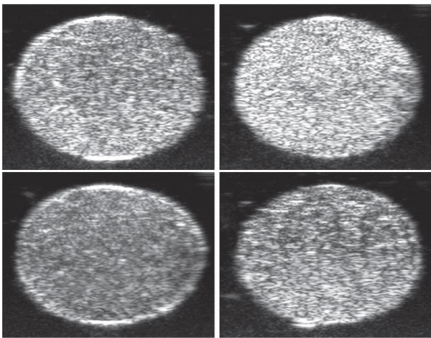
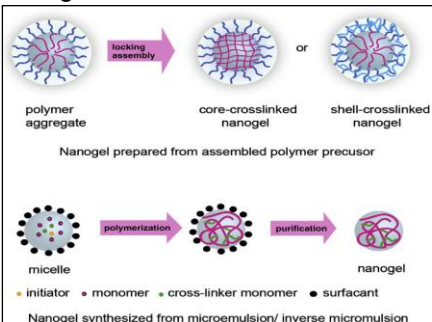


Figure 2.4: A) Nanoparticles that are delivered systemically cross the BBB via either receptor-mediated transcytosis, which requires the presence of specific ligands on the nanoparticle surface, or adsorptive-mediated transcytosis, which utilizes charge-based interactions. B) An innovative mode of drug delivery to the brain using polysorbate 80-coated nanospheres. Polysorbate 80-coated nanospheres adsorb apolipoprotein E (ApoE) (1) from blood plasma and emulate low density lipoprotein, having particular receptors at the surface of endothelial cells of the BBB. The drug may then be released into these cells from the nanospheres and diffuse into the brain (2), or the particles may be transcytosed (3). Other processes such as tight junction modulation or P glycoprotein inhibition may also occur (4) (Radhika et al., 2011). Adapted from Vauthier *et al.*, 2003.

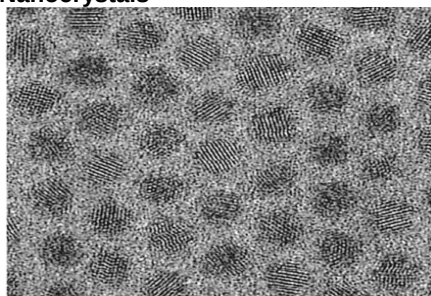
There are several nano-sized drug delivery systems that have been developed for Alzheimer's disease and Parkinson's disease but few have been explored for the treatment of Huntington's disease and ALS. These drug delivery systems are discussed in more detail further down in this paper. ALS though not as common as AD or PD is as damaging to neurons with eventual limb paralysis, loss of speech, swallowing and breathing functions and ultimately death (Mitchell and Borasio, 2007).

Nanotechnological delivery systems have great significance in the development of therapeutics approaches because they can modify the pharmacological profiles of drugs and related therapeutic properties (Prokop and Davidson, 2008) by allowing a more predictable control of drug release kinetics among other things. These systems can achieve targeted drug delivery, where the drug remains in its active conformation, and additionally, achieve a positive alteration in the pharmacokinetics, pharmacodynamics and, biodistribution of the drug (Craparo et al., 2011). These nano-sized delivery systems have been designed in various geometries, configurations and surfaces ranging from nanofibers to polymeric nanomicelles as shown in table 2.2.

Table 2.2: The various structural designs of nanosystems for possible application in ALS drug therapy

Nanosystem	Description
Nanoliposomes  TEM micrographs of nanoliposomes (Imran et al., 2012)	Nanoliposomes are lipid layer/s surrounding an aqueous core. They can be used to deliver both hydrophilic (aqueous layer) or hydrophobic (lipid layer) drugs. Nanoliposomes are prepared by sonicating phospholipids in water (Zaru et al., 2011).
Nanofibers  a) and b) SEM images of hollow nanofibers; c) TEM image and d) selected area electron diffraction (SAED) patterns of nanofibers (Li et al., 2013)	These are fibers that are minimized to nanometers (e.g. 10×10^{-3}–100×10^{-3} mm) and are fabricated by drawing, template synthesis, phase separation, self-assembly, electrospinning, etc (Huang et al., 2003).
Nanobubbles  In vitro ultrasonic images of Nanobubbles at high frequency diagnostic ultrasound in contrast pulse sequencing mode (Yin et al., 2012).	Nanobubbles are nano-scopic soft domains at the liquid-solid interface that resemble spherical caps with heights of ~10nm and diameters of $2R \sim 100\text{nm}$. They correspond to a small contact angle of $\theta \sim 10^\circ$. In other words, they are nano-scale gas bubbles located at the liquid-solid interface (Brenner and Lohse, 2008).
Nanogels  Polymer precursor and emulsion methods for nanogel synthesis (Chacko et al., 2012).	These are networks of cross-linked nanoparticles that consist of ionic and non-ionic polymeric chains prepared using an emulsification solvent. They are able to swell in water as well as integrate drugs (Modi et al., 2010).

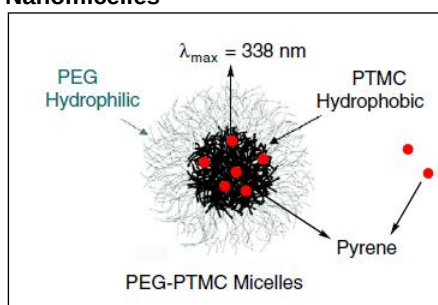
Nanocrystals



HRTEM image showing CdSe nanocrystals (Talpin et al., 2001).

These are nanoparticles with a crystalline character. They can be used as drug nanocrystals where the drug makes up the entire nanocrystal instead of having a carrier. Nanocrystals can also be used as semiconductors and utilized as probes to allow for imaging and diagnostics (Parak et al., 2003).

Nanomicelles



Nanomicelle displaying a hydrophobic inner and hydrophilic outer layer (Kim et al., 2008).

These are formed from amphiphilic block copolymers consisting of hydrophilic and hydrophobic portions (Hyun et al., 2008).

2.6. Lessons learned from nanotechnology in other neurodegenerative disorders

There are several more examples of nanotechnology being employed in testing of potential new treatments in neurodegenerative disorders like Alzheimer's disease and Parkinson's disease than there are for ALS (Table 2.3). We can take lessons from the advances in these disorders and extrapolate them to ALS. Below are certain examples that can be employed in investigating ALS treatment because the delivery systems developed look at aspects of the disorder that are also found in ALS and can therefore be beneficial in finding efficient treatment in ALS. It is important, however, to note that the advances taken in these neurodegenerative disorders are still groundwork. More testing needs to be done to be certain of the actual potential these drug delivery systems possess for ALS patients.

2.6.1. Examples of nanotechnological approaches employed in Alzheimer's disease

2.6.1.1. Gold nanoparticles for the detection of Alzheimer's disease biomarker

Based on the fact that there is currently no absolute cure for Alzheimer's disease, detection of the disease is vital in its very early stages. Early detection will enable the treatments already in the market to work more efficiently and effectively. Tau protein is found in the cerebrospinal fluid (CSF) and is hyperphosphorylated in patients with AD (Neely et al., 2009). Neely and co-workers (2009) demonstrated a label-free, fast and highly sensitive monoclonal anti-tau anti-body (tau-mab) coated gold nanoparticle based two-photon Rayleigh scattering (TPRS) assay for the selective detection of Alzheimer's tau protein in a 1 pg/mL level. It was shown that TPRS is highly sensitive to tau protein (2 magnitudes more sensitive than the conventional colorimetric method) and is able to differentiate between the tau protein and other proteins present in the CSF. The TPRS targets the early stages of AD and is easy and fast, making it a promising tool for diagnosing Alzheimer's disease.

Gold nanoparticles were also used in an *in vitro* study to disrupt the growth of β -amyloid fibrils and plaques that play a crucial role in mental dysfunction. Gold particles were attached to β -amyloid targets and microwave fields were applied, a procedure that allowed a selective supply of energy to dissolve and separate the aggregates. After 7 days of incubation at 37°C the sample was observed for precipitation and no aggregates had formed. The treatment thus showed that it successfully separates the fibrils and decreases the propensity for the β -amyloid proteins to reaggregate (Kogan et al., 2006). Although this was only an *in vitro* study, these gold nanoparticles could possibly be employed in developing a system that could manage the aggregation of the mutant proteins in ALS.

2.6.1.2. Cerium oxide nanoparticles

Cerium oxide nanoparticles have long lasting anti-oxidant properties which makes them a useful tool for the treatment of neurodegenerative disorders caused by oxidative stress. Both Alzheimer's disease and ALS have oxidative stress as a theory for pathophysiology.

Cerium oxide has the ability to transfer and absorb oxygen atoms, oxidise unsaturated hydrocarbons and transfer electrons to hydrocarbon radicals (Singh et al., 2007). Singh and co-workers (2007) treated mixed cultures of cortical brain cells with 10nm cerium oxide particles and this resulted in an increased life span of the cells by up to six fold. This *in vitro*, it was shown that using electron paramagnetic resonance that cerium oxide nanoparticles did not produce free radicals and were exceptional scavengers of both superoxide and hydroxyl radicals. Oxidative stress has also been linked to alterations of glutathione (GSH) metabolism resulting in reduced GSH levels in the brain. GSH delivery to the brain faces the same restriction other agents have and therefore methods to transport it across the BBB have been developed. GSH has been encapsulated in liposomes as well as lipid vesicles made of lecithin and glycerol where the latter formulation was tested on mixed mesencephalic cultures treated with paraquat and maneb in an *in vitro* study (Cacciatore et al., 2012). The liposomal GSH was taken up into neurons, hydrolysed and the amino acids comprising GSH were released from lysosomes and used for GSH biosynthesis which later demonstrated neuroprotection. Other nanocarrier systems for GSH include nanoparticles coated with hydrophilic polymers such as polyethylene glycol or polysaccharides such as chitosan, pectin, hyaluronic acid and dextran. However, a recent *in vitro* study by Brasil and co-workers (2013) showed that GSH may be involved in the activation of FALS-linked mutant SOD1 during chronological aging.¹²⁵ GSH therefore may not be a suitable therapeutic agent in ALS.

2.6.2. Nanotechnologies employed in Parkinson's disease

2.6.2.1. Biodegradable nanofibers in stem cell therapy

A combination of stem cell therapy and polymer-based nanotechnology has been and continues to be tested as a potential future therapy in PD. Biodegradable scaffolds that can promote restoration of damaged neurons by stem cells were developed using chemicals and electrospinning (Nisbet et al., 2008; Modi et al., 2010). This process allowed for the modification of the arrangement of nanofibers to form scaffolds that can be incorporated into

the body (Nisbet et al., 2008). The scaffolds can then be infused into the body and stem cells integrated accordingly. There are various types of scaffolds that can be used for neuron damage repair and the preference of type of materials used is subjected to the cellular reaction to materials. This reaction depends on the physical and chemical properties of the materials and the application for which the scaffolds are employed (Nisbet et al., 2008). Neuron damage is common to all neurodegenerative disorders therefore these scaffolds could potentially be used in the development of new therapies in ALS.

2.6.2.2. Nanoparticles modified with Odorranalectin

Intranasal delivery, as a way to bypass the BBB has long been investigated and there has been a need to improve the drug absorption from the nose to the brain by increasing the resident time of drugs in the nasal cavity. The enhancement of drug absorption can be achieved by introducing bioadhesive compounds, such as lectins, to drug delivery systems in order to target the nasal mucosa and increase absorption at that site. Odorranalectin (OL), the smallest lectin with decreased immunogenicity when compared with the other lectins, was attached to nanoparticles for the advancement of drug delivery to the brain via the nasal route (Wen et al., 2011).

The nanoparticles were formulated using poly (ethylene glycol) and poly (lactic-co-glycolic acid). In this study, hemiparkinsonian rats were given urocortin (UCN) loaded OL-nanoparticles by means of intranasal administration. Rotation behaviour tests, neurotransmitter determination and tyrosinehydroxylase tests were performed. Results indicated that the uptake of the nanoparticles was increased by modification with OL and therapeutic effects of UCN were improved in hemiparkinsonian rats. Such a system could potentially be used in the development of new therapies in ALS by incorporating riluzole, or any other promising therapeutic agents that have been researched but have failed to produce desired results.

2.6.2.3. Lactoferrin-coupled PEG-PLGA nanoparticles

Lactoferrin (Lf), a cationic iron-binding glycoprotein (Naot et al., 2005; Hu et al., 2011), was used to enhance polyethylene glycol-poly(lactide-polyglycolide) (PEG-PLGA) nanoparticles for delivery of therapeutic agents across the BBB (Hu et al., 2011). Lf receptor is found on the BBB and transports Lf across the BBB. Following its thiolation, Lf was attached to the maleimide function enclosing the PEG incorporated nanoparticle thus producing a Lf-nanoparticle complex which was then examined using a fluorescent probe coumarin-6. There was a higher amount of coumarin-6-loaded Lf-nanoparticles in bEnd.3 cells than of unmodified nanoparticles in uptake studies. Moreover, intravenous administration of coumarin-6 containing Lf-nanoparticles yielded quantities 3 fold greater than that of coumarin-6 integrated in unconjugated nanoparticles in the mouse brain. Lactoferrin thus has great potential of ensuring that therapeutic agents are taken up by endothelial cells of BBB therefore crossing the BBB and increasing their yield at their target site. In the study, the drug delivery system was used for the delivery of UCN for therapy in PD but just like the previous system, its potential can be translated to therapeutic agents used for therapy in ALS.

2.6.3. Huntington's disease

Gene therapy has been at the forefront of the search for breakthrough treatment for HD. The development of non-viral, nanoparticulate vectors for gene delivery has received much attention. These vectors could be beneficial for, not only research in HD therapy, but for other disorders that are caused by gene defects including ALS. Below are examples of such innovations in HD.

2.6.3.1. Nanotechnologies employed in Huntington's disease

2.6.3.1.1. Organically modified silica (ORMOSIL)-DNA nanoplexes

Techniques have been developed by Stachowiak and co-workers (2009), to bind DNA on the surface of organically modified silica nanoparticles (ORMOSIL) to create nonviral vectors for the safe and efficient delivery of genes. These methods were developed specifically to target the integrative nuclear FGF Receptor 1 signalling (INFS) pathway which is responsible for the development of neural stem/progenitor cells (NS/PCs) *in vitro* (Stachowiak et al., 2009). ORMOSIL nanoparticles have both hydrophobic and hydrophilic groups on the precursor alkoxy organosilane which plays a role in self-assembly of both normal and reverse micelles under suitable conditions (Modi et al., 2010). These nanoparticles are formed using the oil-in-water micro-emulsion method that evades corrosive solvents and undergoes an intricate purification process. ORMOSIL-DNA nanoplexes were used to induce neurogenesis in transfected NS/PCs of an adult mouse brain. Nanoplexes carrying the recombinant nuclear form of FGF Receptor 1 (FGFR1) stimulate NS/PC withdrawal from the cell cycle and neuronal differentiation *in vivo*. Stachowiak and co-workers (2009) were able to demonstrate that nanoparticle-mediated gene delivery in NS/PCs promotes neurogenesis through the INFS mechanisms *in vivo*.

Table 2.3: A summary of Alzheimer's disease, Parkinson's disease and Huntington's disease. Symptoms, incidences and nanotechnologies investigated for their treatment

Neurodegenerative Disease	Symptoms	Worldwide Incidence	Current Medication	Examples of Nanosystems Used
Alzheimer's disease (AD)	Progressive short-term memory loss, behavioural symptoms, functional deficiencies, mood disorders, anxiety, psychotic episodes and aggression (Gauthier et al., 2005)	33.9 million (Barnes DE, Yaffe, 2011)	Cholinesterase inhibitors, memantine, selegiline, Gingko <i>biloba</i> (Schmitt et al., 2004)	• Gold nanoparticles for detecting AD biomarker (Neely et al., 2009) • Cerium oxide nanoparticles (Singh et al., 2007) • Clioquinol-loaded N-butylcyanoacrylate nanoparticles (Modi et al., 2010) • Inhibition of β-amyloid plaque formation using nanoparticles (Cabaleiro-Lago et al., 2008) • D-penicillamine nanoparticles for the treating AD (Cui et al., 2005)
Parkinson's disease (PD)	Cognitive dysfunction, movement impairments, dementia (Kehagia et al., 2010)	8-18/100000 (de Lau and Breteler, 2006)	Levodopa, selegiline, COMT inhibitors, dopamine agonists, anticholinergic drugs, amantadine, apomorphine (Jankovic and Aguilar, 2008)	• Delivery of dopamine using chitosan nanoparticles (Trapani et al., 2011) • Nanoparticle-based gene therapy (Yurek et al., 2009) • Nanofibers as stem cell therapy (Nisbet et al., 2008) • Carbon nanotube and gold nanoparticle biosensors for detection of PD (Tisch et al., 2013)
Huntington's disease (HD)	Chorea and dystonia, personality changes, cognitive dysfunction (Walker, 2007)	2.71/100000 (Pringsheim et al., 2012)	Antipsychotics, antidepressants, tranquilizers, mood stabilizers, botulinum toxin, tetrabenazine (xenazine) (Gudesblatt and Tarsy, 2011)	• Organically modified silica (ORMOSIL)-DNA nanoplexes (Stachowiak et al., 2009) • Self-assembling β-cyclodextrin nanoparticles (Godinho et al., 2013)

2.6.3.1.2. Self-assembling β -cyclodextrin nanoparticles

Modified amphiphilic cationic β -cyclodextrin (β -CDs) nanoparticles were developed as carriers of short interfering RNAs (siRNAs) for the treatment of HD (Godinho et al., 2013). The use of siRNAs is to target the silencing of the mutant huntingtin protein (Godinho et al., 2013). The positively charged β -CDs are suggested to have an interaction with the anionic

huntingtin targeted siRNAs which in turn produced nanoparticles. These nanoparticles were found to show great stability in simulated cerebrospinal fluid. In addition, the use of this nano-sized delivery system in this study resulted in a decrease in the expression of the huntingtin gene in the rat striatal cells and in human HD primary fibroblasts. Frequent injections of the nanoparticles mitigated some of the motor symptoms in the R6/2 mouse model of HD. These findings point towards β -CD nanoparticles being potentially used in the application of HD treatment. Both delivery systems mentioned for gene therapy in HD treatment have potential in ALS research.

2.7. Drug delivery nanosystems used for the treatment of ALS

Since the BBB restricts the entry of a copious amount of xenobiotic molecules including drugs, it is very difficult to efficiently achieve purposeful recovery in neurodegenerative disorders. Nanotechnology is at the forefront of biotechnology and of overcoming this barricade (Modi et al., 2010). The low bioavailability of some therapeutic agents is possibly one of many problems that the agents tested in clinical trials have faced. Even though riluzole showed some success, it still doesn't reach satisfactory therapeutic levels. Designing a proficient targeted nano-enabled drug delivery system and incorporating riluzole, or any other potential therapeutic agent, as the active agent has the prospective to advance its therapeutic profile. Polymeric nanoparticles are prospective components in developing a drug-delivery system for the treatment of neurodegenerative disorders such as ALS.

Currently, according to our research, there have only been three attempts to develop ALS therapy combining nanotechnology with riluzole treatment. These attempts have all been at foundation stages and require further extensive investigation before reaching clinical implication. Moreover, as mentioned previously, ALS rat models have failed to translate to positive results for human trials. This means that, although promising, these nano-enabled systems would need to be tested on much improved ALS animal models. Since riluzole is an

anti-glutaminergic agent, the nano-enabled systems incorporating this drug would thus be targeting the irregular stimulation of glutamate receptors and block these receptors.

Bondì and co-workers (2010) prepared riluzole-loaded solid lipid nanoparticles for the treatment of ALS. The lipophilic properties of these nanoparticles provided them with the ability to target the brain by endocytosis. The riluzole-loaded nanoparticles and conventional riluzole were administered to rats separately and the two were compared. The riluzole-loaded nanoparticles were able to bypass the BBB and deliver an increased amount of drug to the brain as compared to conventional riluzole. Additionally, it was established that there was minimal drug in organs such as the spleen, liver, kidney and heart when the rats were given riluzole-loaded nanoparticles. Conversely, the drug bio-distribution was increased in these organs in the rats that received conventional riluzole. The entrapped riluzole reduced the concentration of drug in the bloodstream and therefore reduced side-effects that are apparent with conventional riluzole (Bondì et al., 2010).

In a different study, carbon nanotubes (CNTs) were used as carriers for riluzole (Chigumbu et al., 2012). The physicochemical properties of CNTs such as their chemical composition, surface area, length, and diffusion in solution were adapted to enhance their employment in drug delivery. Riluzole was covalently bound to the CNTs after their modification, which in turn resulted in further alterations in CNT chemical structure. The *in vitro* study aimed to investigate the possible toxicity that the riluzole-bound multi-walled nanotubes could introduce to cells. After the comparison between cells treated with these nanotubes and untreated control cells, the cells were still alive and functional. This comparison verified that the riluzole-bound multi-walled nanotubes do not instigate cytotoxicity.

Lastly, in an *in vitro* study by Shanmukhapuvvada and Vankayalapati (2012), chitosan nanoparticles were formulated to carry riluzole to the brain. In this investigation, chitosan

was cross-linked with tripolyphosphate using an emulsification method. The physicochemical properties of chitosan (hydrophilic and cationic) and the size of the nanoparticles permitted the targeting of the central nervous system by endocytosis or transcytosis. The optimised formulation with a size of 418nm showed drug entrapment of 55% and released 86.2% of the drug within 24 hours. Although this study provided no comparisons *in vivo*, it demonstrated that it is possible to design a system that has the potential to carry and deliver riluzole to the central nervous system.

Additional studies incorporating agents other than riluzole have also shown that using targeted therapy or carrier-based therapy increases the chances of producing positive results. Minocycline, an agent that was previously reported to lack the desired efficiency, was encapsulated in lipopolysaccharide modified liposomes to target activation of microglia in SOD1 mice (Wiley et al., 2012). Wiley and co-workers (2012) aimed to prove that introducing nano-encapsulated minocycline intracerebroventricularly would increase site-specific action of the drug and therefore delay the onset of ALS and increase lifespan in SOD1 mice. They also aimed to demonstrate that these minocycline-carrying nanovesicles could delay future disease development by reducing neuro-inflammation achieved by the activation of microglia. The TLR4 receptors on the microglia of SOD1 mice were targeted using the modified nanoliposomes and their efficacy was compared to non-targeted nanoliposomes. The *in vitro* results showed a 29% increase of drug uptake in BV-2 microglia cells when using nanoliposomes targeting the TLR4 receptors on microglia compared to non-targeted nanoliposomes. Both targeted and non-targeted nanoliposomes were able to delay disease onset in SOD1 mice. This study also showed intracerebroventricular administration of the minocycline-loaded nanoliposomes was more effective in the latency to the disease endpoints than when compared to the oral and injected minocycline.

2.8. Future Recommendations

In view of the minimal existing therapy used in the treatment of ALS, the need for more pioneering approaches to unearthing an effective treatment with negligible side effects is highly evident. In addition, the BBB hinders the penetration of therapeutic agents into the brain thus necessitating a delivery system that will bypass this barrier and achieve high bioavailability. Incidentally, the use of nanotechnology as well as stem cells as carriers for therapeutic agents is a potential strategy. As mentioned in this review, a number of attempts have been made to develop treatment for ALS and only a few attempts using nanotechnology have been investigated. With only riluzole being FDA approved, it is clear that clinical trials have failed dismally to produce desirable results.

The ideal brain delivery system should be biocompatible, biodegradable, present extremely low toxicity, require minimal drug to achieve maximum therapeutic levels and be able to carry a therapeutic agent and deliver it to its target site still in its active conformation; that is, be a targeted delivery system. It should also be able to retain the therapeutic agent at the target site for a prolonged period, degrade slowly and thus provide a sustained delivery of the therapeutic agent and also be practicable. Not much is being done to explore such delivery systems in the treatment of ALS. Considering that there are numerous theories to the pathophysiology of ALS, perhaps a multifunctional delivery system could bring about much desired results. Therapeutic agents that have been tested have shown little to no effect therefore a combination of these agents, incorporated in a multifunctional system would be an interesting avenue to investigate. Furthermore research has been done into implantable and/ or injectable polymeric hydrogels for the treatment of tumours and other illnesses (Hatefi and Amsden, 2002; Schmaljohann, 2006). These could possibly be beneficial when designing a delivery system that can provide all the characteristics mentioned earlier. Polymeric hydrogels have been shown to have features that are very

useful in a delivery system, including slowly degrading to release a therapeutic agent activated by an external stimulus such as temperature. A combination of nanoparticles with the hydrogel could bring about great benefits from both systems. This has been attempted for the treatment of other diseases such as cancer (Gong et al., 2012) and could be used to develop a system for ALS.

The issues that have been identified with the process of discovering or inventing an effective treatment include the difficulty in designing and performing clinical trials. This is an aspect that needs to be thoroughly investigated and improved significantly. An improvement in the way clinical trials are designed and run could possibly result in an increase in the amount of FDA approved therapeutic agents. Many clinical trials have been based on a two-arm, parallel study design and a few have attempted the crossover design, a sequential study design, and others for a different option (Levy et al., 2006). However, designs have not produced positive outcomes because of the difficulties associated with managing ALS, the lack of biomarkers for early diagnosis, the research that still needs to be done to measure the effect of a drug and to detect realistic effect size together with methods to minimize missing data and selecting suitable dose, regimen and route. In addition, ALS has a short disease duration therefore patients in the trial will likely not be able to be in the study for a long period of time. Many other considerations such as the ones mentioned need to be made in the designing of ALS clinical trials and this could possibly change the state this disorder is currently in.

2.9. Concluding Remarks

Carrier-based therapy in neurodegenerative diseases has shown to have potential in eradicating the problems associated with the extensive neuron damage of ALS and the BBB. The advantage that nanotechnology offers with regard to nano-scale formation enables it to

be at the forefront of efficiently delivering genes and drugs to affected areas with reduced side effects and less difficulty in crossing the BBB. Biological components such as stem cells provide both a delivery system for therapeutic agents as well as bringing their own benefits in protecting motoneurons. Many therapeutic agents have been found unsuccessful in countless clinical trials possibly as a result of a number of problems including low bioavailabilities and detrimental side effects. The introduction of nanotechnology or stem cells to these agents could yield better results than those achieved previously. ALS has had many failed therapeutic agents and, to our knowledge, investigations into the implementation of nanotechnology as a solution to improving treatment have been minimal. The use of biodegradable and biocompatible polymers to formulate nanosystems that are able to support the regeneration of damaged neurons, promote neuroprotection and transport therapeutic agents should be exploited in developing treatments for ALS. It would be intriguing and valuable to utilise nanotechnology to improve the efficiency of some of the ALS therapeutic agents that provided minimal success in clinical trials previously. The result could possibly be one that translates to positive outcomes in ALS patients.

CHAPTER THREE

PREFORMULATION ANALYSIS OF THE DRUG AND COMPONENTS INTENDED FOR NANOSPHERE PREPARATION

3.1. Introduction

The diverse methods and materials used for the preparation of nanoparticles have improved vastly over the years and the quest for finding the most pertinent and efficient of these is still in progress. The process required to prepare suitable and effective nanoparticles involves researching the properties of different polymers that will maximise the efficiency of those particles, investigating the organic solvent that the polymers dissolve in, the ratio of the concentration of polymers and organic solvent as well as finding a suitable stabiliser.

Polymethacrylates, also known as Eudragits[®], have conventionally been used for the coating of tablets and capsules to achieve masking of taste for increased patient compliance (Nikam et al., 2011) and, more importantly, to prevent the release of active agents before they reach their target site. They are synthetic cationic and anionic polymers of dimethylaminoethyl methacrylates, methacrylic acid, and methacrylic acid esters in different ratios (Evonik Industries: Eudragits, 2013). A number of them are available commercially as either the dry powder, as an aqueous dispersion, or as an organic solution. The ability that these unique polymers have to control the location and time of release of active agents has undoubtedly led to an expansion of their pharmaceutical application to the formulation of nanoparticles.

Likewise, polylactides are well established natural polymers that have been used extensively in the preparation of various nano-sized controlled release drug delivery systems. Their appeal is largely based on their biodegradability and biocompatibility properties (Ahmed et al, 2009). This is highly advantageous as it eliminates the need for surgical removal of foreign material in the body once the drug-load has been exhausted. Lactides can be found in three diastereoisomeric forms (see figure 3.1): L-lactide, D-lactide and DL-lactide (PDLLA) which is an amorphous racemic mixture of the D and L isomer. Any of these forms can be used in the formation of nanoparticles.

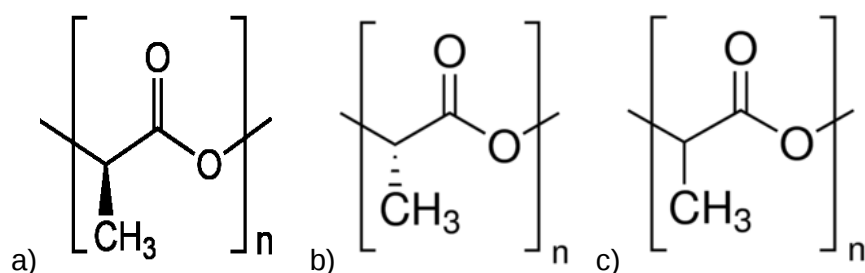


Figure 3.1: a) Poly-D-Lactide b) Poly-L-Lactide c) Poly-D,L-Lactide

The most desirable nanoparticles are stable, very small in size (< 300 nm) and for some applications must be small enough for cellular uptake. A blend of polymethacrylates and polylactides in the preparation of nanoparticles is worthwhile to explore as their properties indicate that a combination of these polymers has the potential to produce highly stable and functional nanoparticles. However, because polymethacrylates can either be cationic or anionic, the charge they carry is vital in how they interact with other polymers and therefore will exhibit different behaviours accordingly. Polylactides are ordinarily uncharged molecules; conversely, because they encompass a carboxyl group they are able to exist as zwitterions or polyelectrolytes at neutral pH level. This means it is possible for them to form polyelectrolyte complexes when mixed with polyions.

Polyelectrolyte complexes (PECs) or interpolyelectrolyte complexes form when oppositely charged polyelectrolytes (polyanions and polycations) are dissolved in an aqueous solution as seen in figure 3.2 (Buchhammer, et al., 2003). An electrostatical interaction occurs between the two polymers resulting in involuntary aggregation. This aggregation is caused by the increasing entropy that occurs when counterions of low molecular weight are discharged from the polymers (Paneva, et al., 2007). Polyelectrolyte complex nanoparticles have been formed using Poly(ethyleneimine) and Poly(acrylic acid) where it was determined that pH, molecular weight, mixing ratios, mixing order and polyelectrolyte concentration are parameters that regulate nanoparticle size (Müller, et al., 2011).

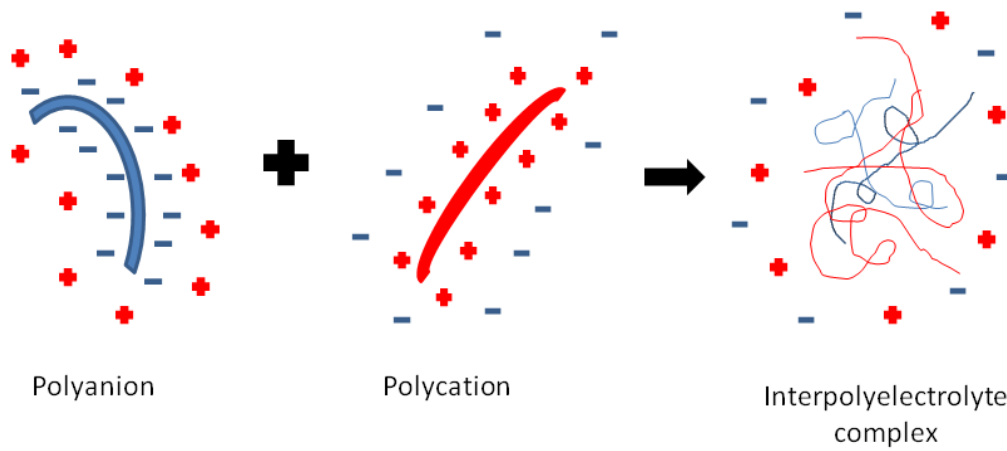


Figure 3.2: Oppositely charged polyelectrolytes mixed in an aqueous solution form an interpolyelectrolyte complex. A Monte Carlo spatial (non-lattice) model can demonstrate this reaction where stiff chains are organised into a “ladder” structure in which chains are located parallel to each other and monomeric units are arranged into ionic pairs according to their position in the chain. Flexible chains form a globular “scrambled-egg” structure with a disordered position of monomer units (Pergushov et al., 2012)

It has been suggested that there are two steps in the formation of polyelectrolyte complex nanoparticles. Primary polyelectrolyte complexes as described earlier are bound by electrostatic interactions and this process is usually followed by the formation of secondary polyelectrolyte complexes which are primary PECs that are bound by short range dispersive interactions. This may lead to secondary aggregation which presents as water-insoluble complexes and thus the formation of unstable nanoparticles.

For the simulation of PEC structures, Lazutin and co-workers (2012) used a Monte Carlo spatial (non-lattice) model. They acknowledged excluded volume interactions, bond-length and bond angle constraining potentials, sticky interaction within ionic pairs as the interactions between monomeric units. The excluded volume interaction of non-bonded monomer units E_{ex} is described by hard sphere potential:

$$E_{ex} = \begin{cases} 0 & r_{ij} > 1 \\ \infty & r_{ij} \leq 1 \end{cases} \quad \text{Equation 3.1}$$

where r_{ij} is the distance between monomeric units i and j . Distances here and below are measured in units of monomer size α .

The chain structure is imposed by bond length constraining potential E_b between neighbouring monomer units; it is taken in the form of a square-well potential:

$$E_b = \begin{cases} 0, & 1 < r < 1.2 \\ \infty & \text{otherwise} \end{cases} \quad \text{Equation 3.2}$$

where r is the distance between neighbouring units. Thus, the bond length b is limited within the interval from $b = 1$ to $b = 1.2$. The value of maximum bond length $b = 1.2$ together with maximal displacement of Monte Carlo step $\Delta r_{\max} = 0.1$ was chosen so that the condition of non-phantom chain was automatically satisfied. To model the macromolecule backbone stiffness, the bending potential E_{stiff} that depends on the angle θ between the adjacent bond vectors b_i and b_{i+1} was introduced,

$$E_{\text{stiff}} = 0.5\epsilon_{\text{st}}\theta^2 \quad \text{Equation 3.3}$$

where ϵ_{st} is the stiffness coefficient. The total energy E_{bond} defining the ionic pair formation is equal to

$$E_{\text{bond}} = -\epsilon_{\text{bond}}n_{\text{bond}} \quad \text{Equation 3.4}$$

where n_{bond} is the total number of salt bonds formed between charged units of a PEC. A salt bond AB is considered to exist if the distance between two units, A and B, belonging to different chains is smaller than r_{bond} .

In an effort to find the best combination of polymethacrylates with PDLLA for the preparation of nanospheres, both cationic and anionic polymethacrylates were investigated and their physicochemical properties were analysed and compared. The anionic polymethacrylates (a.PMA) investigated were Eudragit S100, L100 and L100-55. These are normally used as enteric coatings because they are resistant to gastric fluid. Eudragit E100 and RS PO were the cationic polymethacrylates (c.PMA) investigated. (See Table 3.1)

Table 3.1: Summary of properties of polymethacrylates

Eudragit® (Trade name)	Chemical name	Charge	Solvents or diluents	Supply form	Solubility/permeability
L100	Poly (methacrylic acid, methyl methacrylate) 1:1	Anionic	Acetone, alcohols	Powder	Soluble in intestinal fluid from pH 6
S100	Poly (methacrylic acid, methyl methacrylate) 1:2	Anionic	Acetone, alcohols	Powder	Soluble in intestinal fluid from pH 7
L100-55	Poly (methacrylic acid, ethyl acrylate) 1:1	Anionic	Acetone, alcohols	Powder	Soluble in intestinal fluid from pH 5.5
RS PO	Poly (ethyl acrylate, methyl methacrylate, trimethylammonioethyl methacrylate chloride) 1:2: 0.1	Cationic	Acetone, alcohols	Powder	Low permeability
E100	Poly (butyl methacrylate, (2-dimethylaminoethyl) methacrylate, methyl methacrylate) 1:2:1	Cationic	Acetone, alcohols	Granules	Soluble in gastric fluid to pH 5

Poly-D,L-lactide was selected to form nanospheres together with the above mentioned polymethacrylates respectively. The effects cationic and anionic polymethacrylates have on PDLLA nanospheres were established as well as the elements that contribute to the stability or lack thereof of these nanospheres.

3.2. Materials and Methods

3.2.1. Materials

Poly (D,L-lactide) (PDLLA) Resomer R203H was purchased from Boehringer Ingelheim Pharma GmbH (Ingelheim am Rhein, Germany). Polymethacrylates (Eudragit® L 100; S 100; L100-55; RS PO; E100) were purchased from Degussa, Rohm GmbH, Pharma Polymers (Germany). Amantadine HCl was purchased from Sigma-Aldrich Inc., (St Louis, MO, USA). Isopropyl alcohol and dichloromethane were purchased from Rochelle Chemicals (Johannesburg, South Africa). All other reagents used were of analytical grade and were used as purchased.

3.2.2. Determination of amantadine

It was necessary to firstly establish a method for the determination of the chosen study drug, amantadine. The analytical techniques reported for amantadine HCl consist of high-performance liquid chromatography, gas chromatography, capillary electrophoresis, potentiometry, and fluorimetry. The amantadine molecule lacks chromophores and/ or auxochromes, therefore, shows no distinct absorption in the UV region above 200 nm. Consequently direct UV spectrophotometry is not valuable for its quantitative determination. A number of spectrophotometric methods have been reported for its determination however, these methods have been found to be too complicated to perform, require derivatisation of the drug and are time consuming (Darwish et al., 2005; Mahmoud et al., 2009).

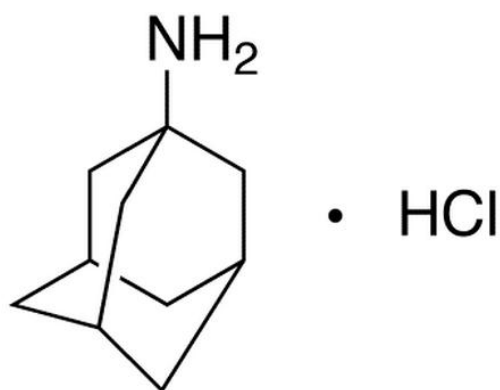


Figure 3.3: Amantadine hydrochloride

Spectrophotometric and fluorimetric analysis are considered more convenient alternative techniques because of their inherent simplicity and high sensitivity. Studies have described the improvement of effortless and sensitive spectrophotometric methods for determination of amantadine HCl. These methods include one based on the oxidation of the drug by either ammonium molybdate (Sridevi and Yusuff, 2007) or potassium permanganate, one based on the charge-transfer complexation reaction between the amantadine base as an electron donor and iodine as a σ -acceptor, as well as one based on the formation of coloured vinylamino-substituted benzoquinone derivative as a result of condensation of amantadine with acetaldehyde-chloranil combination (Darwish et al., 2006).

a) Different methods of Amantadine determination

The charge-transfer complexation reaction and the formation of coloured vinylamino-substituted benzoquinone derivative require slightly more effort than the oxidation method. The preparation of standard solutions for these methods involves accurately weighing out 100 mg of amantadine HCl and dissolving it in 10 ml of distilled water in a 25-ml beaker. The

solution is then transferred quantitatively into a 100 ml separating funnel, rendered alkaline with ammonia solution, and the excess ammonia is evaporated by heating the solution in a water bath. The liberating base is extracted with four 25 mL portions of chloroform. The combined extracts are passed through a small funnel containing anhydrous sodium sulphate (2 g) into a 100 mL calibrated flask. The contents of the separating funnel are washed three times with chloroform. The combined extracts and washings are then diluted to the mark with the same solvent to obtain a stock standard solution of 1 mg.mL^{-1} of amantadine, calculated as hydrochloride salt. This stock solution is then diluted with 1,2-dichloromethane and isopropanol for the charge-transfer complexation reaction and the formation of coloured vinylamino-substituted benzoquinone derivative, respectively, to obtain working solutions in the range of $2\text{-}400 \text{ }\mu\text{g.mL}^{-1}$.

Thereafter, the recommended procedure for the charge-transfer complexation reaction is as follows: 1 mL of the standard or sample solution ($20\text{-}350 \text{ }\mu\text{g.mL}^{-1}$) is transferred into 10 mL calibrated flasks. One milliliter of iodine solution (0.05%, $\frac{W}{V}$ in dichloromethane) is added, and the reaction is allowed to proceed for 20 min at room temperature ($25 \pm 5^\circ\text{C}$). The solution is diluted to volume with dichloromethane and the absorbances of the resulting solution are measured at 290 or 365 nm against reagent blanks treated similarly.

For the formation of coloured vinylamino- substituted benzoquinone derivative: 1 mL of the standard or sample solution ($20\text{-}400 \text{ }\mu\text{g.mL}^{-1}$) is transferred into 10 mL calibrated flasks. One milliliter of the acetaldehyde solution (1%, $\frac{V}{V}$ in isopropanol) and 1 mL of chloranil (0.15%, $\frac{W}{V}$ in isopropanol) are added. The reactions are allowed to proceed for 10 min at room temperature ($25 \pm 5^\circ\text{C}$). The solution is diluted to volume with isopropanol, and the absorbances of the resulting solution are measured at 325 or 685 nm against reagent blanks treated similarly.

b) Method chosen for study

For this study, oxidation of amantadine by potassium permanganate was used for determination of the drug as it was simpler and produced the most practical results (Khedr et al.). Briefly, an accurately weighed amount (1g) of amantadine HCl was transferred into 100 mL calibrated flask, and dissolved in approximately 40 mL of deionised water. The resulting solution was completed to the mark with water to provide a stock solution containing 10mg/mL. Different volumes of this stock solution were then further diluted with water to obtain the working standard solutions of concentrations suitable for analysis by potassium permanganate.

1 mL of the standard solution was transferred into a 10 mL calibrated flask. 1 mL of standardised KMnO₄ reagent was added. 2 mL of sulphuric acid (H₂SO₄ 20%) was added. The contents of the flasks were mixed and the reaction was allowed to proceed for 15 minutes at room temperature (25 ± 5 °C). After completion of reactions, the solutions were completed to volume with water. The absorbances of the resulting solutions were measured at 525 nm against reagent blanks treated similarly.

3.2.3. Synthesis of PDLLA-polymethacrylate nanospheres

The nanospheres were prepared by a double emulsion solvent evaporation method. Briefly, the organic phase was prepared by co-dissolving the polymers PDLLA and Eudragit® (L100, L100-55, S100, E100 or RSPO) in a mixed solvent system comprising of dichloromethane and isopropyl alcohol in a ratio of 1:1. The internal aqueous-phase (1mL of deionised water) and the organic phase were homogenized (Polytron, PT 2000, KINEMATIKA, AG LITTAU, Switzerland) for 3min at room temperature (25±0.5°C) to form a primary emulsion. The external aqueous-phase was prepared by dissolving SPAN80 in phosphate buffer saline (pH 7.4) to form a 0.25%_{v/v} solution. The primary emulsion was added drop by drop to the external aqueous-phase and emulsification was continued for further 30 min using a sonicator to form nanoparticles. The formed emulsion was centrifuged (Nison Instrument (Shangai) Limited, Shangai, China) at 15,000rpm for 10 minutes at 25°C, washed two times with distilled water and then lyophilized (LABCONCO, Kansas City, Missouri, USA) for 24 hours.

3.2.4. Determination of the size and charge of the nanospheres

Particle size is resolved by dynamic light scattering (DLS) that quantifies the Brownian motion of the particles. Brownian motion is the phenomenon where smaller particle sizes move more rapidly than larger particles therefore exhibiting greater Brownian motion.

The particle size is calculated from the translational diffusion coefficient using relaxation time of the Brownian motion (Stokes-Einstein equation) (Ma et al., 2004):

$$T_B = \frac{4\pi\eta r^3}{kT} \quad \text{Equation 3.5}$$

where r is the hydrodynamic radius of the particle, k is Boltzmann's constant, T is the fixed temperature and η is the viscosity of the liquid/ solvent used. Temperature needs to be kept

constant to ensure that the viscosity of the liquid is maintained throughout the measurement process.

The adsorption of a charged surfactant, the ionization of charged groups in the polymers used or the loss of ions from the recently formed nanospheres may result in particle surface charge or, alternatively the zeta potential. The surface charge may control the interactions with electrolytes found in the body and permanence of the formulation.

The Derjaguin, Landau, Verwey and Overbeek (DLVO) theory can be used to determine the stability of particles (Jiang et al., 2009).

$$V_t = V_e + V_d \quad \text{Equation 3.6}$$

where V_e and V_d are the forces of electrostatic born repulsion and van der Waals attraction respectively that exist between the particles.

However, it has been recommended that to incorporate particles suspended in water, which will essentially have Lewis acid-base interactions, an extended-DLVO approach should be employed instead (Chang and Chang, 2002).

$$V'_t = V_e + V_d + V_s \quad \text{Equation 3.7}$$

where V_s is the hydration energy.

A charge of $\pm 25\text{mV}$ is an indication of a stable formulation that will not aggregate.

Polymers are made up of repeat components (monomers) which are chemically bonded into long chains. Chain length is defined by the molecular weight of the polymer chain, pertaining to the relative molecular mass of the monomers and the number of monomers attached to the chain (Rogosic et al., 1996). All synthetic polymers have polymer chains of unequal length which makes them polydisperse. This means that the molecular weight is more than one value (i.e. exists as an average molecular weight of all molecular weights of all the chains in the sample) and consequently, the polymer consists of a distribution of chain lengths and molecular weights. (Polymer molecular weight distribution and definitions of MW averages, 2014).

The number average molecular weight (M_n) is the statistical average molecular weight of all the polymer chains in the sample, and is defined by equation 3.8:

$$M_n = \frac{\sum N_i M_i}{\sum N_i} \quad \text{Equation 3.8}$$

where M_i is the molecular weight of a chain and N_i is the number of chains of that molecular weight. M_n is calculated by polymerisation techniques and is determined by methods that resolve the number of molecules in a sample of a given weight.

The weight average molecular weight (M_w) is defined by equation 3.9:

$$M_w = \frac{\sum N_i M_i^2}{\sum N_i M_i} \quad \text{Equation 3.9}$$

M_w considers the molecular weight of a chain to confirm the contributions to the molecular weight average. The larger the chain, the more the chain contributes to the M_w . M_w is calculated by processes that are perceptive to the molecular size as well as the number, such as light scattering methods.

Commonly, a succession of average molecular weights can be defined by the equation:

$$M = \frac{\sum N_i M_i^{n+1}}{\sum N_i M_i^n} \quad \text{Equation 3.10}$$

where: $n = 1$ gives $M = M_w$

$n = 2$ gives $M = M_z$

$n = 3$ gives $M = M_z + 1$

The higher the averages, the more sensitive they are to high molecular weight polymers and as a result are increasingly more complex to measure meticulously. They are usually associated with methods that measure the motion of polymer molecules, such as diffusion or sedimentation techniques.

For all synthetic polydisperse polymers:

$$M_n < M_w < M_z < M_z + 1$$

The polydispersity index is used as a measurement of the broadness of a molecular weight distribution of a polymer and is defined by:

$$\text{Polydispersity index} = \frac{M_w}{M_n}$$

Equation 3.11

The larger the polydispersity index, the broader the molecular weight.

Determination of the average particle size, polydispersity index (Pdi) and zeta potential of the nanospheres was completed using a Zetasizer NanoZS instrument (Malvern Instruments (Pty) Ltd., Worcestershire, UK) at 25°C. All nanosphere particle size and zeta potential measurements were performed in the same manner. Each sample was diluted (1 in 10) with deionised water using disposable cuvettes for each run. Each test was performed in triplicate and the average value in each case was reported accordingly.

3.2.5. Study of the stability of the nanosphere emulsions

The stability of the emulsions was determined employing a Turbiscan Lab[®] (Turbiscan LabTM, Formulaction SA, L'Union, France). The Turbiscan Lab[®] assessed the phenomena affecting the homogeneity of the emulsions which consists of particle migration resulting in creaming or sedimentation as well as particle size variation or aggregation causing coalescence or flocculation. The Turbiscan Lab[®] Expert software was used to study transmitted (T) and backscattered (BS) light relating to equation 3.12. The transmission of light in a random dispersed medium may be considered as independent when the photon mean path length $\lambda^*(\phi, d)$ is larger than the wavelength λ of the incident radiation as seen in equation 3.13

$$BS = \frac{1}{\sqrt{\lambda^*}}$$

Equation 3.12

$$\lambda^*(\phi, d) = \frac{1}{n (\pi d^2/4) Q_e} = \frac{2d}{3 \phi Q_e}$$

Equation 3.13

$$\phi = n \frac{\pi d^3}{6}$$

Equation 3.14

Where λ^* is the photon transport mean free path, ϕ the particle volume fraction, n is the particle density, d the particle mean diameter and Q_e the extinction efficiency factor for scattering and absorption phenomena. Non-absorbing particles with an extinction efficiency

factor Q_e equals to the scattering efficiency factor Q_s which is an optical parameter given by the Mie theory. A particle can emit anisotropic scattering of light which can be characterised by the asymmetry factor g , which is the average cosine $\langle \cos\theta \rangle$ of the scattering angles weighted by the phase function or scattering diagram $P(\theta)$ of the scatterer ($g = 0$ for isotropic Rayleigh scatterers and $0 < g < 1$ for Mie scatterers of sizes larger than the wavelength).

Poly-DL-lactide polymethacrylate nanosphere emulsions were poured into flat bottomed boroscillated glass cells (27.5x70mm) up to a height of approximately 40mm then placed into the Turbiscan Lab[®] instrument. Synchronous dual measurements were performed by the transmission detector (at 180°) that receives the light, which went through the samples, while the backscattering detector (at 45°) received the light scattered backward by the samples. The light source has pulsed infrared with a wavelength of 880nm. Measurements were performed at 25°C and the Turbiscan Lab[®] was configured to perform scans for 5 minutes for each sample, over a 55mm cell length from bottom to top acquiring transmission and backscattering data every 40 μm . Deviation in particle volume fraction (Φ) on particle migration (sedimentation or creaming) and the mean particle diameter (d) attributable to coalescence resulted in variation in the magnitude of transmitted and back-scattered light. The measured amount of transmitted and backscattered light was then interpreted and used to describe the dispersion state (stability) of the emulsion.

3.3. Results and discussion

3.3.1. Amantadine detection

Potassium permanganate is a strong oxidant with an intense violet colour of λ_{max} 525 nm. The oxidation of organic compounds with potassium permanganate has been found to be pH dependent. During the course of the reaction, the valance of manganese changes and the intermediate ions are suggested to be participating oxidants. The species that are considered as potential oxidants depend on the nature of the substrate and the pH of the medium (Asghar and Fawzy, 2014). In strong acidic medium, potassium permanganate (KMnO_4) produces the Mn^{2+} for a net transfer of five electrons. In neutral or basic media manganese dioxide (MnO_2) is formed with corresponding net transfer of three electrons.

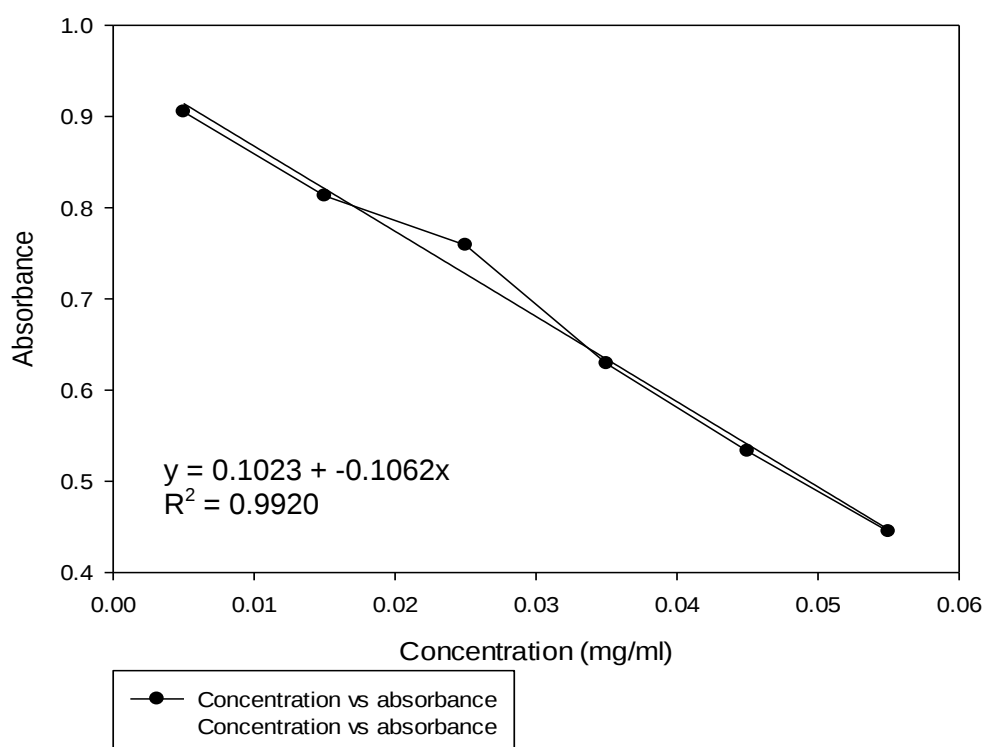


Figure 3.4: Inverse calibration curve for amantadine HCl

Potassium permanganate has an oxidizing effect on amantadine HCl in acidic solutions. This is shown by the decrease in the violet colour of potassium permanganate at 525 nm. This decrease in colour was used as a measure for the concentration of the drug. This is the reason the calibration curve for amantadine HCl is an inverse curve instead of the conventional curve. Amantadine HCl has no absorption capabilities in the region of measurements of potassium permanganate at 525nm.

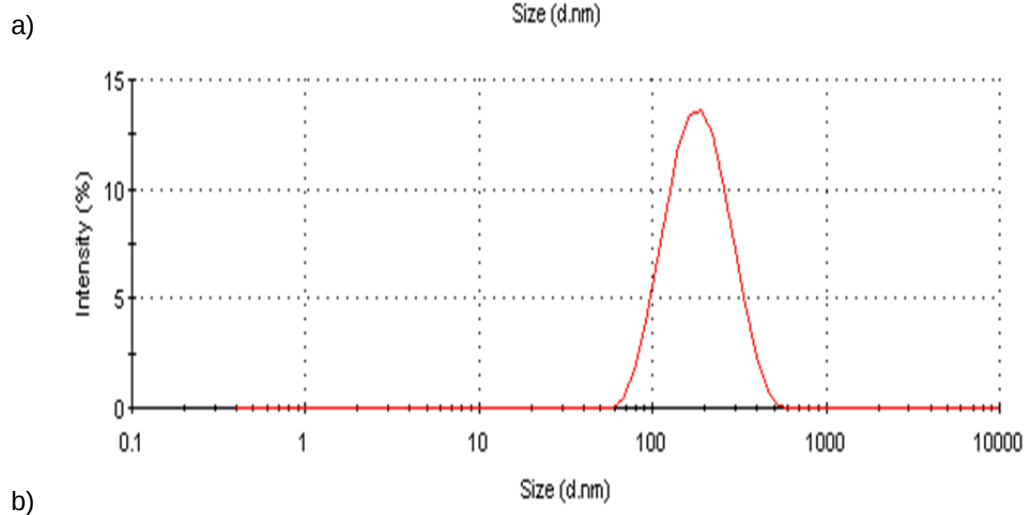
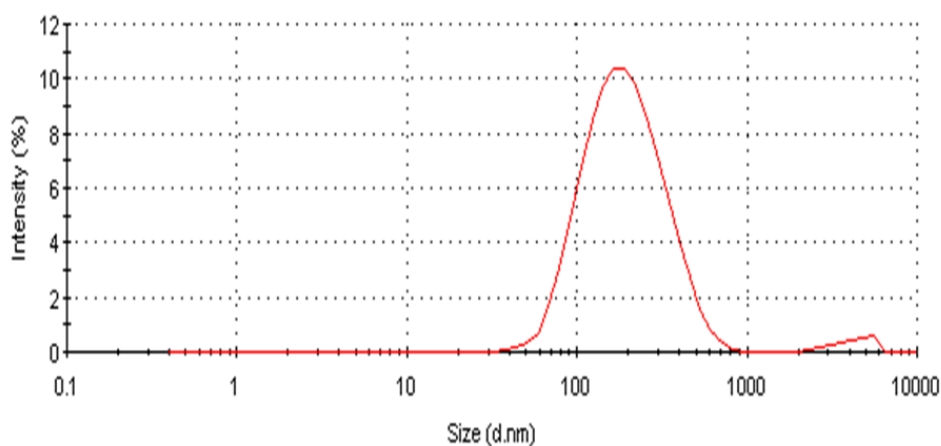
3.3.2. Physicochemical properties of the nanospheres

Physicochemical characterisation of the nanospheres was determined using Dynamic Light Scattering (Zetasizer NanoZS, Malvern Instrument, UK). The average size of the nanospheres formulated with anionic polymethacrylates ranged from 134.1nm to 160.7nm (see table 3.2). However, the two PDLLA-c.PMA combinations yielded average sizes above 300nm (366.8nm and 338.0 for PDLLA-E100 and PDLLA-RSPO respectively). Ideally, any nanoparticles should have sizes below 300nm. The Pdl value of the PDLLA-a.PMA combinations ranged from 0.231 to 0.276 whereas the PDLLA-c.PMA combinations had values much closer to 0.5 (the upper limit for an ideal Pdl value). As mentioned in the introduction of this chapter, in a neutral pH (the organic phase is neutral); PDLLA is able to exist as a polyelectrolyte because it encompasses a carboxyl group. It is thus possible for

PDLLA to form a polyelectrolyte complex when mixed with the charged PMAs. Results show that PDLLA combined with the cationic PMAs in nanosphere preparation resulted in large unstable particles which presented as aggregates during nanosphere formulation. It is possible that unstable polyelectrolyte complexes were formed.

Table 3.2: Physicochemical characterisation of nanospheres

Composite Polymethacrylate	DLS size (nm)	Polydispersity Index (0-1)	Zeta Potential (mV)
PDLLA-L100	160.7	0.276	-24.9
PDLLA-L100-55	150.0	0.231	-25.9
PDLLA-S100	134.1	0.249	-29.2
PDLLA-E100	366.8	0.465	-12.9
PDLLA-RS PO	338.0	0.447	-27.1



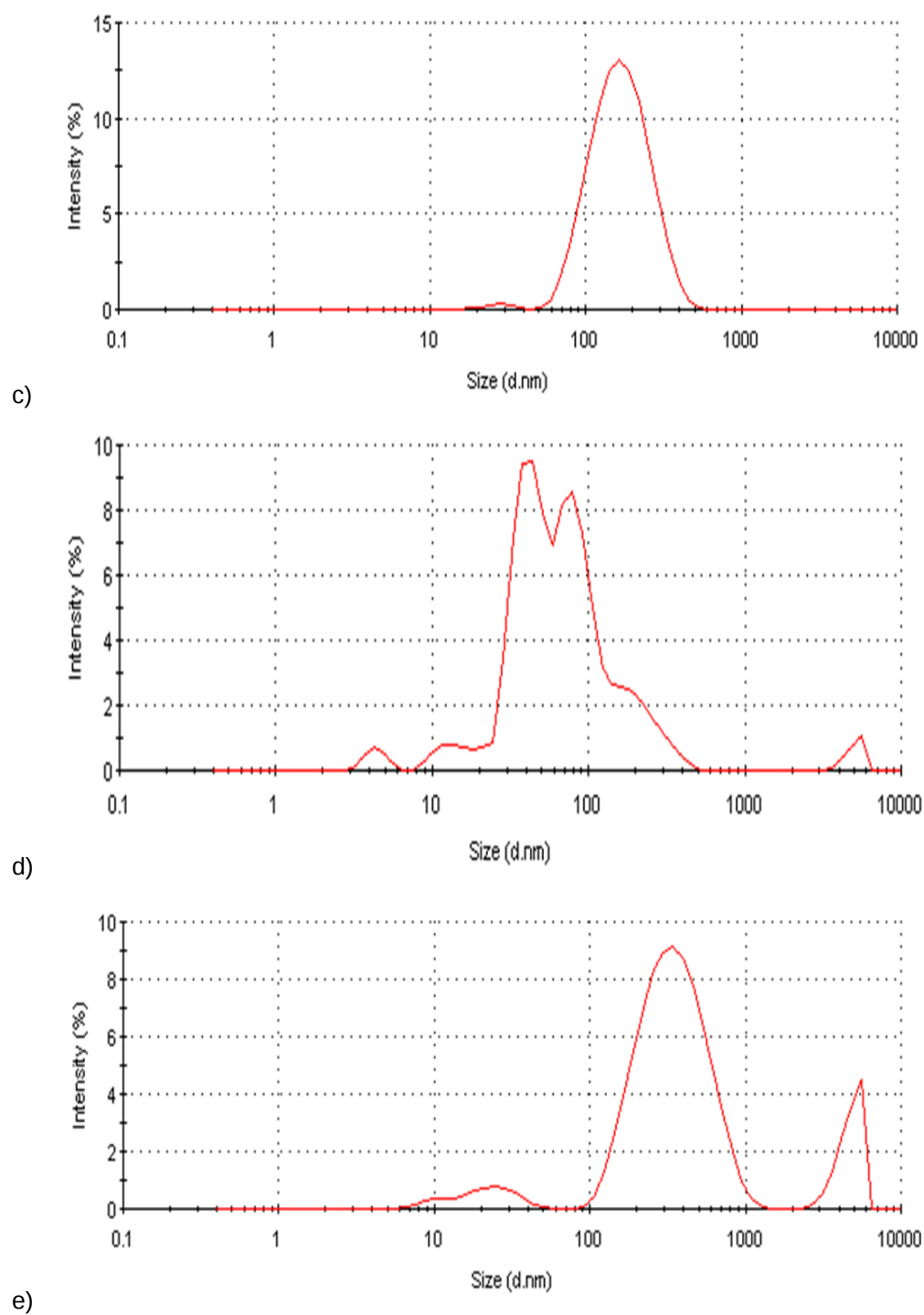


Figure 3.5: Size distributions by intensity showing the more stable PDLLA-a.PMA nanospheres a) PDLLA-L100 nanospheres; b) PDLLA-L100-55; c) PDLLA-S100 and the less stable PDLLA-c.PMA nanospheres d) PDLLA-E100 and e) PDLLA-RSPO

3.3.3. Morphological characterisation of the nanospheres

Distinctive TEM images of the PDLLA-a.PMA nanospheres verified that nanosized and spherical particles were formed. Figure 3.6a-c shows the characteristic images of PDLLA-a.PMA nanospheres. Figure 3.6d and 3.6e show the larger PDLLA-c.PMA nanospheres. The zwitterionic nature of PDLLA resulted in a negative charge on the polymer in aqueous solution (the hydrogen on the carboxyl group gets released and leaves the carboxyl group negatively charged). The addition of cationic polymethacrylates to the PDLLA resulted in the interaction of a positively charged polyelectrolyte (carried by the nitrogen) with a negatively charged polyelectrolyte thus forming unstable polyelectrolyte complexes.

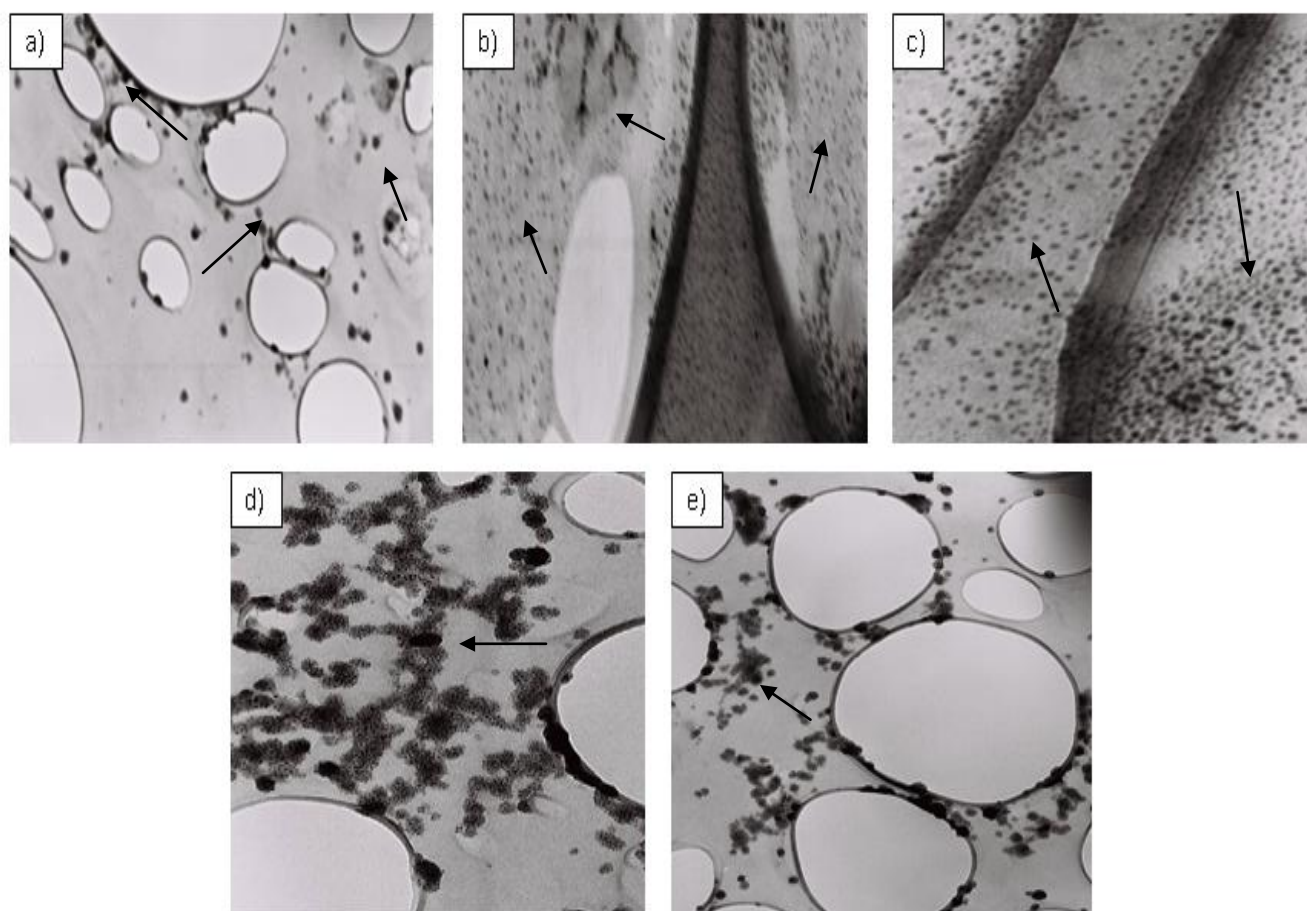


Figure 3.6: TEM micrographs of a) PDLLA-L100; b) S100; c) PDLLA-L100-55; d) PDLLA-E100 and e) PDLLA-RS PO nanospheres. Arrows indicating individual and clusters of nanospheres.

3.3.4. Stability of the nanosphere emulsions

The migration behaviour and aggregation propensity of all the nanosphere formulations were observed using the Turbiscan[®]. Results indicated that the PDLLA-L100, PDLLA-L100-55 and the PDLLA-S100 nanospheres had minimal motion in the suspension and no aggregation (Figure 3.7a-c). However, PDLLA-L100 nanospheres (Figure 3.7b) were the most stable of the PDLLA-a.PMA nanospheres with a small amount of local destabilisation which can be seen by the slight wavering in the delta backscattering signal at the top end of the glass cell. The destabilisation is more prominent in the PDLLA-S100 nanospheres.

Comparably, the PDLLA-E100 as well as the PDLLA-RSPO nanospheres gave off fluctuating backscattering signals throughout the entire length of the borosilicated glass cells (Figure 3.7d-e). This confirms that some of the polymer did not dissolve completely during the formation of the nanospheres and the fluctuating backscattering signals are indicative of free flowing polymer in solution. These results point towards the combination of PDLLA with the anionic polymethacrylates as being the more suitable composite for the formation of these nanospheres. The difference in the formulations is also shown in Figure 3.8 with high levels of aggregation in the PDLLA-c.PMA nanospheres.

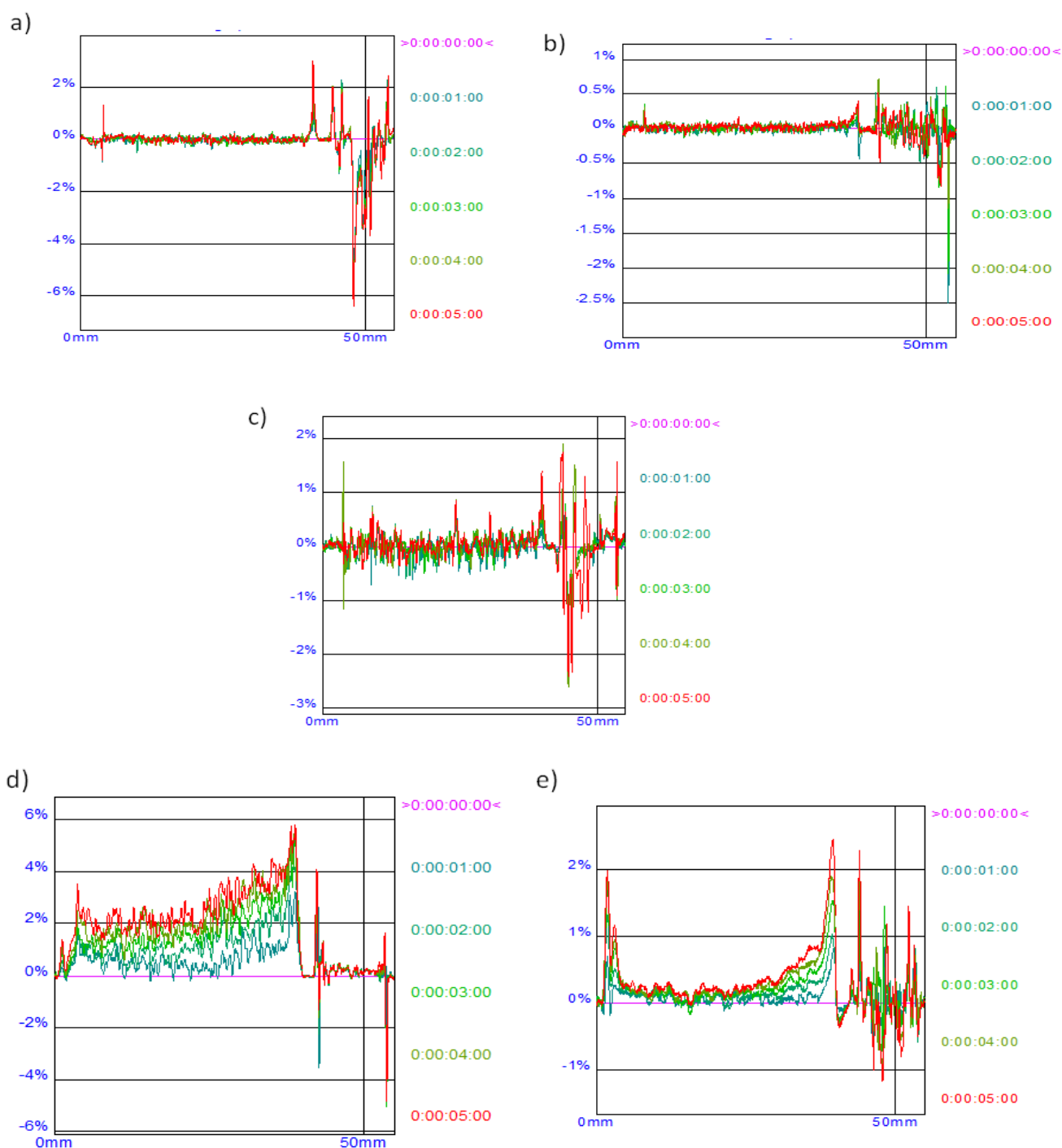


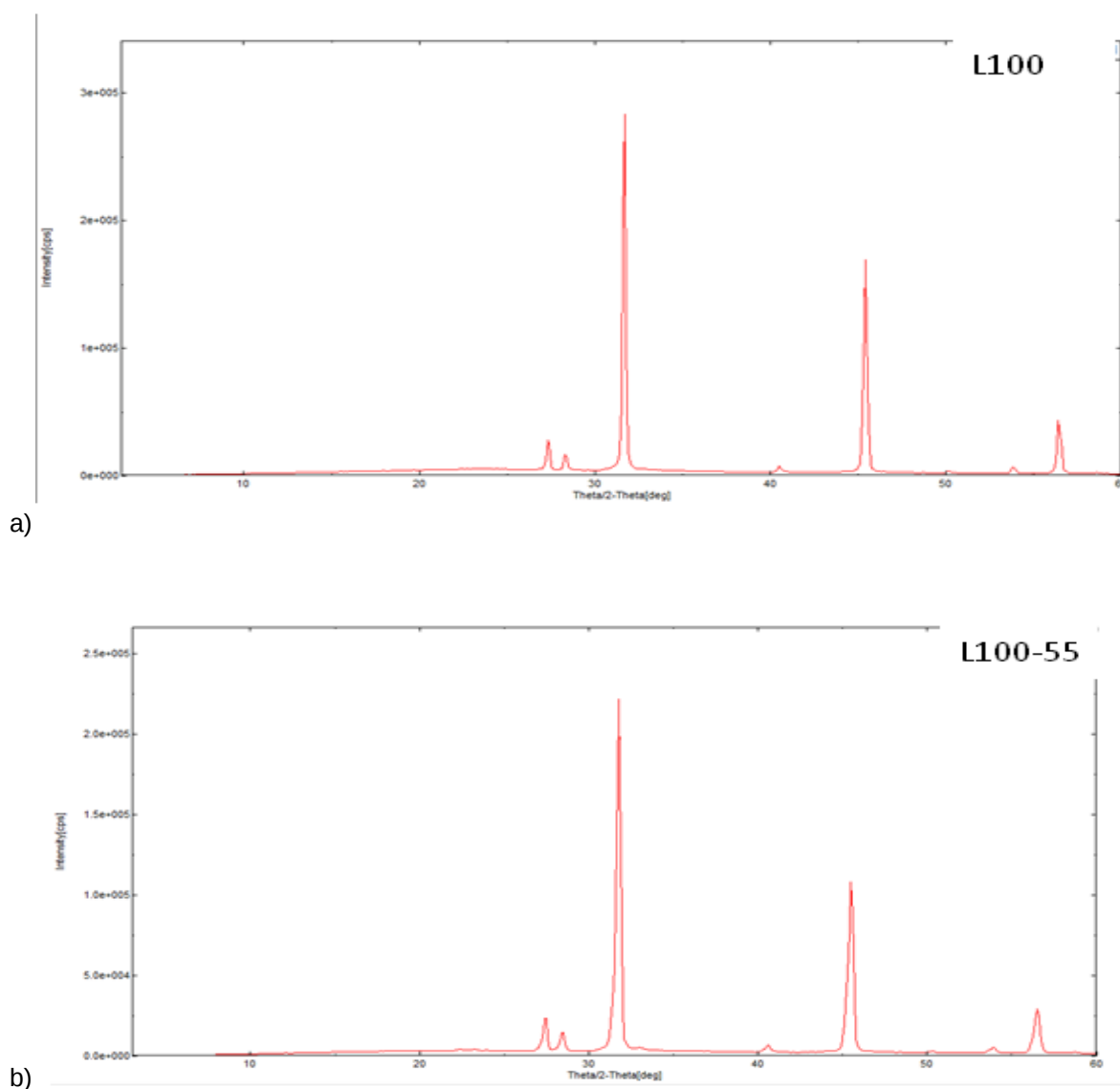
Figure 3.7: Turbiscan graphs of delta backscattering 3.7a – 3.7c anionic eudragits and 3.7d & 3.7e cationic eudragits: 3.7a) PDLLA-L100-55 nanospheres; 3.7b) PDLLA-L100 nanospheres; 3.7c) S100 nanospheres; 3.7d) E100 nanospheres; 3.7e) RS PO nanospheres



Figure 3.8: Different nanosphere formulations in the beakers they were prepared in. Figure 3.8a shows the nanospheres prepared with anionic polymethacrylates. They appear completely homogenous without any residual polymer. Figure 3.8b and 3.8c show nanospheres prepared with cationic polymethacrylates. These had some polymers that did not completely dissolve or aggregated during the formation of the nanospheres. The arrows are pointing to the residual polymers that accumulated around the beakers and fluctuated to the top of the solution.

3.3.5. X-ray diffraction (XRD) of the nanosphere formulations

X-ray diffraction (XRD) analysis of the various nanosphere formulations was performed to determine peak intensity, position and width. Figure 4.10 depicts the XRD diffractograms of; nanospheres prepared with PDLLA and L100, S100, L100-55, RS PO and E100. The diffractograms show similar peaks but at different intensities. The diffractograms also show that the formulations were mostly amorphous as a result of the amorphous polymers used. The two major peaks observed at 32.374° and 46.093° , however, are a contribution from polymethacrylates which have been found to have slight crystallinity. It has also been reported that the sharpness of the peaks indicate that the particles are in the nano-arrangement (Saxena et al., 2012). It is possible, however that the resulting (common) diffractograms are due to the sample holder and not the actual sample.



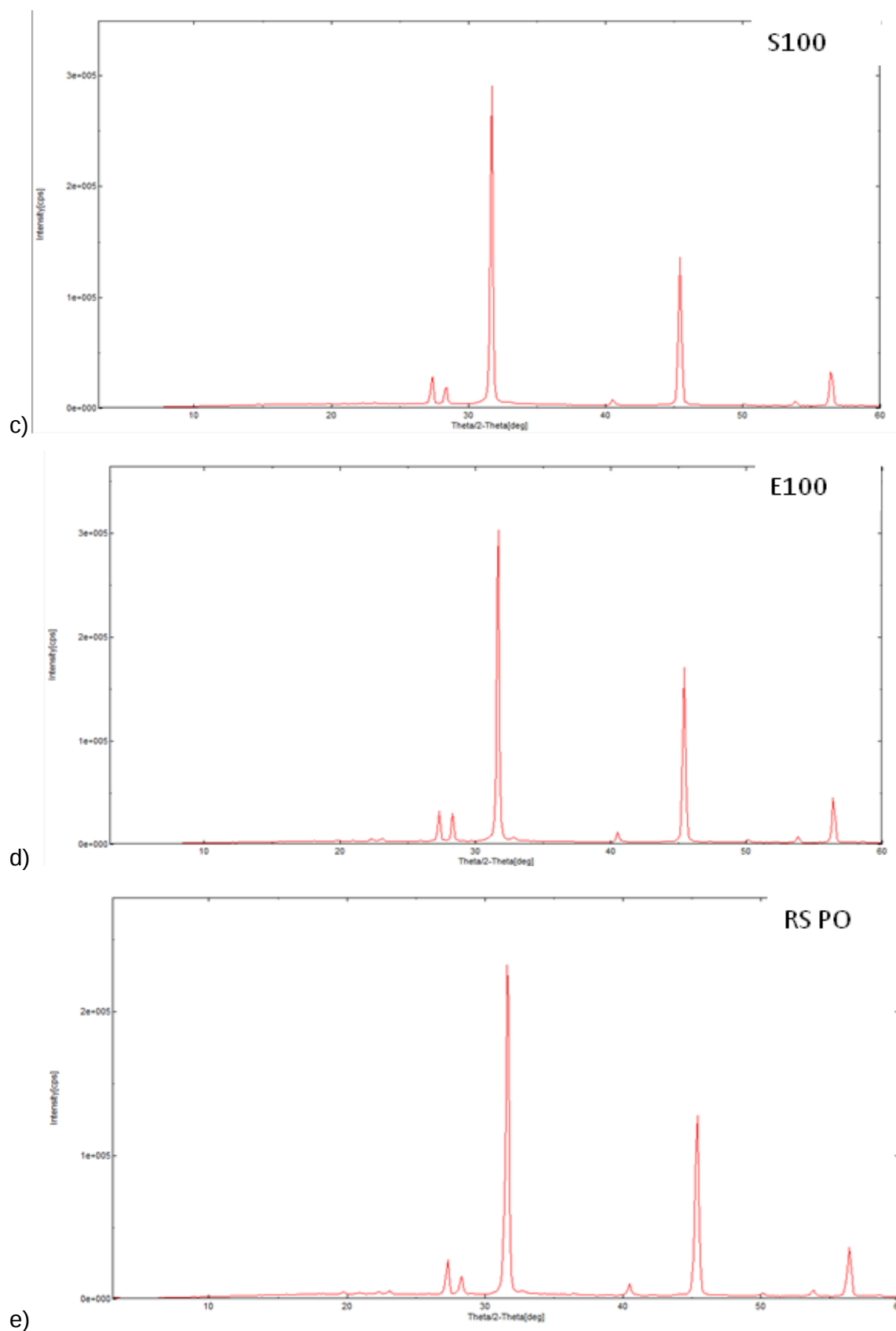


Figure 3.9: XRD pattern of the different nanosphere formulations. They all have similar sharp peaks due to the slight crystallinity found in the polymethacrylates and are indicative of particles in the nano-scale.

3.4. Concluding remarks

Physicochemical characterisation of the various PDLLA-PMA nanospheres was undertaken using Dynamic Light Scattering. Results showed that nanospheres prepared with cationic polymethacrylates were highly unstable with polydispersity indexes that were close to 0.5. Cationic polymethacrylates resulted in nanospheres with sizes over 300nm and unstable size distributions. Conversely, nanospheres prepared with anionic polymethacrylates were stable and had much smaller sizes. The negatively charged nanospheres had polydispersity indexes closer to zero and narrow and stable size distributions.

Additionally, stability studies were performed using the Turbiscan[®] Lab instrument. These studies showed that PDLLA-c.PMA nanospheres produced fluctuating backscattering signals throughout the entire length of the borosilicated glass cells indicating unstable particles most likely to be polyelectrolyte complexes. PDLLA-a.PMA nanospheres, however, were shown to be more stable with PDLLA-L100 showing the most stability of the nanosphere emulsions.

Essentially, the results indicate that the PDLLA combination with anionic polymethacrylates is far more desirable than the combination with cationic polymethacrylates. Anionic polymethacrylates form much weaker bonds with PDLLA which allows for some repelling of the particles and therefore decreased chances for aggregation. On the contrary, cationic polymethacrylates form stronger bonds with PDLLA which leads to a propensity for aggregation. The PDLLA-a.PMA combination, therefore, has the potential of contributing towards the formation of physically stable nanospheres in terms of size, Pdl and zeta potential.

CHAPTER FOUR

FORMULATION AND CHARACTERISATION OF AMANTADINE-LOADED PDLLA-EUDRAGIT® NANOSPHERES

4.1. Introduction

MND is a term describing a rare group of progressive neurodegenerative diseases (Burvill, 2009). It is divided into several classes, such as Amyotrophic Lateral Sclerosis (ALS), Spinal Muscular Atrophy (SMA), Primary Lateral Sclerosis (PLS) and Progressive Bulbar Paralysis (PBA) (Wu, 2005). ALS is the most common form of MND with an incidence of 2 in 100,000 in most populations (Barber et al., 2006). It is caused by degeneration of both upper motor neurons in the motor cortex and lower motor neurons connecting the spinal cord and brain stem to muscle fibers (Barber and Shaw, 2010). The onset of majority of ALS patients typically develops between 40-60 years of age, resulting in eventual paralysis, speech deficits and ultimately death due to respiratory failure within 2–5 years of clinical onset (Julien, 2001).

There are currently many theories concerning ALS pathogenesis, one of them being oxidative stress (Barber and Shaw, 2010). Familial ALS (FALS) is associated with mutations to the copper, zinc dismutase protein (SOD1) which results in a toxic gain of function (Raoul et al., 2005; Kabashi et al., 2010; Saccon et al., 2013; Vanden Broeck et al., 2014). A variety of anomalous oxidative reactions catalyzed by mutant SOD1 have been proposed to contribute to this new toxic function. These commonly presuppose that the conformation of mutant SOD1 is more open than the wild-type protein, allowing substrates other than superoxide to penetrate the active site and react with the copper and zinc ions enclosed inside (Bruijn et al., 1998; Barber et al., 2006). Usually, SOD1 catalyzes dismutation of superoxide radicals to hydrogen peroxide and oxygen in a redox reaction entailing reduction and reoxidisation of the copper ion in the active site of the dimeric protein. On the other hand, many ALS associated SOD1 mutants have been reported to show altered zinc binding, and the geometry of zinc-deficient SOD1 enables reducing agents other than superoxide (for example, ascorbate and glutathione) to react rapidly with the oxidised Cu^{2+} at the active site (Barber et al., 2006). It has been proposed that the reduced SOD1- Cu^+ can produce superoxide in the reverse of its usual dismutase reaction, which reacts with nitric oxide within the active site to produce peroxynitrite, which then causes tyrosine nitration.

A number of therapeutic metal chelators such as ethylenediaminetetraacetic acid (EDTA), histidine residues and zinc acetate have been investigated for the prevention or reversal of A β aggregation in Alzheimer's disease (Liu et al., 2009; Zatta et al., 2009). The aggregation is believed to be caused by a reaction of A β peptide with metal ions in the body (e.g. copper and zinc ions). These chelating ligands are easily taken up by cells and are able to remove unwanted ions. Chelating therapy has been approved by the US FDA for Alzheimer's disease. It is useful to investigate it in ALS as well because of the similar oxidative stress pathogenesis.

Although chelating therapy holds great potential in managing neurodegenerative disorders, factors such as bypassing the blood-brain barrier and toxic side effects remain problematic. Therapeutic agents, such as riluzole, the only FDA approved drug for ALS (Bellingham, 2011), can have an improved bioavailability if carried by a nanoparticulate delivery system.

Nanotechnology is the study of structures and materials at the nanoscale, comprising their fabrication, characterization and application. Natural or synthetic polymers are the most widely used materials for drug delivery and are used in a series of designs and formulations for the application of nano-carriers in pharmaceutical aspirations primarily because of their biodegradable and biocompatible properties. Other materials central to nano-carrier fabrication for drug delivery include macromolecules, lipids and proteins (Xu et al., 2010). Nanoscale drug delivery technologies consist of liposomes, dendrimers, nanotubes, nanomicelles, polymeric nanoparticles, nanospheres and nanoshells (Hughes, 2005).

Nanoparticulate systems are beneficial in improving bioavailability of specific drugs that show a significantly low bioavailability in comparison to nanoscale drug delivery systems. Nanostructures are fabricated employing various techniques which include salting out, polymerisation (emulsion or interfacial) (Reis et al, 2006), coacervation or ionic gelation (Agnihotri *et al*, 2004) solvent displacement and interfacial deposition, emulsification solvent diffusion, supercritical or compressed fluid technology and emulsification solvent evaporation (Reis et al 2006). The emulsification solvent evaporation technique is a relatively simple and efficient way to prepare nanoparticulate systems that involves the dissolving of the drug, followed by the emulsification of the drug in an organic polymer solution. The resulting emulsion is then homogenised in an external aqueous or oil phase. While the organic solvent is eliminated by evaporation, the drug and polymer are precipitated in the droplets, thus forming nanoparticles. This technique can be performed under mild conditions in room temperature and constant stirring, thus resulting in a homogenous emulsion that evades the altering of the drug. A number of formulation dimensions and materials specifications are

implicated in the emulsification solvent evaporation method that influence the measured responses. These parameters include energy employed; aqueous phase volume; polymer and drug concentration in the organic phase, solvent volume and surfactant concentration.

The aim of this study was to design and develop a nanoparticulate delivery system small enough to bypass the blood-brain barrier and one that can enhance cellular uptake and biodistribution of therapeutic agents without presenting cytotoxicity. L100 was the polymethacrylate chosen to prepare and optimise the nanospheres (in combination with PDLLA) as it gave the most desirable results of the anionic polymethacrylates in chapter 3. Amantadine-loaded nanospheres were prepared and bound with the chelating ligand diethylenetriaminepentaacetic acid (DTPA), a polyaminocarboxylic acid (Kontoghiorghes, 1995) that is able to remove several ions including copper and zinc. Various processing parameters on particle size and the characteristics of the nanospheres were investigated. The processing parameters include polymer concentration in the organic phase, volume of solvent and the sonication time of the aqueous and organic phase. The drug used was amantadine, which is an antiviral drug that has anti-parkinsonian effects.

The nanospheres were characterised by utilising techniques such as size and surface analysis; morphology analysis through transmission electron microscopy (TEM); FTIR spectroscopy; and *in vitro* drug release studies.

4.2. Materials and Methods

4.2.1. Materials

Polymethacrylate (PMA): Methacrylic acid copolymer (Eudragit L100) was purchased from Degussa, RohmGmbH, Pharma Polymers (Germany). Poly(D,L-lactide) (PDLLA), Diethylenetriaminepentaacetic acid (DTPA), *N*-Hydroxysuccinimide (NHS), *N,N'*-Dicyclohexylcarbodiimide (DCC) and amantadine hydrochloride were all purchased from Sigma-Aldrich (St Louis, MO, USA). Membrane filters (0.22 μ m) were purchased from Millipore[®] (Billerica, MA, USA). All other chemicals used in the experiments were of analytical grade and were used as purchased.

4.2.2. Box-Behnken design optimisation for the preparation of amantadine-loaded nanospheres

A three-factor, three-level Box-Behnken statistical design on MINITAB[®] (V14, State College, Pennsylvania, USA) was used to optimise the preparation of amantadine-loaded PDLLA-PMA nanospheres. Upper and lower levels of three independent parameters that included;

ultrasonication time, solvent volume and amount of polymer (L100) were selected as they highly influenced the changes in the fabrication of the nanospheres with a constant quantity of PDLLA (40mg). The dependent responses were; particle size, zeta potential, drug entrapment efficiency and mean dissolution time (MDT). Fifteen formulations were generated from the Box-Behnken design (Table 4.1) and were prepared and tested. The results obtained were inserted into the MINITAB[®] design software which produced independent parameter values for the optimised formulation as well as the expected responses (Table 4.2).

Table 4.1: 3-factor Box-Behnken experimental design for PDLLA-PMA nanosphere formulation.

Formulation	Eudragit L100 (mg)	Sonication time (min)	Solvent volume (mL)
1	210	20	15
2	160	20	20
3	210	30	20
4	210	20	15
5	210	20	15
6	260	20	10
7	260	10	15
8	260	20	20
9	160	10	15
10	210	30	10
11	160	20	10
12	210	10	10
13	210	10	20
14	260	30	15
15	160	30	15

Table 4.2: Responses for the Box-Behnken statistical design

Response	Objectives
Particle size (nm)	Minimise
Zeta potential (mV)	Minimise
Drug entrapment efficiency (%)	Maximise
Mean dissolution time (hours)	Minimise

4.2.3. Preparation and optimisation of the amantadine-loaded Eudragit® nanospheres

Nanospheres were prepared using the double-emulsion solvent evaporation technique utilizing sonication. Briefly, the internal aqueous phase was prepared by dissolving 100mg of amantadine in 1mL of deionised water. The organic phase was prepared by dissolving both polymers, PDLLA and Eudragit L100, in a solvent mixture of dichloromethane and isopropyl alcohol in a 1:1 ratio. The internal aqueous phase and organic phase were sonicated for 3min at room temperature to form a primary emulsion. The external phase was prepared by dissolving span80 in phosphate buffer saline (PBS) to form a 0.025%^{V/V} solution. The primary emulsion was then added drop-wise to the external aqueous phase followed by sonication to form nanospheres. The formed nanoemulsion was centrifuged at 1500rpm for 20 minutes at room temperature to recover the nanospheres, washed twice with distilled water and thereafter lyophilized (Lanconco, Kansas City, MS, USA) for 24 hours.

The effects of various processing parameters on particle size, drug entrapment and mean dissolution time were investigated. The processing parameters that had an effect on particle size were polymer concentration, volume of solvent and the time taken to apply an external energy (sonication). A 3-factor Box-Behnken design was employed to generate various amantadine-loaded PDLLA-PMA nanosphere formulations (Table 4.1).

4.2.4. Determination of the stability of the optimised nanosphere emulsion

The stability of the nanosphere emulsion was determined employing a Turbiscan Lab® (Turbiscan Lab™, Formulaction SA, L'Union, France) as described in section 3.2.5. Briefly, Poly-DL-lactide polymethacrylate nanosphere emulsion was poured into a flat bottomed boroscillated glass cell (27.5x70mm) up to a height of approximately 40mm then placed into

the Turbiscan Lab[®] instrument. Synchronous dual measurements were performed by the transmission detector (at 180°) that receives the light, which went through the sample, while the backscattering detector (at 45°) received the light scattered backward by the sample. The light source has pulsed infrared with a wavelength of 880nm. Measurements were performed at 25°C and the Turbiscan Lab[®] was configured to perform scans for 5 minutes, over a 55mm cell length from bottom to top acquiring transmission and backscattering data every 40 µm. Deviation in particle volume fraction on particle migration and the mean particle diameter attributable to coalescence resulted in variation in the magnitude of transmitted and back-scattered light. The measured amount of transmitted and backscattered light was then interpreted and used to describe the dispersion state (stability) of the emulsion.

4.2.5. Determination of particle size distribution, zeta potential and polydispersity index

The average size of the nanospheres was measured by dynamic light scattering (DLS) on a Zetasizer NanoZS instrument (Malvern Instruments, Worcestershire, UK) at 25°C, followed by the analysis of zeta potential and polydispersity index (Pdl). Measurements were performed by firstly filtering the nanosphere suspension through a 0.22µm filter (Millipore, Billerica, USA) to remove any polymer agglomerates and using disposable cuvettes for each run (quartz cuvettes). Each test was performed in triplicate and the average value in each case was reported accordingly.

4.2.6. Analysis of the surface morphology of the nanospheres

Transmission electron microscopy (TEM) was used to examine the shape and size of the nanospheres. Samples were dispersed in PBS (pH 7.4) and a drop of the diluted sample placed on a carbon-coated copper grid and left to dry for 20 minutes. The copper grid was placed in the TEM and viewed at various magnifications at room temperature.

4.2.7. Determination of the amantadine-loading capacity from the nanospheres

In order to determine the drug entrapment efficiency of amantadine-loaded nanospheres, accurately weighed samples of each of the 15 formulations were resuspended in 10ml of phosphate buffer saline (PBS; pH 7.4) (at 37°C) accordingly. The samples were then centrifuged at 2500rpm for 30mins followed by a reaction of the supernatant with potassium permanganate for 15 minutes at room temperature. The absorbances of the resulting solutions were measured at 525nm by UV spectrophotometry (Lambda 25, UV/VIS Spectrometer, PerkinElmer[®], Waltham, MA, USA) against a reagent blank and computed

from a standard linear curve of the drug in PBS to determine amantadine content. Equation 4.1 was used to compute the DEE. All tests were performed in triplicate. The theoretical amount of amantadine was considered as the proportional amount of amantadine in 10mg of nanospheres in reference to the loading dose.

$$\% \text{ DEE} = \frac{D_a}{D_t} \times 100, \quad \text{Equation 4.1.}$$

where % DEE is the percentage of drug entrapped, D_a is the actual drug quantity (mg) measured by UV spectrophotometry and D_t is the theoretical drug (mg) added to the formulation.

4.2.8. *In vitro* drug release studies

In vitro drug release studies were carried out for 72 hours using an orbital shaking incubator set at 25rpm. The amantadine-loaded nanospheres contained in dialysis tubing, were immersed in 50 mL phosphate buffer saline (PBS) at pH 7.4, 37°C in glass jars. At predetermined time intervals 2mL samples of the release media were removed and replaced with fresh buffer of the same volume to maintain sink conditions. It was reported that this method can only be used for the release of formulations with drug release times of greater than 1 hour. Samples were then centrifuged and the supernatant analysed by UV spectroscopy at a maximum wavelength of λ_{525} , after leaving it to react with potassium permanganate reagent. Drug quantity was determined using a standard calibration curve.

Dissolution profiles were produced from the amantadine released over 72 hours and these profiles were used to characterise *in vitro* amantadine release kinetics from the nanospheres. Mean dissolution time (MDT) was used to analyse the ability of the nanospheres to control amantadine release using equation 4.2

$$\text{MDT} = \frac{\sum_{i=0}^n t_j \Delta M_j}{\sum_{i=0}^n \Delta M_j} \quad \text{Equation 4.2.}$$

The dissolution efficiency (DE) of a pharmaceutical dosage form is defined as the area under the dissolution curve up to a certain time, t , expressed as a percentage of the area of the

rectangle described by 100% dissolution in the same time. It is represented in Figure 4.1, and can be calculated by the following equation:

$$DE = \frac{\int_0^t y \times dt}{y_{100} \times t} \times 100\% \quad \text{Equation 4.3.}$$

where y is the drug percentage dissolved at time (t)

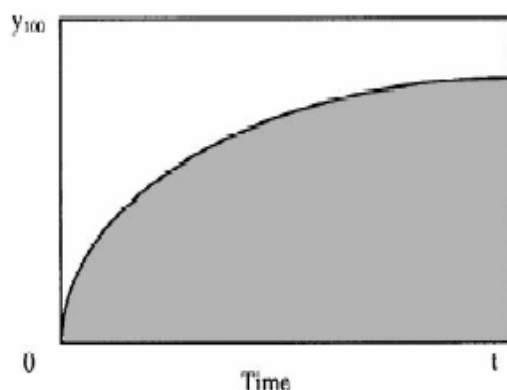


Figure 4.1: Illustration of dissolution efficiency which is defined as the area under the dissolution curve up to a certain time (Costa et al., 2001)

$$DE (\%) = \frac{SA}{R} \times 100 \quad \text{Equation 4.4.}$$

where SA is the shaded area and R is the rectangle area ($y_{100} \times t$).

4.2.9. Analysis of chemical structure of nanospheres

Amantadine-loaded and drug-free nanosphere samples were scanned at high resolution over wavenumbers ranging between 4,000 and 650 cm^{-1} on a Nicolet Impact 400D FTIR Spectrophotometer combined with Omnic FTIR research grade software (Nicolet Instruments Corp., Madison, WI, USA). The samples were compressed into 1x13mm disks using a Beckmann Hydraulic Press (Beckmann Instruments Inc., Fullerton, USA) to prepare them for analysis.

4.2.10. Thermal analysis using differential scanning calorimetry (DSC)

The thermal properties of PDLLA, L100, amantadine and the amantadine-loaded nanospheres were analysed by DSC. The analysis was conducted on a differential scanning calorimeter (Mettler Toledo, DSC1, STARe System, Schwerzenback, Switzerland) which was dually calibrated for temperature and enthalpy using indium and zinc. All experiments were performed at a heating rate of $10^{\circ}\text{C min}^{-1}$ under a dry nitrogen atmosphere (Afrox, Germiston, Gauteng, South Africa) which flowed at a rate of 200mLmin^{-1} acting as the purge gas in order to reduce sample oxidation. Samples were placed in $40\mu\text{L}$ aluminium pans and heated at various temperature ranges.

The samples were then cooled down to -10°C at a rate of $20^{\circ}\text{C min}^{-1}$. The midpoint melting point (T_m) which was used for characterisation was obtained from the melting point depression of the peaks generated on the experimental DSC curves when samples were heated.

4.2.11. Surface modification of the nanospheres using DTPA

Nanospheres were resuspended in 20ml of phosphate buffer saline (PBS; pH 7.4) and DCC was dissolved in 5ml of dichloromethane. The two solutions were mixed under magnetic stirring for 1 hour. The dichloromethane was removed by rotary evaporation (Rotavapor® RII, Büchi Labortechnik AG, Switzerland) maintained at 65°C for 1 hour. Simultaneously, NHS and DTPA were dissolved in PBS (pH 7.4) under magnetic stirring for 3 hours. The two solutions were then combined and stirred for a further 4 hours. The final solution was stabilised at 4°C before it was centrifuged and washed twice to remove unbound DTPA.

4.2.12. Assessment of the conjugation efficiency of DTPA on the nanosphere surface

The evaluation of the conjugation efficiency of DTPA on the surface of nanospheres was done using a UV spectrophotometer. The absorbance of the conjugated nanospheres in solution was read at $\lambda_{\text{max}} = 260\text{nm}$ against a blank sample of unmodified nanospheres and calculated using equation 4.5:

Conjugation efficiency (%) = Actual quantity of DTPA on nanospheres

Theoretical quantity of DTPA employed

Equation 4.5.

4.3. Results and Discussion

4.3.1. Preparation and optimisation of the amantadine-loaded Eudragit® nanospheres

Amantadine-loaded nanosphere formulations were obtained using the different preliminary variables predetermined by the 3-Factor Box-Behnken experimental design (Table 4.1). Organic solvents were chosen according to the solubility of the polymers. The double emulsion evaporation technique was used based on its advanced encapsulation of water soluble drugs in comparison to other incorporation methods (Dhanaraju et al., 2003; Ravi et al., 2008). Double emulsion solvent evaporation involved two steps where the polymer solution containing the drug was emulsified, which determined the size distribution of the nanospheres, followed by the solidifying of the nanospheres through solvent evaporation and polymer precipitation. The addition of the primary emulsion to the external aqueous phase resulted in an instant change of the solution into a turbid mixture, indicative of the spontaneous formation of nanospheres.

The shear stress produced by the sonicator induced the breaking up of the polymer solution into nano-droplets while the addition of the span 80 in the external phase increased the stability of the formulations. Adding the primary emulsion intermittently and drop-wise into the external phase prevented aggregation of the polymers as well as assisted in producing smaller sizes of the nanospheres. Larger sizes were produced when formulations were prepared utilising parameters lying outside the predetermined limits.

The subsequent responses obtained for the various formulations: the nanosphere size, zeta potential, amantadine entrapment and mean dissolution time were employed for the optimisation process. The results obtained from the experimental design (Table 4.3) were inserted into the MINITAB® software to yield ten potential optimised formulations and the selected, most suitable optimised formulation had a composite desirability (D), size desirability, zeta potential desirability and MDT desirability of 1.000. The most optimum parameters were those that displayed a desirability of 1.000 and those that had a desirability closer to 0.000 were considered the least optimum parameters. The optimal formulation had independent parameters; sonication time of 30.0 minutes, Eudragit L100 amount of 260.0 mg and solvent volume of 20.0 mL which produced predicted nanosphere responses of; size of 79.03 nm, zeta potential of -27.24 mV, drug entrapment of 37.28 % and a MDT of 18.27 hours (Figure 4.2). The correlation of the independent parameters; sonication time, solvent volume and amount of Eudragit L100 to the responses; nanosphere size, zeta potential, drug entrapment and MDT was represented as response mesh plots presented in Figure 4.3.

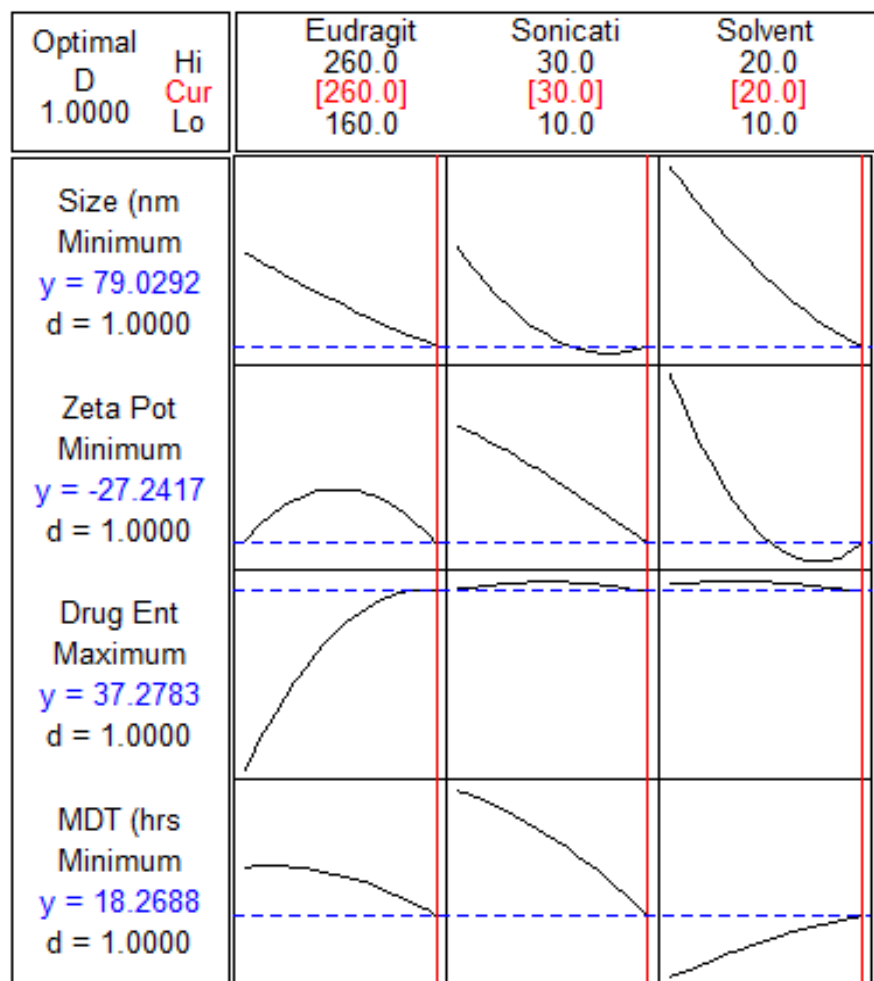


Figure 4.2: Optimisation plots displaying factor levels and desirability values for the optimised amantadine-loaded nanospheres

Table 4.3: Response data obtained for the 3-factor Box-Behnken experimental design PDLLA-PMA nanosphere formulations.

Formulation	Size (nm)	Zeta Potential (mV)	Drug Entrapment (%)	MDT (hrs)
F1	81.94	-24.7	35.84	21.07
F2	104.4	-21.1	19.43	21.34
F3	81.98	-26.4	33.31	20.12
F4	69.08	-23.2	34.89	21.91
F5	88.22	-27	35.48	19.33
F6	99.99	-25.9	39.75	14.07
F7	75.8	-25.9	39.29	22.5
F8	78.4	-22	38.62	22.47
F9	73.78	-27.1	19.45	12.71
F10	101.2	-14.3	34.08	20.4
F11	68.31	-26.5	19.56	19.54
F12	76.32	-22.8	35.53	15.57
F13	138.1	-23.7	32.97	21.79
F14	112.8	-27.9	38.13	18.1
F15	113.6	-29.2	19.48	21.94

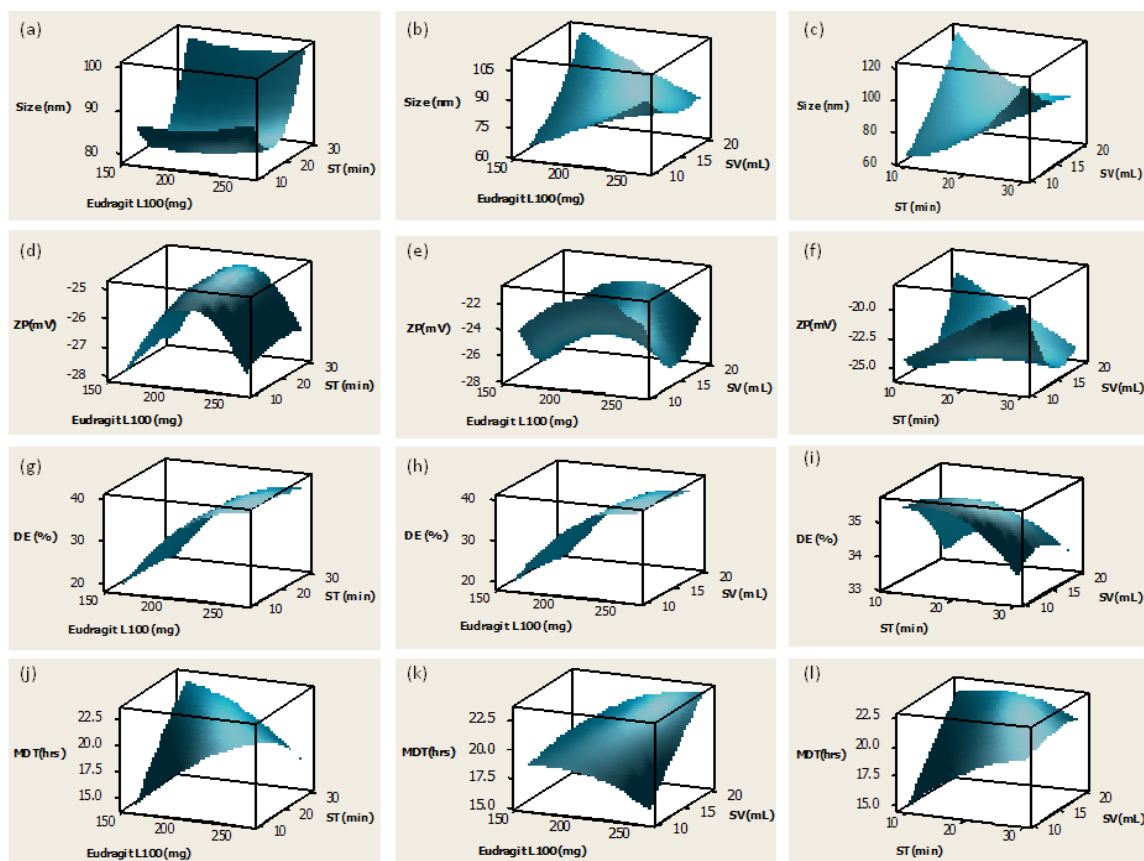


Figure 4.3: Response mesh plots correlating dependent formulation parameters; nanosphere size, zeta potential (ZP), drug entrapment (DE) and MDT to their independent parameters; sonication time (ST), solvent volume (SV) and amount of Eudragit® L100.

4.3.2. Effects of Formulation Variables on Measured Parameters

Nanosphere size has an effect on drug loading, drug release, and ultimately targeted delivery of amantadine across the BBB. Prepared formulations produced sizes of between 68 and 113nm (Figure 4.4) which is small enough for cellular uptake considering vesicles between 100-150nm can be endocytosed at the site of action (Kim et al., 2007). The formulations displayed poly dispersity index (Pdl) values of less than 0.5 which was an indication of a uniform nanosphere size distribution. The zeta potential values ranged from -14.3mV to -29.2mV (Table 4.4). The increased zeta potential values indicate the high stability of the nanospheres by electrostatic repulsion forces and the net negative charge is as a result of the anionic PMA. The experimental design showed moderate entrapment efficiency of the nanospheres ranging from 19.43 to 39.75%. A large quantity of amantadine was still in solution after phase separation.

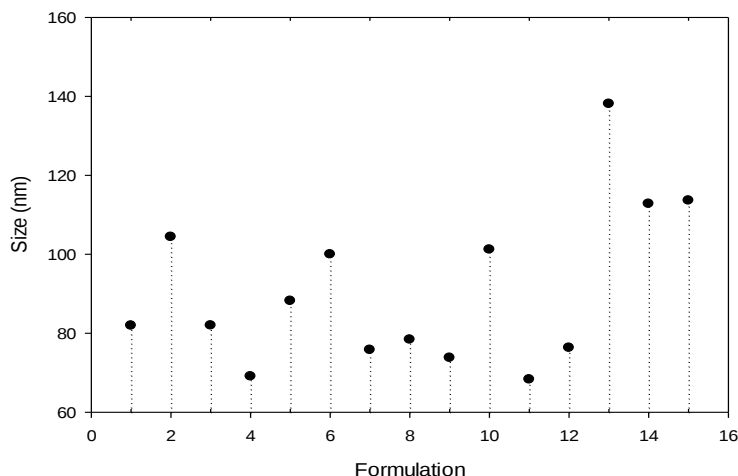


Figure 4.4: Size plot depicting the sizes of various PDLLA/PMA nanosphere formulations

The assessment of the residual error is crucial in determining the effectiveness of an experimental design. Various residual plots were used for the evaluation of the design. It must be taken into consideration, however, that a small sample size (less than 50) was used. In such a case a histogram is not regarded as the best selection in the assessment of normality however, normal probability plot has a much higher level of sensitivity. Figure 4.5 shows the different residual plots for the dependent formulation parameters. All the parameters produced points that roughly form a straight line in their normal probability plots i.e. all points are in close proximity to the straight line, signifying a normal distribution of residuals. Even distribution on either side of the zero line is apparent in the fitted residual versus fitted value plots. A bell-shaped histogram indicates normal distribution of data. None of the responses produced bell-shaped histograms which may suggest the presence of data points with large residuals. In the residuals versus the order of the data, there is regular oscillation on either side of the centre line representative of error terms that are not related with one another and are therefore independent.

Dissolution studies indicated a controlled release profile of amantadine from the formulations. Minimal drug was released at $t = 0.5$ for all 15 formulations (Figure 4.6). This initial release could be attributed to drug that has been adsorbed on the surface of the nanospheres followed by the release of the drug entrapped in the core of the nanospheres. After 24 hours about 50% of the drug was released from the nanospheres and the graphs show that there was prolonged drug release for over 72 hours.

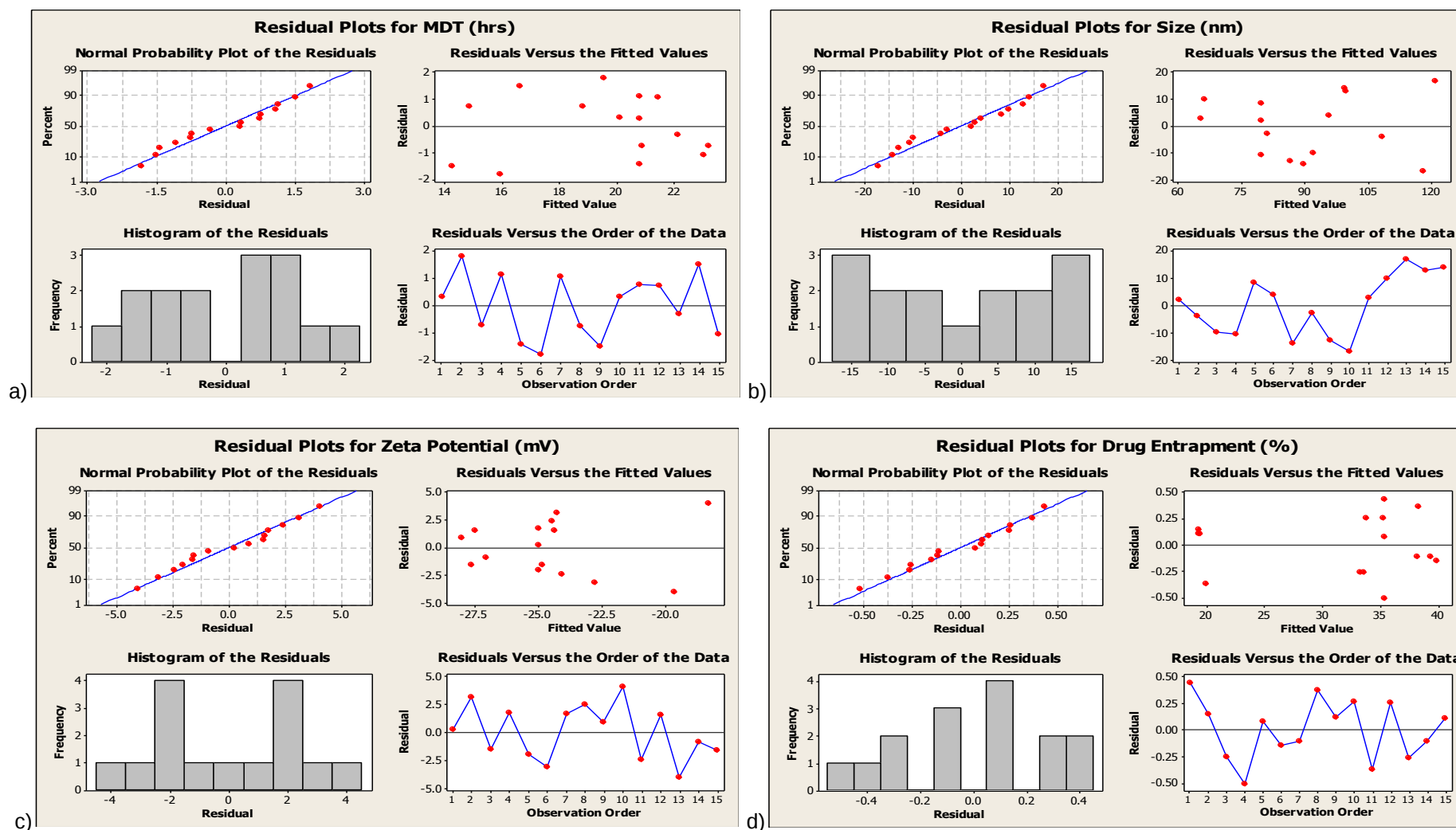


Figure 4.5: Residual plots of the data for MDT, particle size, zeta potential and drug entrapment for the nanosphere formulation

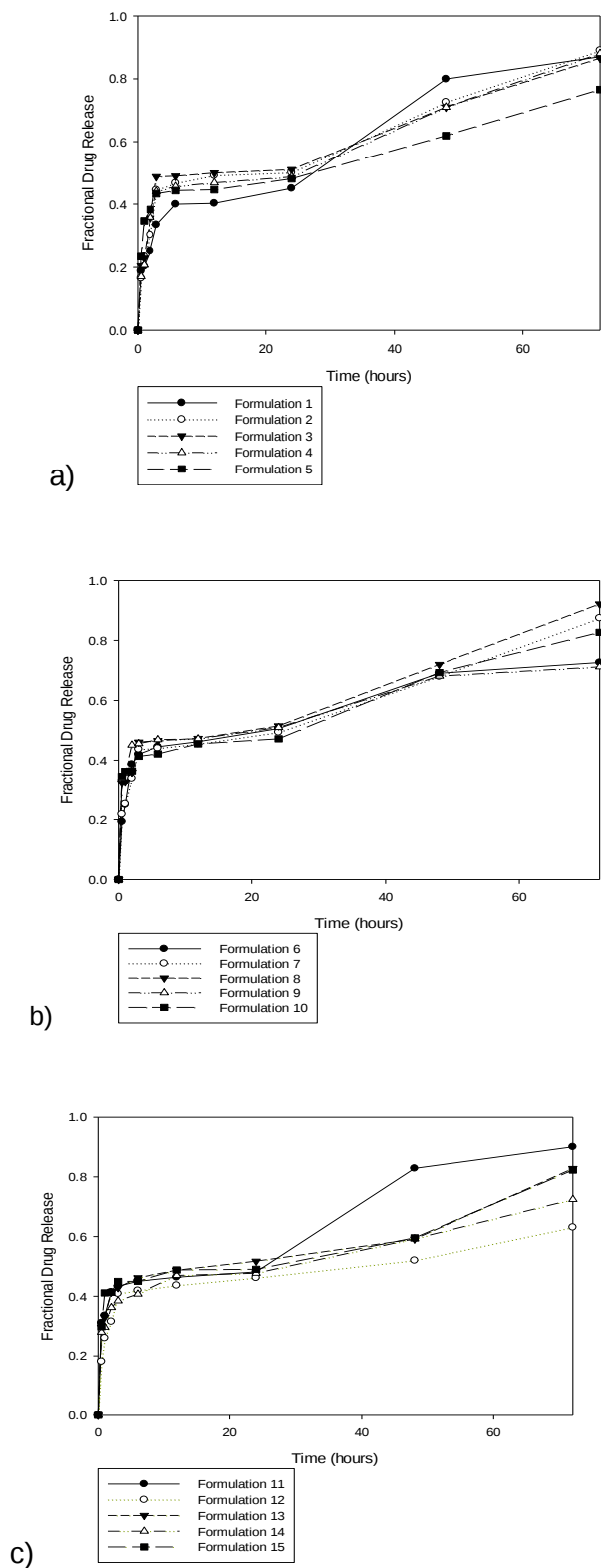


Figure 4.6: Drug release profiles of the 15 formulations in the experimental design (Standard Deviations (SD): ± 0.2693 ; ± 0.2622 ; ± 0.2512 ; ± 0.2566 ; ± 0.2060 ; ± 0.2195 ; ± 0.2434 ; ± 0.2454 ; ± 0.1971 ; ± 0.2185 ; ± 0.2545 ; ± 0.1809 ; ± 0.2132 ; ± 0.1944 ; ± 0.2085 respectively)

4.3.3. Optimised formulation

The optimised formulation had an average zeta size of 80.41nm (Figure 4.7), zeta potential of -27.7mV, drug entrapment of 50.92% and a Mean Dissolution Time (MDT) of 19.3 hours. All these values were exceedingly close to the values predicted (see Figure 4.2). This shows that the box-behnken design is excellent at predicting responses. Drug entrapment, however, had a noticeable improvement to the predicted value.

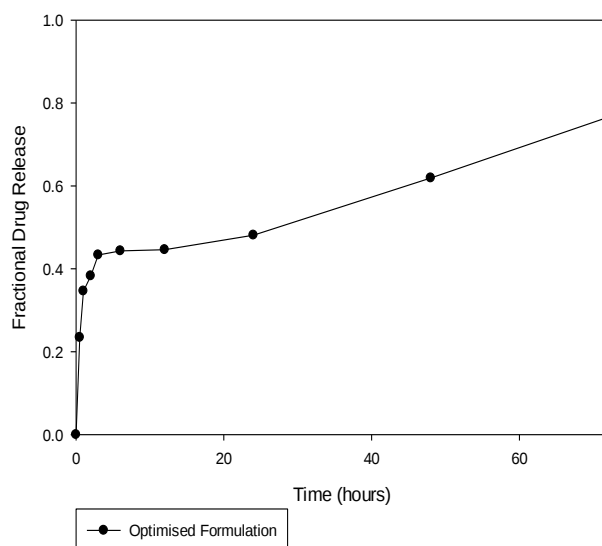


Figure 4.7: Drug release profile of the optimised nanosphere formulation (SD ± 0.206)

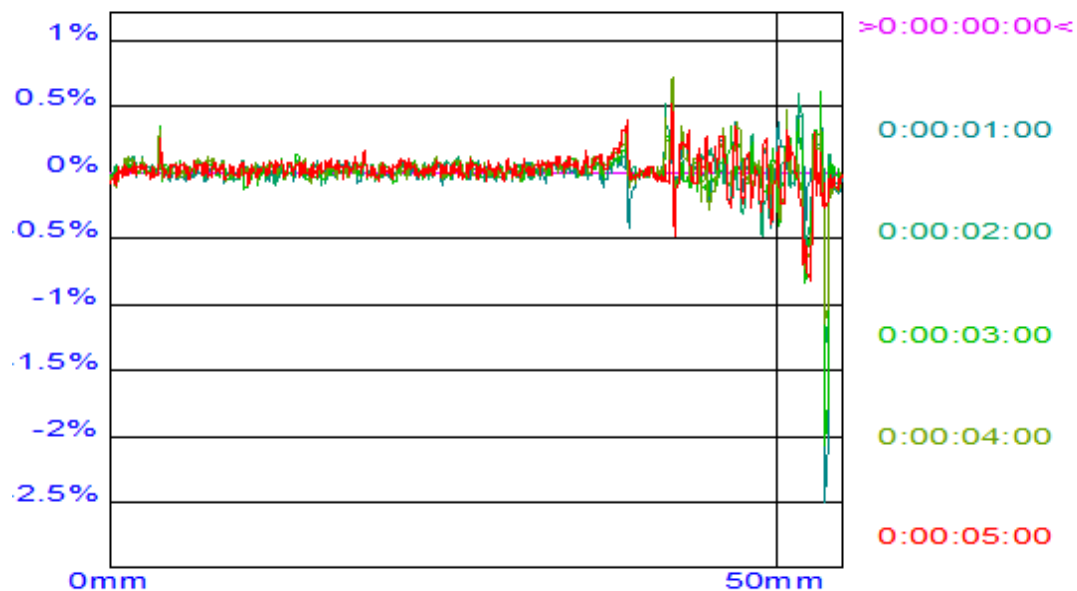
4.3.4. Nanosphere emulsion stability

The delta back scattering profile of the optimised nanosphere emulsion depicted in Figure 4.8a shows that the nanosphere emulsion was largely stable. The middle and the bottom of the profile remained consistent which meant that the emulsion was stable over the test duration and no flocculation or coalescence occurred to the nanosphere emulsion during the entire time the experiment took place. There was, however, a small amount of local destabilisation which can be seen by the slight wavering in the delta backscattering signal at the top end of the glass cell.

Figure 4.8b shows that between 32.4mm and 48.1mm (a section in the middle of the profile) backscattering in percentages versus time remained steady and further confirms that the nanosphere emulsion was stable. Figure 4.9 depicts the phase thickness of the clear phase

from middle to the beginning of the top profile. The emulsion slightly increases in thickness between 36.7mm and 52.5mm showing the minor destabilisation at the top profile.

a)



b)

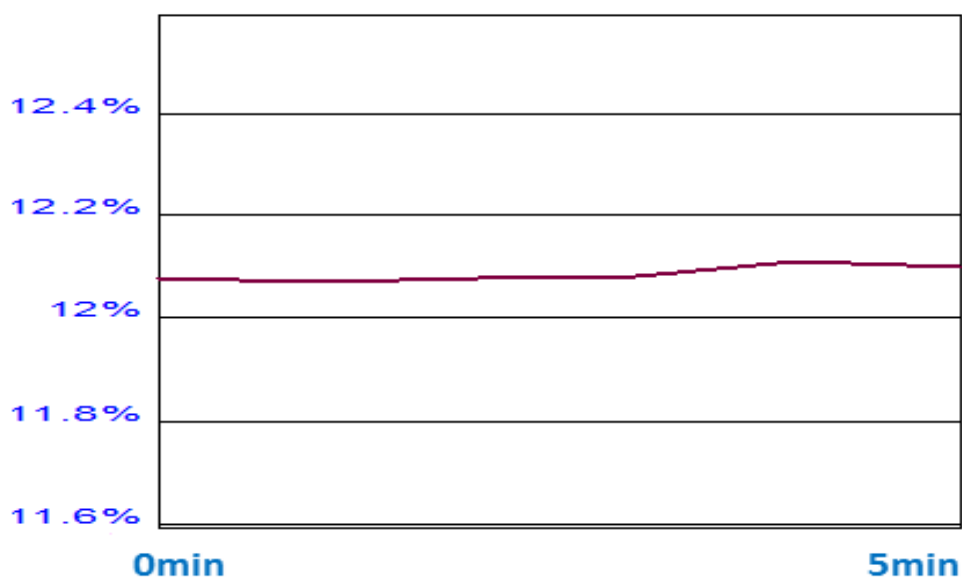


Figure 4.8: a) Delta backscattering (DeltaBS) for optimised amantadine-loaded PDLLA-L100 nanospheres; b) Backscattering presented in percentage versus time: BS (t), at the following section on the cell: 32.4mm – 48.1mm

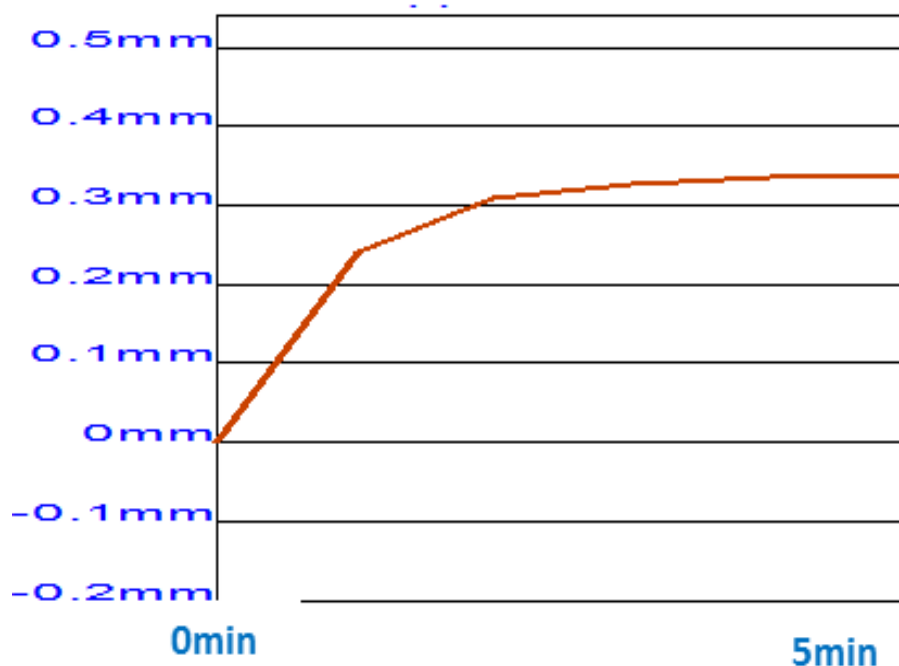


Figure 4.9: Delta H (t), which is the phase thickness from transmission (36.7mm – 52.5mm)

4.3.5. Differential scanning calorimetry of the drug and nanosphere components

DSC thermograms of L100, PDLLA, amantadine and amantadine-loaded PDLLA-L100 nanospheres are depicted in figure 4.10. L100 had an onset melting point at 196.66°C and an exothermic crystallization peak at 214.42°C (Figure 4.10a). PDLLA had a melting point represented by an endothermic peak maximum at 54.02°C as well as an exothermic crystallization peak at 58.18°C (Figure 4.10b). Amantadine, the study drug, had an endothermic peak with a melting point peak maximum at 320.53°C and an exothermic crystallization peak at 323.44°C (Figure 4.10c). The thermal DSC thermogram of amantadine-loaded PDLLA-L100 nanospheres (Figure 4.10d) confirmed that L100, PDLLA and amantadine were all present and contributed to the composition of the nanospheres.

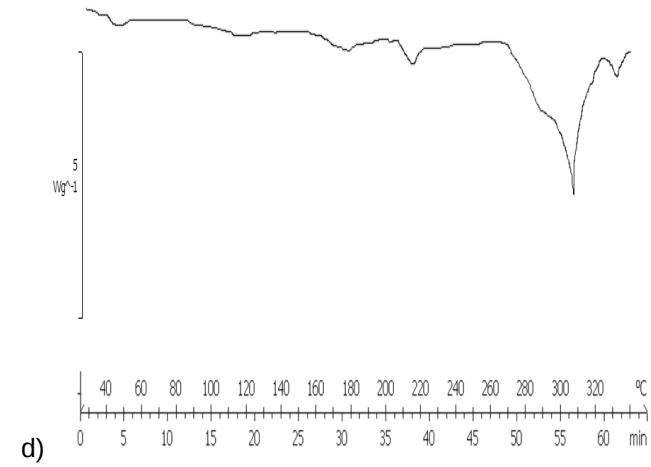
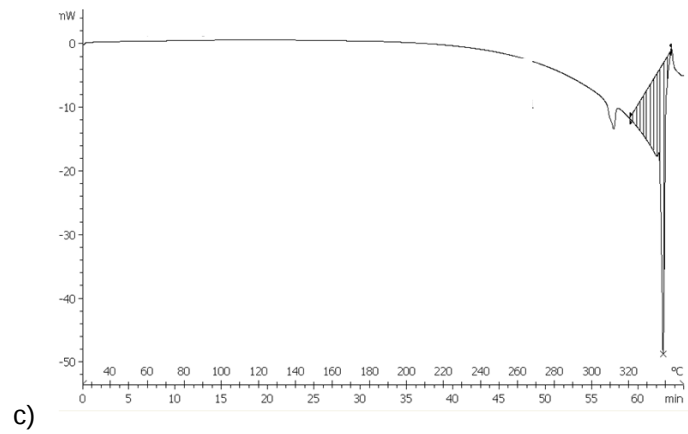
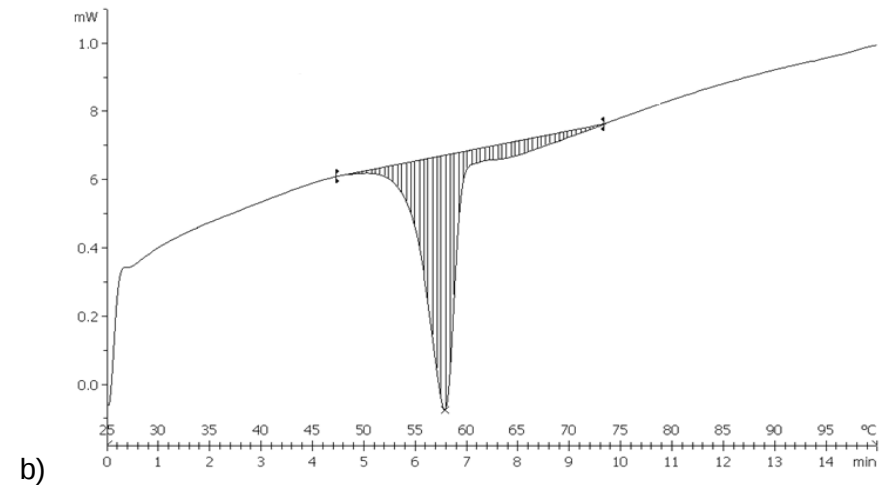
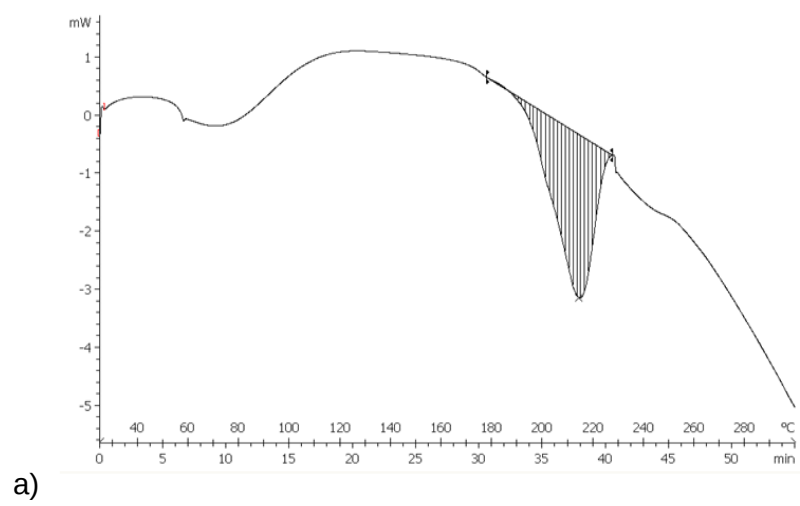


Figure 4.10: DSC thermograms of a) L100; b) PDLLA; c) amantadine and d) amantadine-loaded PDLLA-L100 nanosphere

4.3.6. Conjugation Efficiency of DTPA on the Surface of the Nanospheres

The conjugation efficiency of the modified DTPA-bound nanospheres was determined using a NanoPhotometer™ spectrophotometer (Implen GmbH, Munich, Germany). Results showed a conjugation efficiency of 74%. This outcome shows that the method of conjugation is highly proficient and thus the modified DTPA-bound nanospheres are suitable for enhancing delivery of therapeutics into brain cells.

The DTPA-bound nanospheres had a size of 105.6nm (Figure 4.11b), a Pdl of 0.240 and zeta potential of -31.0mV. The results indicated that the DTPA-bound nanospheres provided a slight increase in the net negative charge for the zeta potential value and a comparative increase in particle size. Accordingly, the zeta potential of the modified nanospheres provided certainty that the synthesised nanospheres were physically stable.

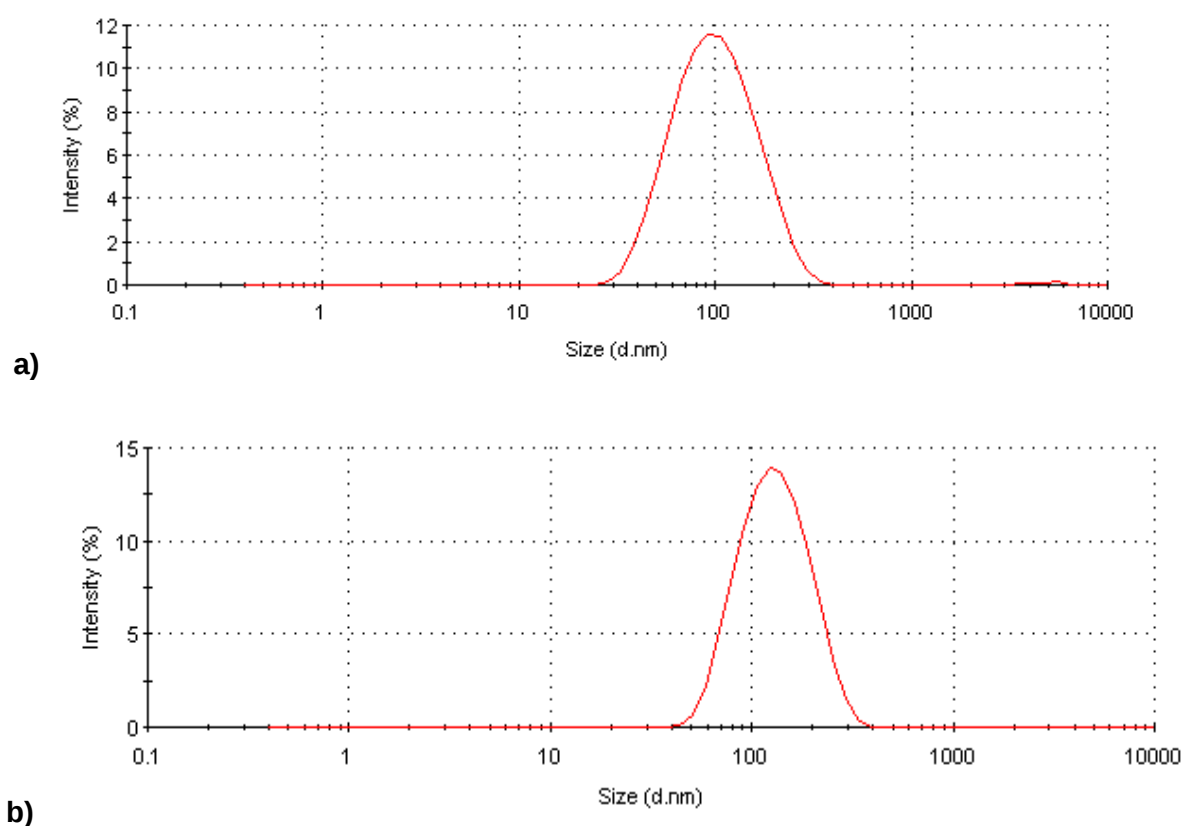


Figure 4.11: Comparison of the size distributions of a) the unmodified optimised nanospheres and b) DTPA-bound optimised nanospheres

4.3.7. Morphological Characterisation of the Modified Nanospheres

TEM micrographs of modified DTPA-bound nanospheres are shown in Figure 4.12. TEM images revealed spherical and uniform unmodified nanospheres and modified nanospheres. In addition, the images show that the unmodified nanospheres and modified nanospheres synthesized were of a nano size range. DTPA was conjugated onto the nanospheres and was incorporated in the surface morphology.

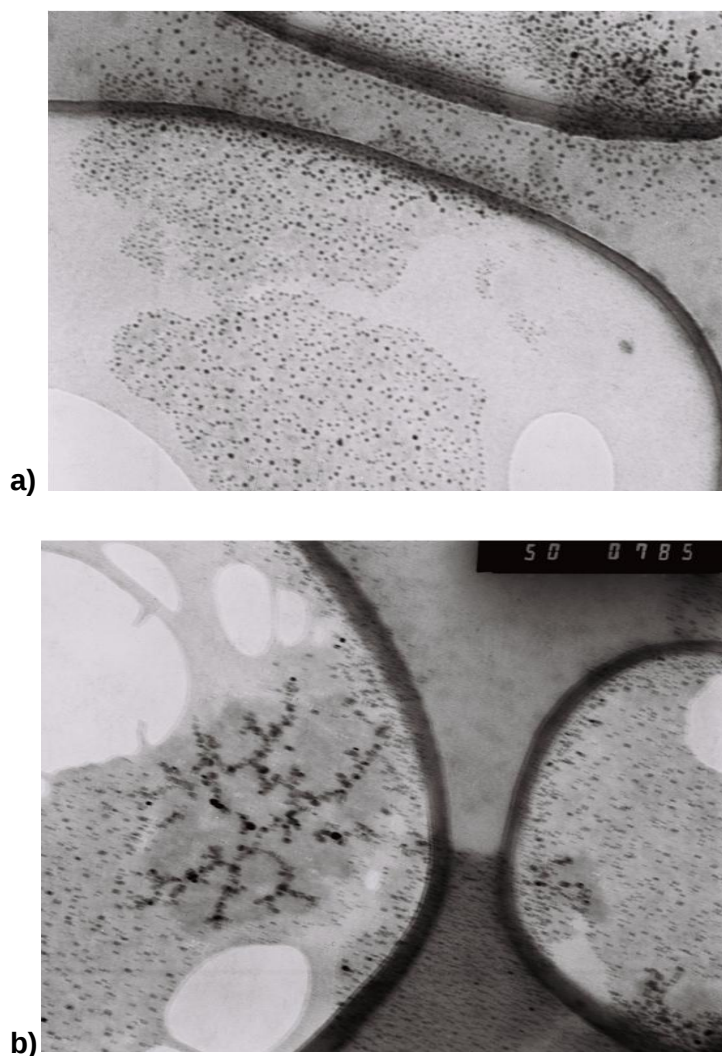


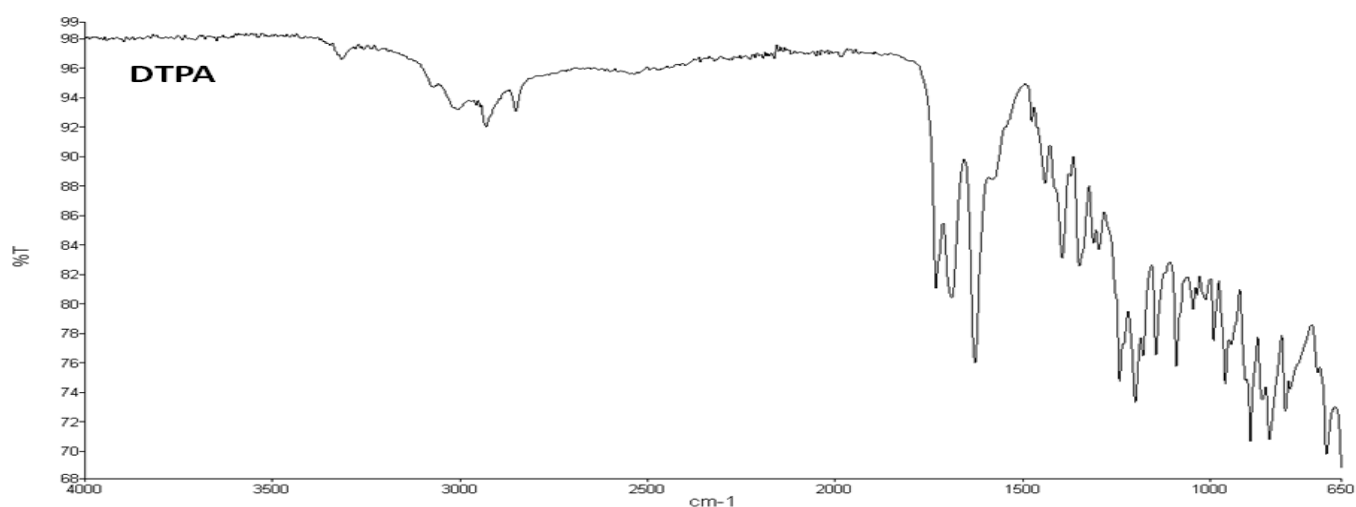
Figure 4.12: TEM images of the a) unmodified optimised nanospheres and b) DTPA-bound nanospheres

4.3.8. Assessment of the Modified Nanosphere Chemical Structure Variations

The qualitative features of infrared spectroscopy are one of the most powerful aspects of this varied and resourceful analytical technique. FTIR spectra were generated to characterize the

potential interactions of the nanospheres and DTPA. As clearly depicted in Figure 4.13, FTIR spectroscopy confirmed that there were molecular structural changes in the modified nanospheres compared to the unmodified nanospheres. The FTIR spectra of modified nanospheres displayed a characteristic bond formation at a wavenumber of 3321.09 cm^{-1} which was assigned to --OH and --NH stretch due to the presence of the DTPA ligand. This peak was absent in FTIR spectra of the unmodified nanospheres. Results showed that there were interactions between the nanospheres and DTPA, which culminated in the formation of the new modified nanospheres.

a)



b)

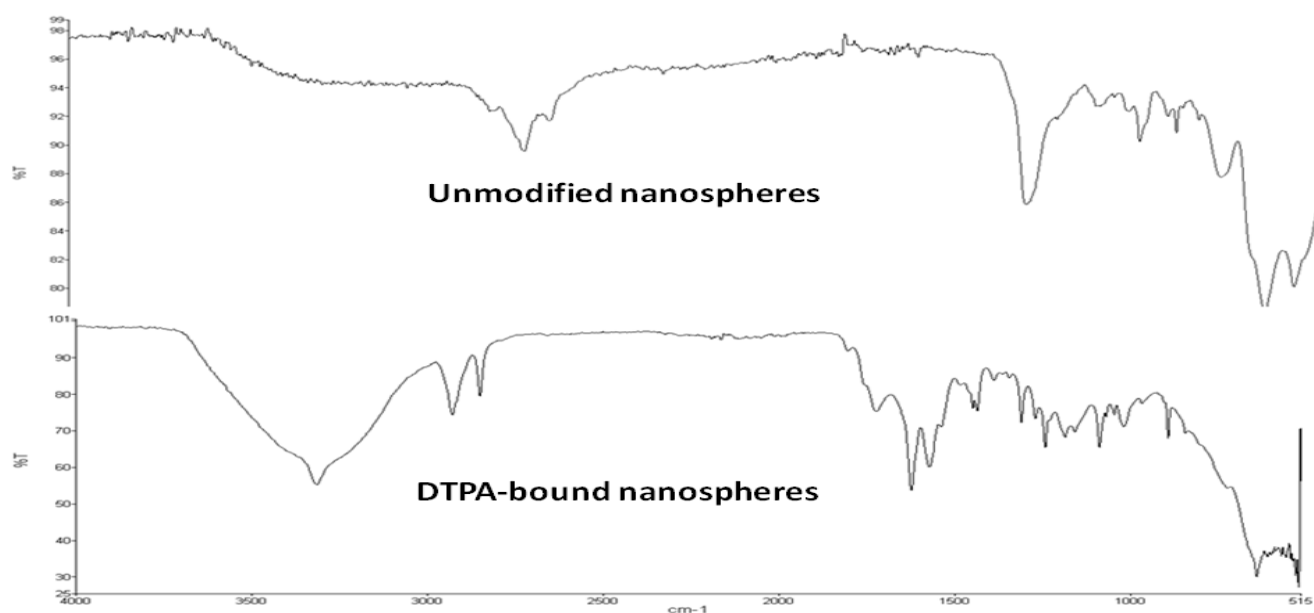


Figure 4.13: FTIR spectra of (a) native DTPA and (b) unmodified nanospheres and modified DTPA-bound nanospheres

4.4. Concluding remarks

Amantadine-loaded DTPA-bound PDLLA-L100 were successfully designed, characterised and optimised employing the randomised Box-Behnken statistical design. Desirability plots were used to elucidate the efficacy of the design. The prepared nanospheres had response values very close to those of the predicted values with a noticeable improvement in the drug entrapment efficiency. The double-emulsion solvent evaporation method was used which produced small, spherical and stable nanospheres. Dynamic light scattering, TEM and the backscattering profiles from the Turbiscan Lab[®] were used to confirm these results respectively. Dissolution studies implemented in PBS (pH 7.4) showed that minimal drug was released in the first 30 minutes of the study. Thereafter, there was prolonged drug release for over 72 hours. The nanospheres were also successfully conjugated to the chelating ligand DTPA with a conjugation efficiency of 74% and this was observed on FTIR spectra.

Essentially, biocompatible nanospheres which are able to deliver amantadine over 72 hours, are small enough to be internalised by cells and are modified with a chelating ligand were effectively formulated with the aim to employ them in ALS. These nanospheres would benefit from being embedded in an implant that would further prolong the release of amantadine and ensure that the site of action is sufficiently exposed to the drug with vastly decreased dosing. Further studies incorporating these amantadine-loaded DTPA-bound nanospheres into a biocompatible thermosensitive hydrogel are presented in Chapter 5. The hydrogel is to serve as a carrier for the nanospheres and further control the release of the drug.

CHAPTER FIVE

FORMULATION AND *IN VITRO* TESTING OF A THERMOSENSITIVE HYDROGEL AS A CARRIER FOR THE AMANTADINE-LOADED LIGAND-BOUND NANOSPHERES

5.1. Introduction

Implantable systems are valuable in biomedical applications such as regional tissue engineering for mechanical support as well as drug and cell delivery functions (Figure 5.1). These implants are beneficial in keeping patients compliant in their treatments. For example, in the treatment of patients with a mental illness, where cognitive impairment may affect compliance or in tuberculosis where a number of pills have to be taken over a long period, implants are advantageous. Implants that are designed to gradually release therapeutic agents over several weeks or months reduce the number of times patients must visit the hospital or the frequency medication needs to be administered.

Therapeutically, implants could achieve increased levels of remedial agents at the implantation site. Specifically, injectable thermally responsive polymers with a lower critical solution temperature (LCST) below body temperature offer promising biomaterials for a variety of applications in the human body (Ma et al., 2010). Thermosensitive polymers are characterised by the presence of hydrophobic groups, such as methyl, ethyl, and propyl groups (Satish et al., 2006). Commonly, the LCST-based phase transition in these polymers occurs upon increased temperature *in situ* as a result of dehydration of polymer components, leading to the disintegration of polymers (Aseyev et al., 2010). Above LCST, hydrogen bonds (between amide groups in polymer and water molecules) are broken and water molecules are expelled from the polymer, resulting in precipitation of the polymer (Aseyev et al., 2010). A range of naturally derived and synthetic polymers displaying this characteristic and others have been employed and some can be seen in Table 5.1. Natural polymers consist of elastin-like peptides and polysaccharide derivatives, while notable synthetic polymers include those based on poly(Nisopropylacrylamide) (PNIPAAm), and amphiphilic block copolymers, often containing poly(ethylene glycol) (Schmaljohann, 2006). Not only are these thermoresponsive polymers used for implants or gels, but for various other delivery systems prepared in a variety of ways, achieving solidification *in situ* through mechanisms depending on the delivery system (Table 5.2). These delivery systems have distinctive challenges related to their development that are linked to drug stability, drug

release kinetics and the environment under which the system is delivered to the body (Hatefi and Amsden, 2002).

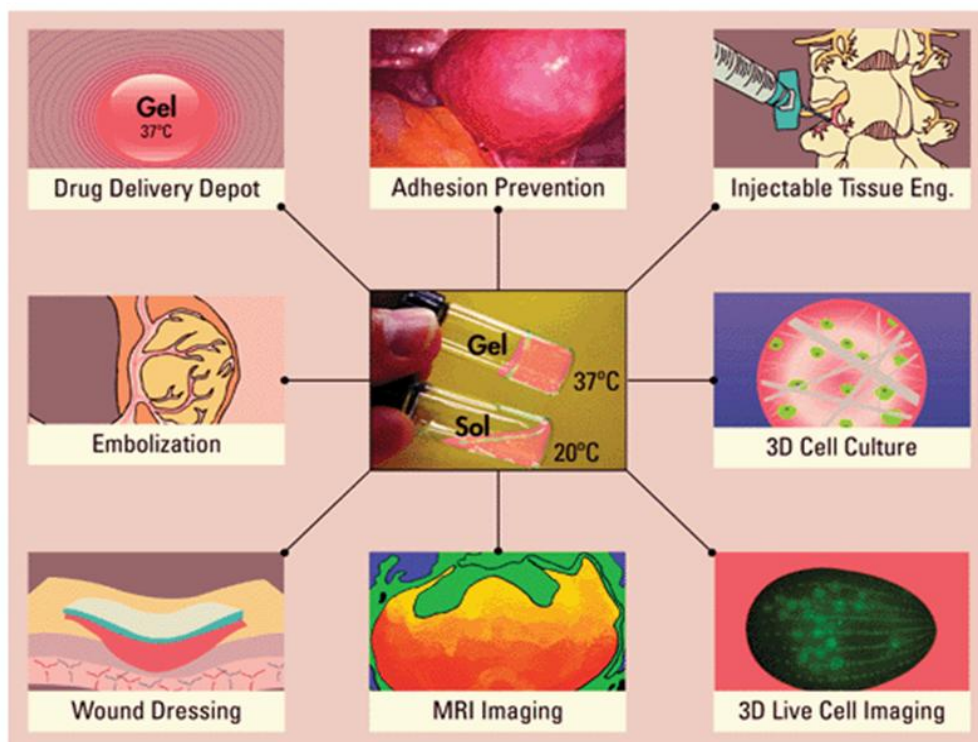


Figure 5.1: Uses of *in situ* forming thermally responsive hydrogels (Moon et al., 2012) (<http://pubs.rsc.org/en/content/articlelanding/2012/cs/c2cs35078e#!divAbstract>)

Table 5.1: Common natural polymers and synthetic monomers employed in hydrogel preparation (Ganji and Vasheghani-Farahani, 2009)

Natural polymers	Synthetic monomers/ polymers
Chitosan	Hydroxyethylmethacrylate (HEMA)
Alginate	N-(2-Hydroxy propyl)methacrylate (HPMA)
Fibrin	N-Vinyl-2-pyrrolidone (NVP)
Collagen	N-Isopropylacrylamide (NIPAMM)
Gelatin	Vinyl acetate (VAc)
Hyaluronic acid	Acrylic acid (AA)
Dextran	Methacrylic acid (MAA)
	Polyethylene glycol acrylate/methacrylate (IPEGA/PEGMA)
	Polyethylene glycol diacrylate/dimethacrylate (PEGDA/PEGDMA)

Table 5.2: Biodegradable *in situ* solid forming delivery systems (Hatefi and Amsden, 2002)

Delivery system	Common problems	Common components
Thermoplastic paste	High temperature at the time of injection	PLA, PLGA & PCL. Alcohols as initiator.
<i>In situ</i> crosslinked systems		Stannous Octoate as catalyst.
Thermosets	- Unacceptable level of heat released during reaction - Burst in drug release - Toxicity of un-reacted monomers	Oligomers of PLA, PDLLA & PCL, Polyols as initiator and peroxides as curing agent.
Photocrosslinked gels	Shrinkage and brittleness of the polymer due to high degree of crosslinking	PGA, PLA, PCL & PEG, initiators such as eosin dye, light source (e.g., UV or laser)
Ion mediated gelation	- Low shelf life - Burst in drug release - Long degradation time	alginate with Ca ²⁺ as gelling agent
<i>In situ</i> polymer precipitation	Burst in drug release	PDLLA, PCL & PLA. Solvents such as DMSO or NMP.
Solvent-removal	Burst in drug release	
Precipitation	Application of organic solvents	
Thermally induced sol-gel transition	Stability of oils and purity of waxes	NIPAAm, PEG, PLA, PLGA & Chitosan, Pluronics
Organogels	- Lack of toxicity data - Phase separation	Oils such as peanut oil & Labrafil, waxes (e.g., Beeswax & Pericrol).

PLA = Polylactic acid; PLGA = Poly(lactic-co-glycolic acid); PCL = poly(ϵ -caprolactone); PDLLA = poly(D,L-lactide); PGA = Polyglycolic acid; PEG = poly(ethylene glycol); DMSO = dimethyl sulfoxide; NMP = *N*-methyl-2-pyrrolidone

Implants that form *in situ* exist as solutions at room conditions and transform into a gel like phase when in contact with a particular stimulus. There are various stimuli which may be ionic or chemical cross-linking or photopolymerisation or a local environmental stimulus such as pH, ionic strength or as mentioned previously, temperature. Temperature sensitive polymers can exhibit one of two behaviours; they either display upper critical solution temperature (UCST) or a lower critical solution temperature (LCST) (Zhang et al., 2004; Aseyev et al., 2011). Polymers, which become insoluble upon heating, have a LCST and those that become soluble upon heating, have an upper critical solution temperature. An observable advantage of thermal gelation is its nearly instantaneous gelation subsequent to the polymer reaching the LCST.

Gelation can either be physical or chemical (Gupta et al., 2006). Physical gels can be further classified as strong physical gels and weak physical gels (An et al., 2010). Strong physical gels possess strong physical bonds between polymer chains and are permanent at a given set of experimental conditions. Consequently, strong physical gels are equivalent to chemical gels. Examples of strong physical bonds are lamellar microcrystals, glassy nodules or double and triple helices. Weak physical gels have fluid networks formed from transitory links between chains. These links have finite lifetimes, splitting and being repaired constantly (Rubinstein and Dobrynin, 1997). Examples of weak physical bonds are hydrogen bond, block copolymer micelles, and ionic associations (Rubinstein and Dobrynin, 1999). On the other hand, chemical gelation involves formation of covalent bonds and always results in a strong gel. The three main chemical gelation processes include condensation, vulcanisation, and addition polymerisation (Gulrez et al., 2011).

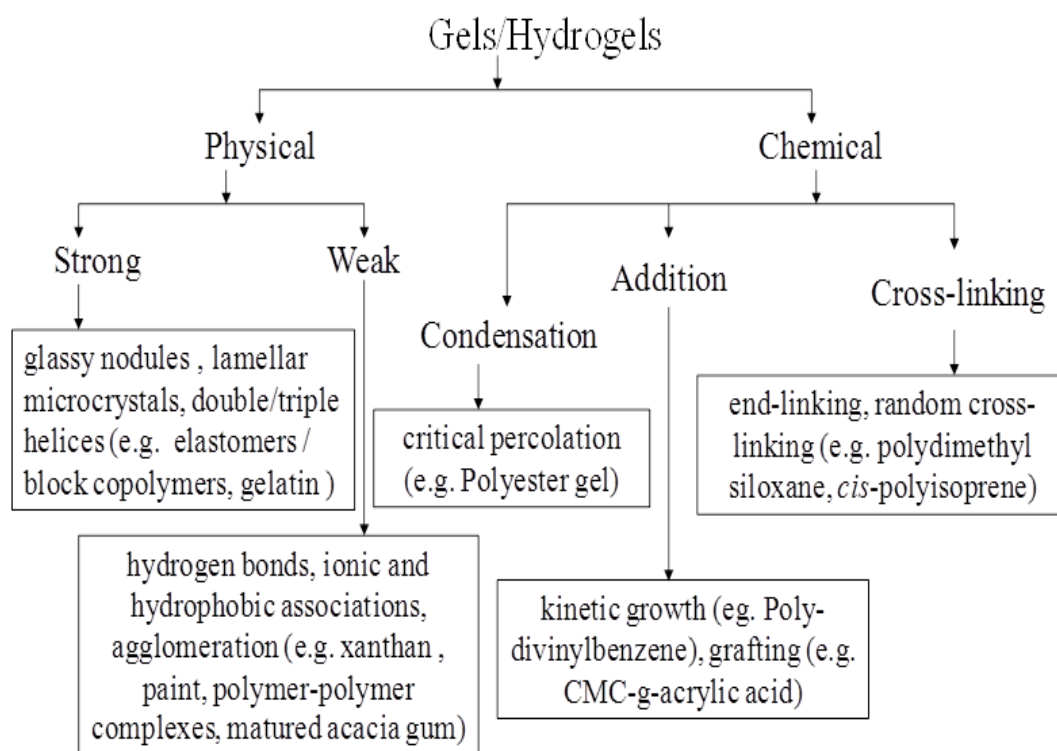


Figure 5.2: Classification of gelation mechanism and relevant examples (Gulrez et al., 2011).

(<http://www.intechopen.com/books/progress-in-molecular-and-environmental-bioengineering-from-analysis-and-modeling-to-technology-applications/hydrogels-methods-of-preparation-characterisation-and-applications>)

5.1.2. Viscoelasticity

Viscoelastic materials are materials that exhibit both viscous and elastic characteristics when external forces are applied to them at different strain rates and temperatures (Ozkaya et al., 2012). These materials resist shear flow and strain linearly with time when a stress is applied. This means a viscoelastic material will return to its original shape after any force that transformed its structure has been withdrawn (i.e., it will display an elastic reaction (Banks et al., 2010)) even though it will take time to do so (i.e., it will have a viscous component to the reaction). There are three ways in which a material can react in response to external forces in a physically stressful setting. It can add the load directly onto the forces that hold the essential atoms or molecules together, as occurs in simple crystalline (including polymeric crystalline) and ceramic materials – such materials are classically extremely firm; or it can transfer the energy into large modifications in shape (the main mechanism in noncrystalline polymers) and flow away from the force to deform either semi-permanently (as with viscoelastic materials) or permanently (as with plastic materials).

Hydrogels are viscoelastic materials as they exhibit both elastic and liquid characteristics. For elastic materials, Hooke's Law of Elasticity (Figure 5.3) states that the force needed to extend or compress a spring by some distance is proportional to that distance. Hooke's Law is used to describe the flow properties of a material with elastic characteristics and the term shear modulus (G) is used to relate the relationship between the shear stress (τ) and the deformation (γ) (Equation 3.2). Liquids are defined by Newton's Law of Viscosity (Equation 3.3) whereby the shear stress (τ) is proportional to the shear rate of the liquid ($\frac{\Delta\gamma}{\Delta t}$).

$$\tau = G\gamma \quad \text{Equation 5.1}$$

$$\tau = \eta \frac{\Delta\gamma}{\Delta t} \quad \text{Equation 5.2}$$

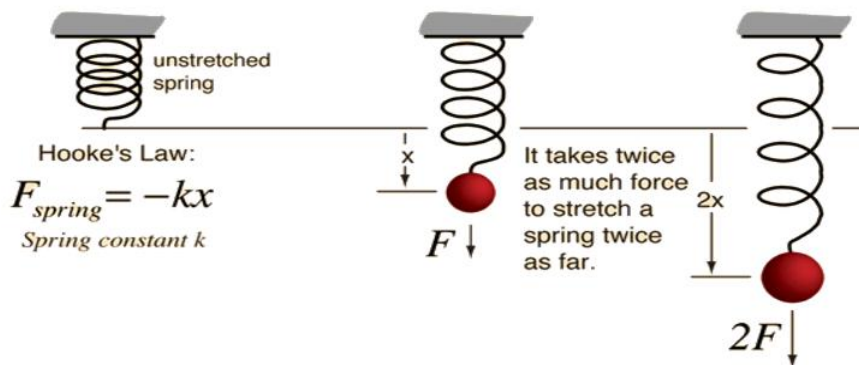


Figure 5.3: Illustration of Hooke's Law showing the relationship between force and distance when applied to a spring. This can be expressed mathematically as $F = -kX$, where F is the force applied to the spring (either in the form of strain or stress); X is the displacement of the spring, with a negative value demonstrating the displacement of the spring once it is stretched; and k is the spring constant and details just how stiff it is (What is Hooke's Law, 2015). (<http://www.universetoday.com/>)

Oscillatory rheology is required for the characterisation of hydrogels. The fundamental principle of an oscillatory rheometer is to generate a sinusoidal shear deformity in the sample and compute the resultant stress response; the time scale examined is determined by the frequency of oscillation, ω , of the shear deformation (Oscillatory rheometer, 2015). As shown in Figure 5.4 the application of a sinusoidal strain results in different resultant stress patterns in Hookean solids, Newtonian fluids and a viscoelastic solid. The viscoelastic solid shows a phase shift that lies between the phase shift of 0 for an elastic solid and $\frac{\pi}{2}$ for a viscous fluid (Banks et al., 2010).

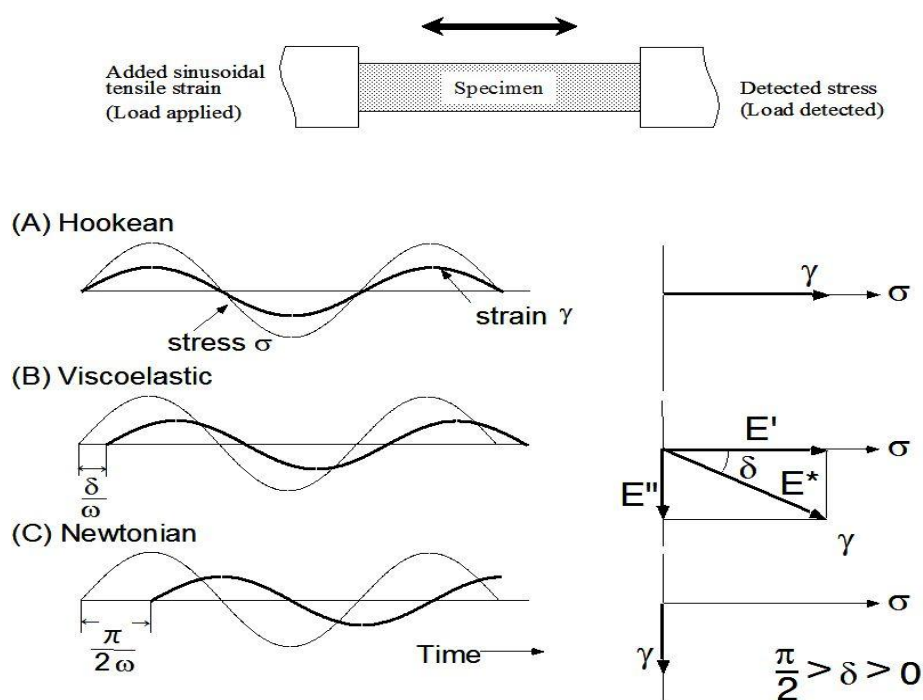


Figure 5.4: Schematic representation of dynamic mechanical test instrument and relationship between stress and strain of completely elastic solid (Hookean solid) (A), viscoelastic material (B) and completely viscous liquid (Newtonian fluid) (C) with sinusoidally varying stress (Murata, 2012).

(<http://www.intechopen.com/books/polymerization/rheology-theory-and-application-to-biomaterials>)

In Figure 5.4, E' and E'' are used as symbols for the elastic modulus component or storage modulus and loss modulus respectively instead of the commonly used G' and G'' . Equation 5.3 is used to determine the total resistance of the sample to oscillatory shear which is represented by the complex modulus, G . The complex viscosity (η^*) is the flow resistance of the sample and is determined using Equation 5.4 and Equation 5.5.

$$G = G' + iG'' = (G'^2 + G''^2)^{1/2} \quad \text{Equation 5.3}$$

$$\tan \delta = \frac{G''}{G'} \quad \text{Equation 5.4}$$

$$\eta' = \frac{G}{\omega} \quad \text{Equation 5.5}$$

where δ is the phase angle, η' is the complex viscosity and ω is the angular frequency.

5.2. Materials and methods

5.2.1. Materials

N-vinylcaprolactam (VCL), ϵ -Caprolactone and 2,2'-Azobis (2-methyl-propionitrile) (AIBN) were all purchased from Sigma-Aldrich Inc., (St Louis, MO, USA). All other chemicals used in the experiments were of analytical grade and were used as purchased.

5.2.2. Preparation of the thermosensitive hydrogel

Several techniques have been reported for the synthesis of hydrogels. These include, firstly, an approach comprising copolymerization or cross-linking of co-monomers employing multifunctional co-monomer, which plays the role of a cross-linking agent. The polymerisation reaction is triggered by a chemical initiator. This form of hydrogel synthesis can be sub-divided into solution polymerisation and suspension polymerisation. The second technique entails the cross-linking of linear polymers by irradiation or by chemical compounds. The monomers used in the preparation of the ionic polymer network usually contain a group that is able to go through a substitution reaction after the polymerisation is completed. This leads to fabricated hydrogels enclosing weak acidic groups like carboxylic acids, weak basic groups like substituted amines, or strong acidic and basic group like sulfonic acids, and quaternary ammonium compounds. Some of the frequently used cross-linking agents include N, N'-methylenebisacrylamide, divinyl benzene, and ethylene glycol dimethacrylate. (Satish et al., 2006)

For this study, solution polymerisation was used. N-vinylcaprolactam and ϵ -Caprolactone were the co-monomers and AIBN was the cross-linker. Briefly, 2.5g of N-vinylcaprolactam

(VCL) and 0.2ml of ϵ -Caprolactone were dissolved in 20ml of isopropanol. The reaction mixture was flushed with nitrogen for 30 minutes. The solution was then dissolved in distilled water and the isopropyl alcohol removed by rotary evaporation. 0.2ml of AIBN was added to the remaining solution followed by overnight incubation in a sealed vessel at 60°C to form a sol. This reaction is depicted in Figure 5.5.

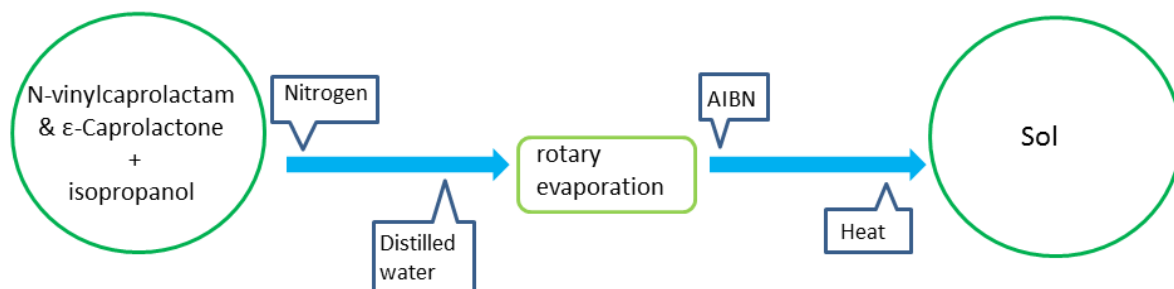


Figure 5.5: Reaction scheme for solution polymerisation of the hydrogel

5.2.3. Establishing the gelation temperature of the polymeric formulations employing oscillatory rheology

Investigating the flow properties was important to this study as it is central to the mechanisms by which the hydrogel functions. At room temperature the hydrogel remains in the liquid state to allow delivery through injection and as the temperature increases so does the thickness of the gel until it forms a solid-like structure. In order to characterise and analyse the flow behaviour and determine the gelation temperature of the hydrogel, rheology studies were conducted using a Haake Modular Advanced Rheometer System (ThermoFisher Scientific, Germany).

5.2.3.1. Determination of the viscoelastic region of the thermosensitive hydrogel

Viscoelastic solids should be tested for their properties within the viscoelastic region. In order to determine the viscoelastic region of the hydrogel formulation, the sample was placed on the lower plate of the rheometer and the yield stress of the formulation was first determined, as this is the point at which a minimum critical shear stress is exceeded and therefore the point at which a viscoelastic substance begins to flow. Stress sweeps at 0.01Hz, 1Hz and 10Hz were then conducted ensuring that the minimum strain applied was less than the yield stress value obtained. The stress sweep plots were then analysed and the plateau or linear viscoelastic region where both G' (storage modulus) and G'' (loss modulus) were independent of the stress amplitude, was used as the viscoelastic region. The first point of deviation from the plateau and the frequency at which it occurred was used

for subsequent temperature ramping tests. The yield stress was also determined, which is the amount of force needed to be applied for a sample to change its formation. For instance, some gels remain in their solid form until a force is applied where they then change to liquid.

5.2.3.2. Determination of the gelation temperature of the hydrogel

In order to determine the lower critical solution temperature (LCST) and hence the gelation temperature of the hydrogel formulation, the temperature of the sample was ramped from 10-40°C at a rate of 0.25°C/min while applying the predetermined stress obtained from the stress sweeps previously described, at the frequency observed in that test. The gelation temperature was determined as the temperature at which the cross-over of G' and G'' occurred i.e., the point at which the formulation was no longer acting as a liquid (G'') but the solid phase (G') was dominating.

5.2.4. Chemical structure analysis of the thermosensitive hydrogel employing Fourier transform infrared spectroscopy

FTIR spectroscopy was performed on the polymers used to formulate the hydrogel as well as the hydrogel in its liquid and sol state in order to characterize the potential interaction between the polymers as well as the effect temperature has on the bonds in the hydrogel. Samples were compressed into 1x13mm disks using a Beckmann Hydraulic Press (Beckman Instruments Inc., Fullerton, USA), and then analyzed at high resolution with wave numbers ranging from 4,000–650 cm^{-1} on a Nicolet Impact 400D FTIR Spectrophotometer coupled with Omnic FTIR research grade software (Nicolet Instrument Corp., Madison, WI, USA).

5.2.5. Morphological characterisation by scanning electron microscopy of a lyophilised sample of the thermosensitive hydrogel

A lyophilised sample of the thermosensitive hydrogel was mounted on a specimen stub and gold coated using a SPI-ModuleTM sputter coater (SPI Supplies, STRUCTURE PROBE INC, West Chester, Pennsylvania, USA) and then observed at various magnifications under a scanning electron microscope (SEM) (PHENOMTM Desktop SEM, FEI Company, Oregon, USA) operated at 10KV in the electron imaging mode.

5.2.6. Preparation of the amantadine-loaded DTPA bound nanospheres carried by the thermosensitive hydrogel

A homogenous solution was formed by blending the previously prepared amantadine-loaded DTPA-bound nanospheres with the thermosensitive hydrogel. The NVL/ ϵ -Cap hydrogel was completely dissolved in water at a certain temperature followed by slightly heating it to form a sol. The nanosphere suspension was then mixed with the hydrogel to form a homogenous solution.

5.2.7. *In vitro* drug release behaviour of amantadine from the thermosensitive hydrogel

In vitro drug release studies to determine the release behaviour of amantadine from the prepared drug delivery system were carried out for 72 hours using an orbital shaking incubator set at 25rpm. The nanosphere-hydrogel composite was placed in dialysis tubing and immersed in phosphate buffer saline (PBS) at pH 7.4, 37°C in a glass jar. At predetermined time intervals 2ml samples of the release media were removed and replaced with fresh buffer of the same volume to maintain sink conditions. It was reported that this method can only be used for the release of formulations with drug release times of greater than 1 hour. Samples were then centrifuged and the supernatant analysed by UV spectroscopy at a maximum wavelength of λ_{525} , after leaving it to react with potassium permanganate reagent. Drug quantity was determined using a standard calibration curve.

Dissolution profiles were produced from the amantadine released over 72 hours and these profiles were used to characterise *in vitro* amantadine release kinetics from the nanosphere-hydrogel composite. Mean dissolution time (MDT) was used to analyse the ability of the nanosphere-hydrogel composite to control amantadine release.

5.3. Results and Discussion

5.3.1. Rheological analysis of the thermosensitive hydrogel

Rheology studies were performed on the hydrogel. Stress sweep was conducted at 0.1Hz to determine the linear viscoelastic region. This is the region where the viscoelastic properties observed are independent of imposed stress or strain levels. At the point where the graph plunges, is where the hydrogel breaks (Figure 5.6a). Yield stress (Figure 5.6b) is the amount of force required for the deformation of the hydrogel i.e. stretch from its solid phase until the yield point where it will start flowing. Studies showed that 4.558 Pa was the yield stress required for the hydrogel to flow. The irregular section of the graph (in blue) is background noise.

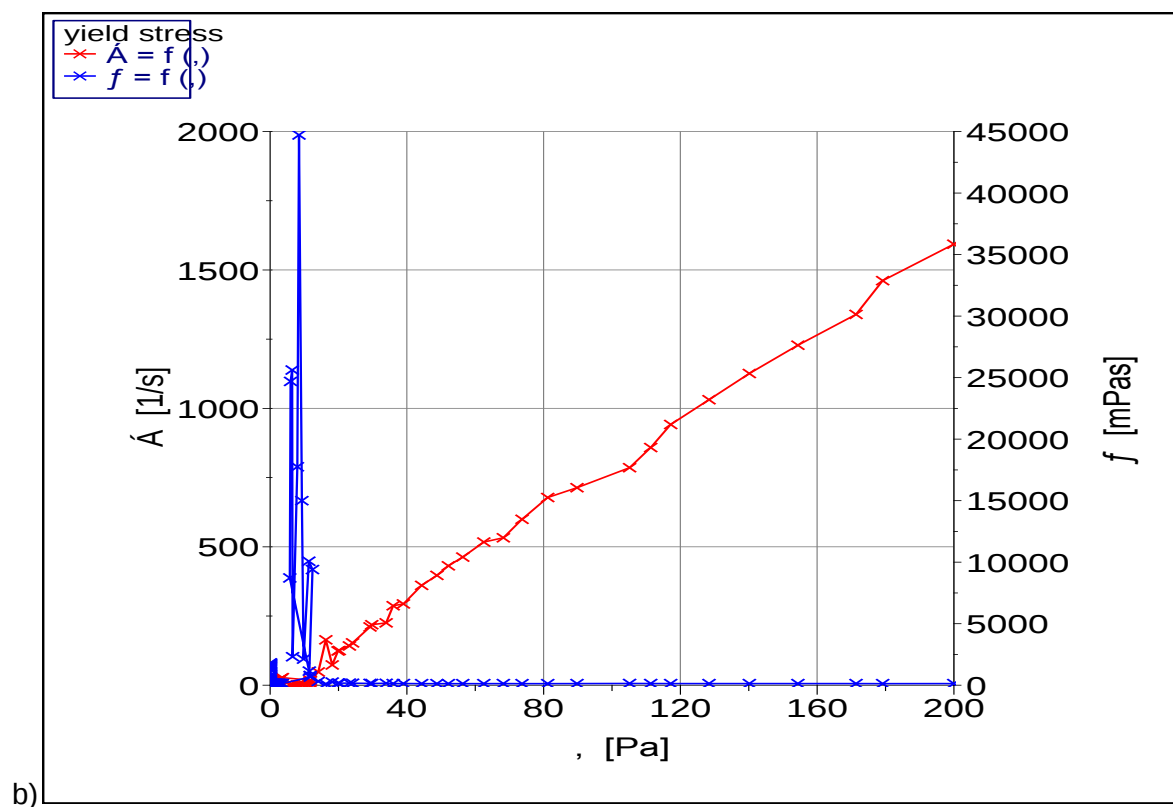
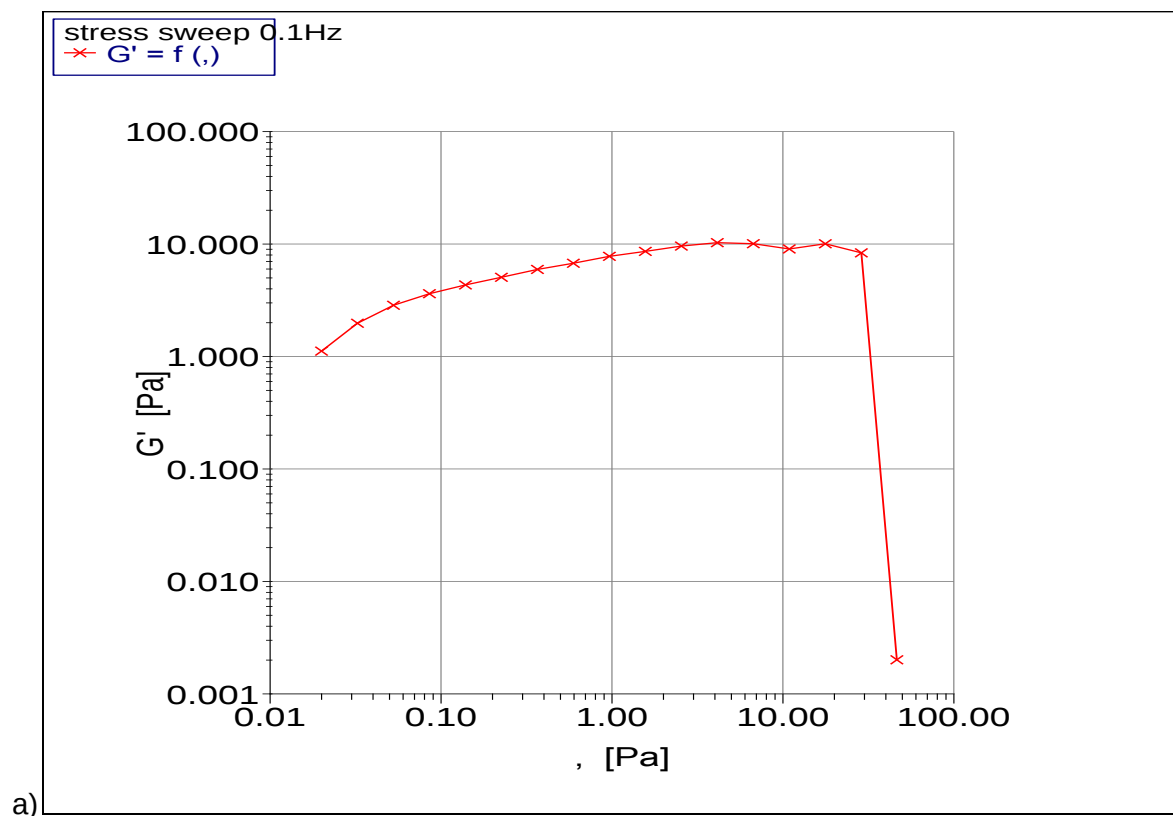


Figure 5.6: a) Rheological profile illustrating the stress sweep of the hydrogel; b) Rheological profile illustrating the yield stress of the hydrogel

The formulation displayed the characteristic pattern of a thermosensitive system with a Lower Critical Solution Temperature (LCST). As clearly illustrated in Figure 5.7, the point at which the storage and loss modulus crossover (dark green vertical line) was considered the gelation temperature ($T^{\circ}g$) for the formulation. The storage modulus (G') of a viscoelastic solid is described as the point where the hydrogel exhibits solid properties or the elastic energy storage properties which signify that the removal of the deformational force will result in the hydrogel returning to its original form. Conversely, the loss modulus (G'') describes the nature of the viscoelastic solid when it exhibits liquid characteristics. At the point at which the storage modulus exceeded the loss modulus, the formulation was therefore, exhibiting more solid properties than it was exhibiting liquid properties.

Initially, G'' is larger than G' , which is expected since the sample was still in liquid state where viscous properties dominate, and therefore most of the energy is lost as viscous heat. As the solution starts to gel and a cross-linked network is formed, both G' and G'' begin to increase; however, the rate of increase of G' is much higher than that of G'' due to the elastic properties of the gelling hydrogel taking over and beginning to dominate. As a result, there is a crossover point where G' becomes larger than G'' . The temperature required for this crossover to occur is referred to as the gelation temperature for the solution, which was 18.45 °C. This was much lower than expected and therefore the formulation had to be stored in the fridge to prevent gelation when handled normally. At room temperature the hydrogel was slightly thick and this thickness increased with increased temperature. This showed that the gel would certainly form a solid implant when inside the body.

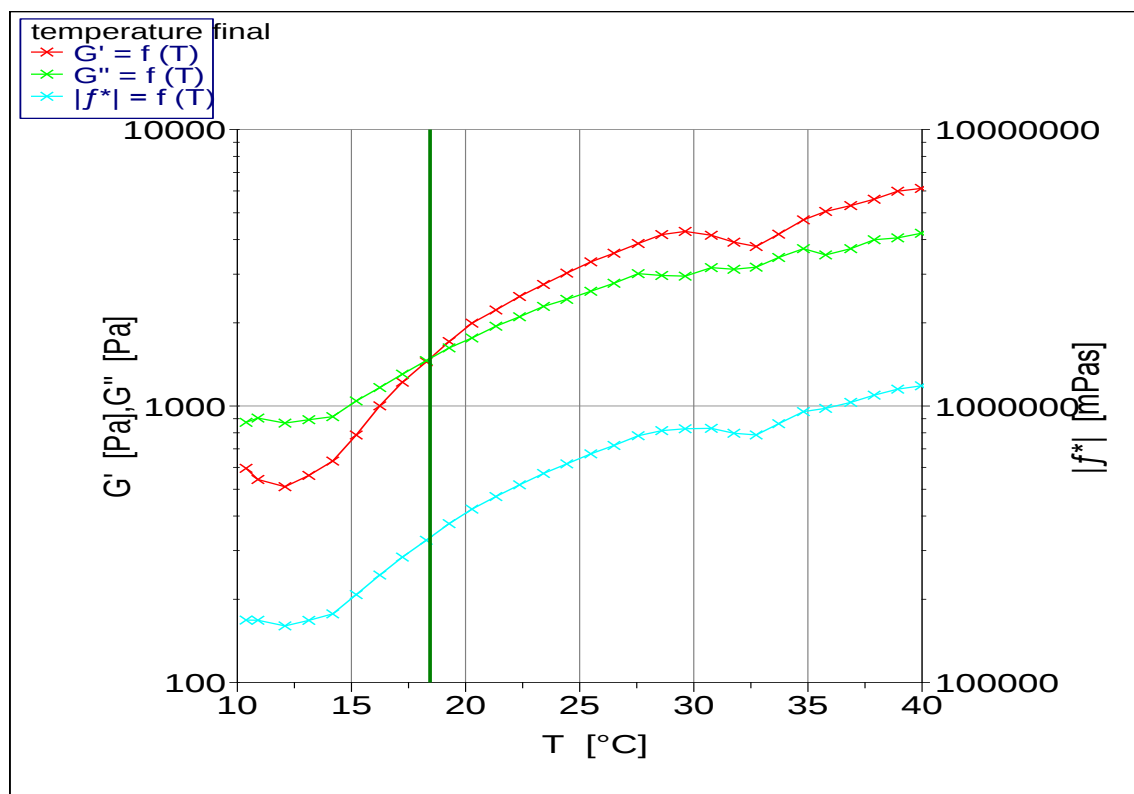


Figure 5.7: Rheology profile of the temperature gelation. The dark green vertical line is illustrating the point at which the storage modulus (G') and the loss modulus (G'') crossover which is considered the gelation temperature (18.45°C)

5.3.2. Chemical structure analysis of the thermosensitive hydrogel employing Fourier transform infrared spectroscopy

FTIR was used to determine the chemical structure of the two polymers used to formulate the hydrogel as well as the hydrogel in its liquid and sol state. The FTIR shows that the two polymers formed new bonds in the formation of the hydrogel (Figure 5.8). A strong absorption peak ($\sim 1600\text{cm}^{-1}$) was observed on both the liquid and sol hydrogel spectrum which may have been contributed to by the strong stretching vibrations of the amide I band in N-Vinylcaprolactam. An ester linkage ($\sim 1700\text{cm}^{-1}$) can also be observed on the hydrogel spectra which is typical of ϵ -caprolactone and thus may be due to its presence. The stretching vibration peak observed at $\sim 3300\text{cm}^{-1}$ in both hydrogel states may have been due to the N-H stretching vibration in N-Vinylcaprolactam. Results also indicate that there is a slight change in the bonds present in the hydrogel when it transitioned from liquid to sol state. Weak physical bonds are driven by intermolecular forces that can be created and broken over experimental time frames such as hydrogen bonding or van der Waals forces. Hydrogel polymer networks depend on chemical bonds for structure and physical bonds to facilitate volume-phase transition (Mah and Ghosh, 2013). Weak physical bonds that are

formed or broken through a phase transition are a collective consequence of supportive polymer-polymer interactions and polymer-solvent interactions. A phase transition then becomes a balance between repulsive forces that decrease polymer-polymer interactions and progress to swell the gel and attractive forces that decrease the polymer-solvent interactions and progress to disintegrate the gel.

With regards to polymer-polymer interactions, attractive forces are hydrophobic interactions and van der Waals interactions while electrostatic interactions and hydrogen bonding may act as both attractive and repulsive forces depending on the environmental conditions.

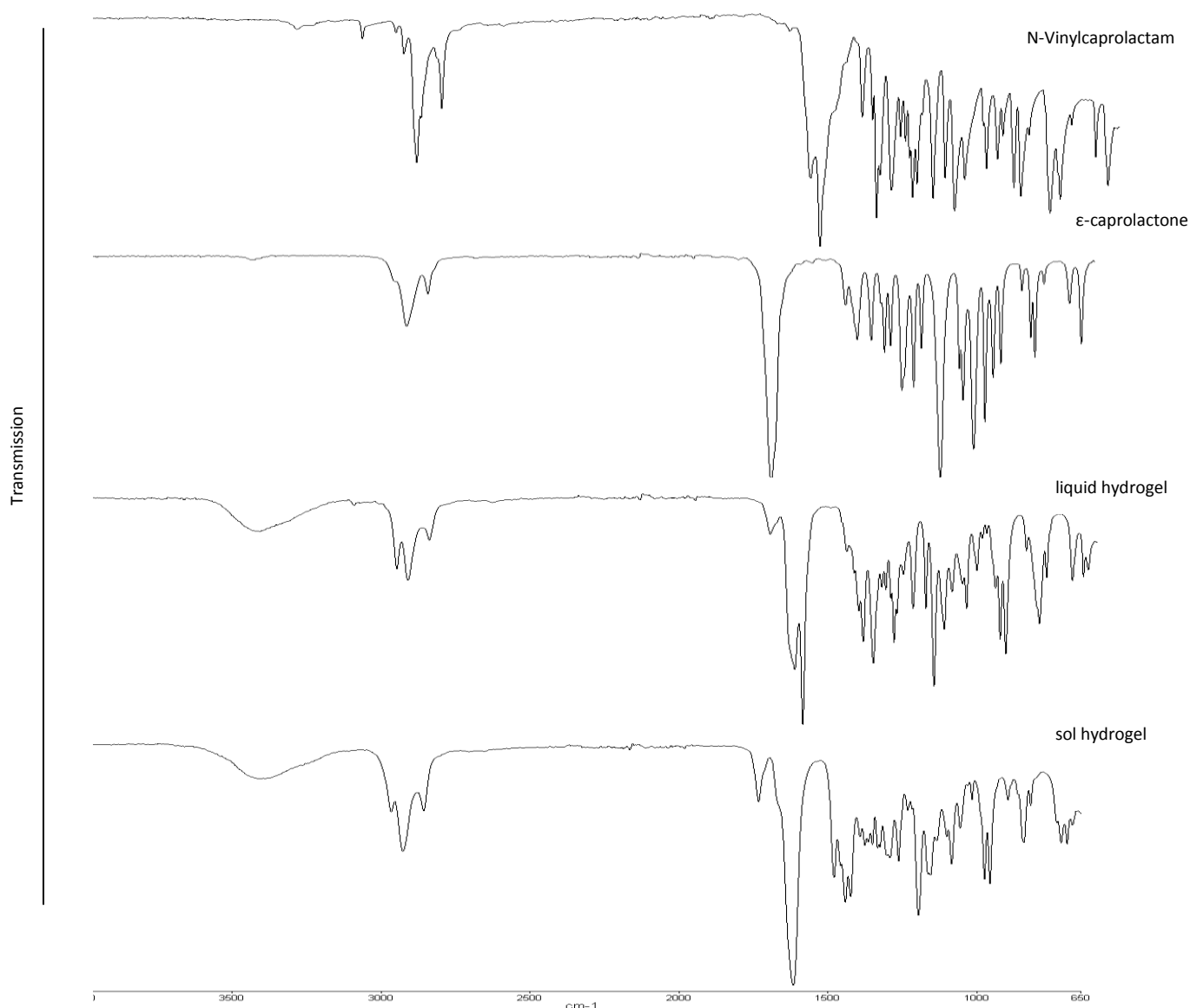


Figure 5.8: FTIR spectra of N-Vinylcaprolactam, ϵ -caprolactone, liquid hydrogel and sol hydrogel respectively.

5.3.3. Morphological characterisation by scanning electron microscopy of a lyophilised sample of the thermosensitive hydrogel

Scanning electron micrographs of the thermosensitive hydrogel revealed the surface morphology and architectural integrity of the lyophilised structure as depicted in Figure 5.9. Figure 5.9a depicts a porous membranous scaffold with thin undefined smooth surfaces. The pores appear large and widespread across the surface of the scaffold. Another view of the hydrogel, however, shows a multidirectional membranous scaffold with some smooth surfaces and pores. This view depicts the hydrogel with uneven sheets embedded with a few pores. These two views of the hydrogel show that the scaffold did not have a homogenous morphology throughout its composition.

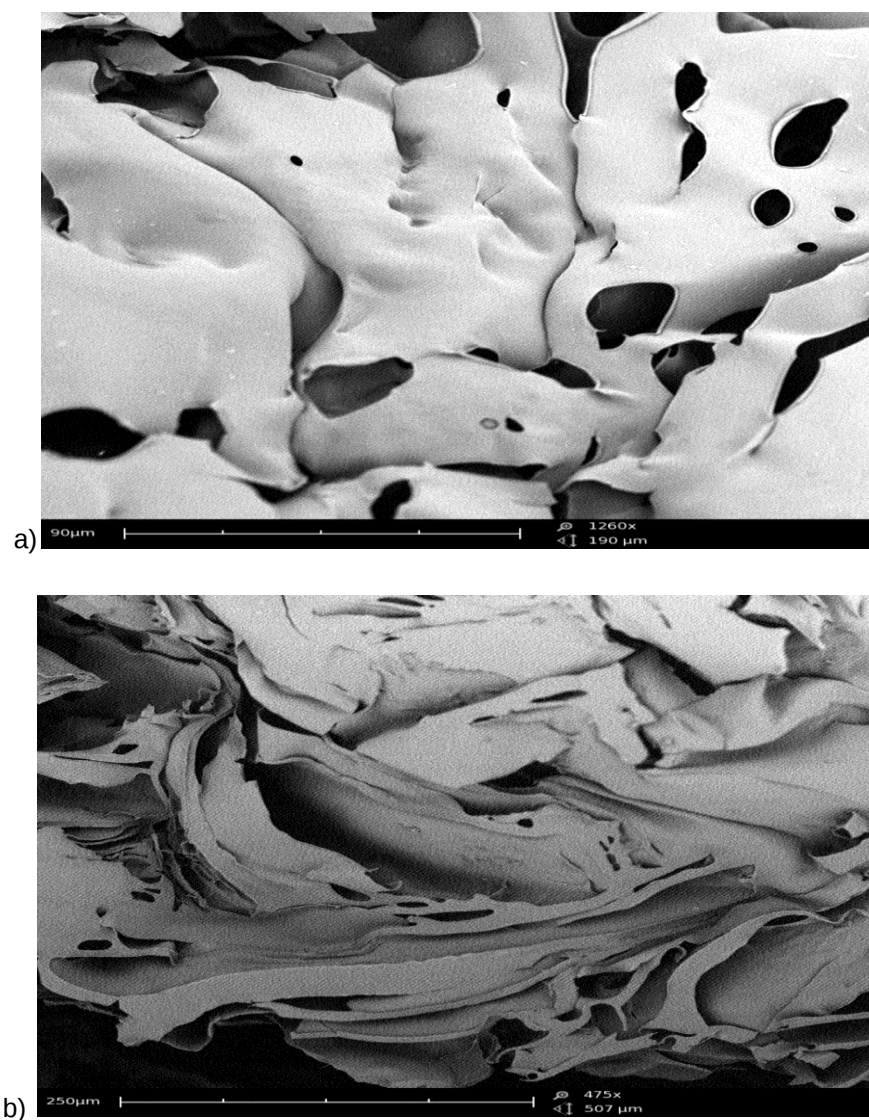


Figure 5.9: SEM images of the lyophilised thermosensitive hydrogel

5.3.4. *In vitro* drug release behaviour

Release behaviour of amantadine from amantadine-loaded nanospheres was first investigated in chapter 4. In section 4.3.5 it was reported that amantadine could be released from the optimised nanospheres over a sustained period. The *in vitro* release profile of amantadine from the nanosphere-hydrogel composite was further examined, which is presented in Figure 5.10. Amantadine was released from the nanosphere-hydrogel composite in a sustained manner over an extended period. The hydrogel gradually released the nanospheres as it degraded followed by the release of the drug by the nanospheres. This resulted in the drug being released at a slower rate than when released solely by the nanospheres (without the hydrogel). This delay of drug release indicates the potential applicability of the nanosphere-hydrogel composite to minimise the exposure of tissues to the drug while increasing the accumulation of therapeutic drug in the focus area.

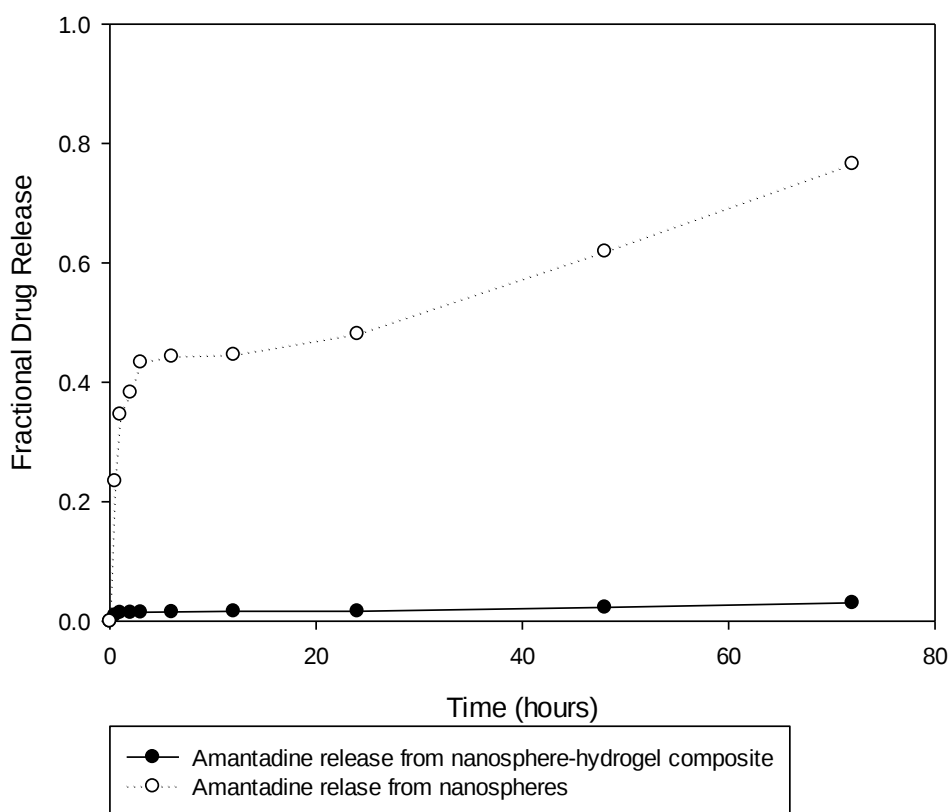


Figure 5.10: Comparison of amantadine released from nanospheres and nanosphere-hydrogel composite with standard deviations of $\pm 7.9481e^{-3}$ and ± 0.2060 respectively. The hydrogel gradually released the nanospheres as it degraded followed by the release of the drug by the nanospheres. This resulted in the drug being released at a slower rate than when released solely by the nanospheres (without the hydrogel).

5.4. Concluding remarks

A thermosensitive hydrogel formulation was prepared employing solution polymerisation and tested using oscillatory rheology and FTIR. The formulation displayed typical thermoresponsive behaviour with a Lower Critical Solution Temperature (LCST) when tested for its gelation temperature. FTIR spectra showed the blending of the two polymers to form the hydrogel. It was also revealed that there was a minor alteration in the bonds present in the hydrogel when it transitioned from liquid to sol state due to weak physical bonds. SEM showed that the hydrogel did not have a uniform morphology throughout its composition.

The hydrogel formulation was blended with the previously prepared amantadine-loaded DTPA-bound nanospheres to form a homogenous solution. The nanosphere-hydrogel composite was tested for drug release. The hydrogel was able to greatly decrease the rate of release of amantadine and proved to be an efficient carrier of the amantadine-loaded nanospheres.

In order to evaluate the safety of the composite system, the hydrogel formulation, the nanospheres and the nanosphere-hydrogel composite with their components would need to be tested for possible cytotoxicity. Chapter 6 describes the toxicity studies performed on motor neuron cells (NSC-34). In addition, cell uptake studies were undertaken to confirm the efficacy of the chelating ligand, DTPA, in increasing nanosphere internalisation by NSC-34 cells.

CHAPTER SIX

IN VITRO TESTING OF THE THERMOSENSITIVE HYDROGEL AND DTPA-BOUND NANOSPHERES ON A MOTOR NEURON CELL LINE

6.1. Introduction

Extensive research has been carried out to develop and investigate *in vitro* models of neurotoxicity. Organotypic and dissociated embryonic tissue are some of the tissues included in primary culture models. Primary culture is valuable because the cells mature morphologically and physiologically ultimately to resemble the cells naturally growing in the body. However, the disadvantage of primary culture is that these neurons require several days in culture to develop and subsequently serial passage is not viable. Various tumour cell lines have also been investigated. Nonetheless, despite the fact that these cells are exceedingly more expedient than primary culture, their extrapolative capacity of neurotoxicity is dependent on whether or not those cells express the applicable target for particular neurotoxic agents. Consequently, in an effort to produce cells that not only preserve neuronal properties but also proliferate, primary neurons have been fused with tumour lines (Cellutions Biosystems Inc, 2014).

NSC-34 is a hybrid cell line that was formed with this technique, where motor neuron enriched, embryonic mouse spinal cord cells were merged with mouse neuroblastoma. Cultures contain two populations of cells: Undifferentiated cells with short neurites and almost no branches, and differentiated cells with long branching processes (Figure 6.1). (Maier et al., 2013) These cells encompass many properties of motor neurons, including choline acetyltransferase, acetylcholine synthesis, storage and release and neurofilament proteins (Matusica et al., 2008).

NSC-34 cells have been examined following exposure of cultures to a range of chemicals known to be neurotoxic to motor neurons. NSC-34 cells react to agents that have an effect on voltage-gated ion channels, cytoskeletal organisation and axonal transport. The sensitivity of action potential production to various ion channel blockers is similar to that in primary motor neurons in culture. Therefore these immortalised motor neuron-like cells have utility as a model for the investigation of neurotoxicity.

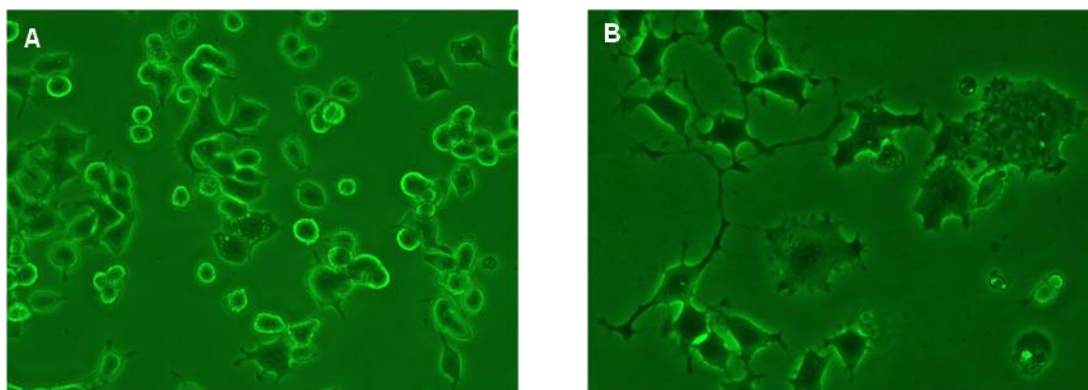


Figure 6.1 Differentiation-related alterations in murine neuronal cells, NSC-34. **(A)** NSC-34 contains small cells derived from mouse motor neurons and large multinucleated cells derived from mouse neuroblastoma. **(B)** Differentiated NSC-34. Small cells with extended neuronal processes. (Cellutions Biosystems Inc, 2014) (<http://www.cellutionsbiosystems.com/>)

Cytotoxicity experiments measure the degree to which a compound is toxic to cells, possibly resulting in cell death. *In vitro* cytotoxic testing methods include the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), trypan blue (TB), sulforhodamine B (SRB) and (4-[3-(2-methoxy-4-nitrophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate sodium salt) (WST) assays and clonogenic assays (Grunert et al., 2011). These tests are vital in the research and development process and, within the context of the current study, allows for cytotoxicity levels to be evaluated prior to *in vivo* studies.

An MTT assay, which is the chosen method for this study, is a colourimetric assay which provides a quantity of cell viability by supplying a measure of the proliferation of the cells (Riss et al., 2013). The tetrazolium salt, MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) is added to the cells followed by a reduction to a purple formazan in the mitochondria of living cells (Figure 6.2). Dimethyl sulfoxide (solubilisation solution) is then added to the cells to dissolve the product formed by formazan which subsequently results in a coloured solution. Evaluation of the treated cells and the untreated control wells provides an indication of the cell viability. Treated cells that generate higher absorbances than the control cells signify enhanced proliferation of the cells and lower absorbance values signify a decline in cell growth.

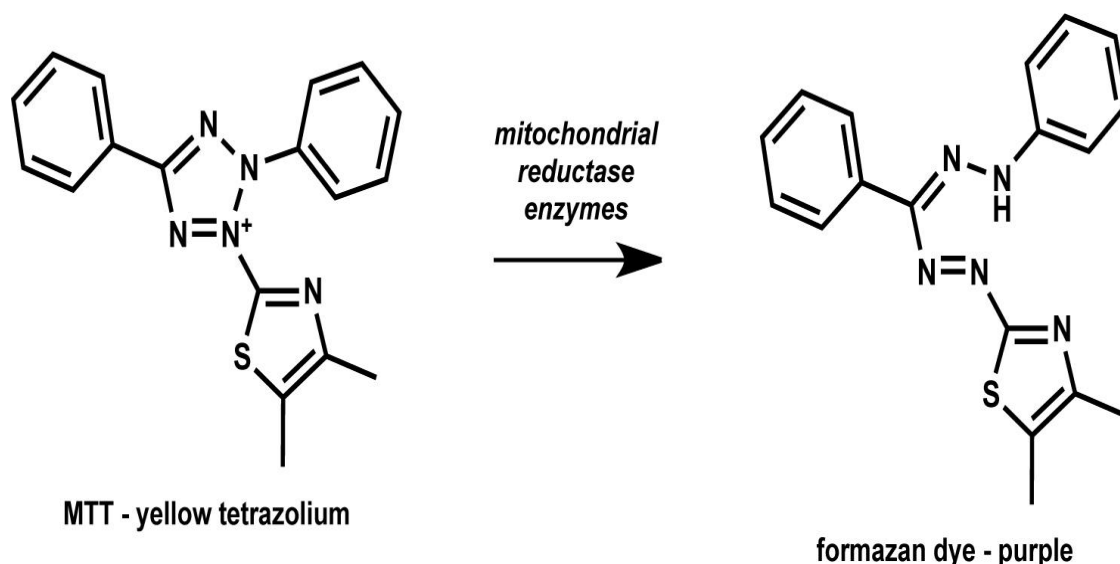


Figure 6.2. Yellow tetrazolium (MTT) is reduced to purple formazan crystals by mitochondrial reductase enzymes.

Another experiment important to this study is the testing of the uptake of the nanospheres by the motor neuron cells. The nanospheres were conjugated to diethylenetriamine pentaacetic acid (DTPA), a chelating ligand that also increases cell uptake of nanoparticles. Vranic and co-workers investigated the mechanisms through which nanoparticles are taken up by cells. To determine whether cell uptake of nanoparticles is a passive or an active process, they depleted the energy of cells employing sodium azide (NaN_3) which has been shown to inhibit the respiratory chain in mitochondria, consequently resulting in the impairment of the generation of ATP in the cell leading to the impairment of the uptake of active ATP. Sodium azide hindered the uptake of 50 nm-FITC SiO_2 nanoparticles up to 76%. They then compared the inhibition of uptake by NaN_3 to the inhibition at 4°C that prevents not only active uptake but also the passive uptake by increasing the rigidity of the plasma membrane. At 4°C the percentage of inhibition was higher (85%) than for the cells treated with NaN_3 (76%), suggesting that some 50 nm-FITC- SiO_2 nanoparticles may enter by passive diffusion. This was verified by confocal microscopy revealing that nanoparticles were on the cell surface as well as inside the cells subsequent to treatment with sodium azide compared to treatment at 4°C . The uptake of nanoparticles by a non endocytic pathway was already proposed and nanoparticles have been observed in red blood cells that lack endocytic properties. This passive uptake by red blood cells has been shown to involve adsorption of nanoparticles on the cell surface and strong local membrane deformations leading to uptake.

Ligands or antibodies have shown to further increase the uptake of nanoparticles by cells. Additionally, the size and shape of the nanoparticles play a role in the quantity of nanoparticles internalised by the cells.

6.2. Materials and Methods

6.2.1. Materials

NSC-34 cells were obtained from Cedarlane Laboratories (Burlington, Ontario, Canada). Fetal bovine serum (FBS) and Penicillin-Streptomycin (Pen-Strep) were purchased from Highveld Biological (Modderfontien, South Africa). Dulbeccos Minimum Essential Medium (DMEM) Trypan Blue Solution (0.4%), MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), Fluorescein isothiocyanate (FITC) and dimethyl sulphoxide (DMSO) were purchased from Sigma-Aldrich (St. Louise, MO, USA).

6.2.1. Methods

6.2.2.1. Aseptic technique

Aseptic techniques were employed continuously in the culturing of cells. Briefly all surfaces of the horizontal laminar flow unit (Labotec, Midrand, South Africa) were wiped with 70% ethanol prior to experiments with cells. Laminar flow units are checked and certified twice a year. Gloves were worn at all times and were regularly sanitised with ethanol and when leaving the laminar flow unit. Laboratory coats and face masks were worn at all times in the cell culture room. All tissue culture bottles and flasks were sanitised with 70% alcohol before placing them in the unit and were flamed on opening and closing. All used disposable flasks, pipettes and culture equipment were incinerated, and all non-disposable items were treated with bleach for 2 hours before being discarded to the drain with profuse amounts of water. Cell medium was periodically tested for contamination by incubating media at 37°C for 24 hours.

6.2.2.2. Culturing of cells

NSC-34 cell line was grown using DMEM (with 4500 mg/L glucose, L-glutamine (0.584 g/L), sodium bicarbonate (3.7 g/L) without sodium pyruvate) supplemented with 10% fetal bovine serum and 0.25% Pen-Strep in a 25cm³ flask. When cells had reached confluency (~ 80%), media in the culture flask was discarded and 2 mL Trypsin-EDTA was added to each flask and the flasks were placed in the incubator for 3-4mins. When cells had detached, 3 mL of fresh media was added and the entire volume was centrifuged at 1000rpm for 3 minutes. The supernatant was discarded and the cell pellet was resuspended in 8 mL of fresh media

and plated into two flasks. When required, cells were frozen in a mixture of 60% growth medium and 30% FBS supplemented with 10% sterile dimethylsulfoxide (DMSO) at -80°C in vials overnight. The vials were then transferred and stored in liquid nitrogen.

6.2.2.3. Cell counting using the trypan blue exclusion assay and a haemocytometer

Following trypsinisation as described in Section 6.2.2.2., and the discarding of the supernatant, the cell pellet was resuspended in 3mL of fresh media. The number of cells per mL of media was then determined using the trypan blue exclusion assay. Briefly, the cell suspension (10µL) was added to 20µL of trypan blue. A disposable chamber from a haemocytometer was then filled and its contents examined using light microscopy (Olympus CKS microscope, Olympus, Japan). The haemocytometer allowed the counting of the cells. Trypan blue was taken up by dead cells only and consequently by counting stained cells as well as unstained cells allowed the estimation of dead cells in the sample. Only samples that showed viability of greater than 95% were utilised in subsequent testing.

6.2.2.4. Controls

Positive and negative controls were used during the testing of samples. Table 6.1 summarises the types of controls used and the rationale for the addition.

Table 6.1 Summary of the controls used in the study

Type of control	Method	Value in the study
Positive	Standard solution of amantadine added to cells	Assessment of susceptibility of the cells to amantadine
Negative	1. Cells grow without the addition of other material	To establish the viability of the cells and confirm that the tested material is responsible for any transformation of the cells
	2. Incubation of blank medium with no cells	To verify that the media is uncontaminated

6.2.2.5. MTT assay

6.2.2.5.1. Preparation of the MTT solution

A 0.5%w/v of MTT was prepared as follows: 5g of MTT was added to 1 L of phosphate buffered saline, pH 7.4. The solution was filtered through 0.22µm filters and stored in the fridge (4°C) in a foil covered flask until further use.

6.2.2.5.2. Determination of the effects of amantadine, polymers, nanospheres, hydrogel and the nanosphere-hydrogel composite on NSC-34 cells using the MTT assay

6.2.2.5.2.1. Preparation of test samples

The total amount of solution in all wells typically has to be 100µL therefore concentrations were calculated to make up 100µL when added to the cells and MTT solution. Solutions of the polymer as well as solutions of amantadine were added directly to the wells of the plate in a concentration range of 25µg/ml to 200µg/ml. Nanospheres were suspended in media. The hydrogel and nanosphere-composite were first submerged in PBS pH 7.4 and the elutes released into the PBS at various time periods over 96 hours were tested. A control formulation was also prepared.

6.2.2.5.2.2. Testing of samples

Culture media (60µL) was added to each well of the 96 well plate. Cells were allowed to attach and grow for 24 hours in an incubator (37°C, 5% CO₂). Test samples were then added to the wells and the plate was incubated for 24 hours.

Positive and negative controls were used as described in Table 6.1. The MTT solution (5mg/mL) was added to each well and the cells were left to incubate in the dark at 37° C for 2-4 hours. After incubation the medium was removed from each well and 200µl of dimethylsulphoxide (DMSO) was added to dissolve the formazan crystals. The plates were read at 540nm with a reference wavelength of 690nm using a plate reader.

6.2.2.6. Utilisation of confocal microscopy to confirm the extent of cell uptake of nanospheres by NSC-34 cells

6.2.2.6.1. Growing cells on cover slips

Cells were grown on sterile cover slips (22 x 22mm). Briefly cover slips were sterilised by flaming and placed in petri dishes. Cells were diluted (1 in 20) with complete media and centrifuged. The supernatant was discarded and the pellet resuspended in complete media. Less than 1 mL of cell suspension was added to each cover slip and monitored over 12 hours. Once the cells had attached to the cover slips, 2 mL of complete media was added.

6.2.2.6.2. Fixing of motor neuron cells onto cover slips

A fixative was prepared using a 3% paraformaldehyde solution (using PBS, pH 7.4). This solution was prepared in a fume hood (not in sterile room) as it is a toxic substance that could contaminate cells.

Media was aspirated from the petri dishes. The cover slips were rinsed with PBS 3 times to get rid of unwanted protein. After rinsing with PBS, a thin layer of PBS was placed on the petri dishes followed by an addition of 2 mL of fixative to cease all biological processes and preserve the cells. Cells were fixed for 15-30 minutes followed by a rinse with PBS 3-4 times. The petri dishes were covered with parafilm and left in a fridge at 4°C for 48 hours. Cover slips were then mounted using 10% glycerol in PBS.

6.2.2.7. High speed fluorescence microscopy for live imaging of uptake of nanospheres by NSC-34 cells

A high speed fiber-optic fluorescence microscope (Cellvizio® LAB, coupled with Microprobes and ImageCell™ Software; Visualsonics and Mauna Kea Technologies, USA) was utilised for *ex vivo* cellular imaging to determine nanosphere uptake by NSC-34 cells. It provides an *in situ* dynamic fluorescence microscopy solution, generating key information on the manner and location in which a molecular compound is taken up at the cellular level. Fluorescein-conjugated nanospheres were prepared (Figure 6.3). Each nanosphere suspension (0.5mL) was placed in a graduated sterile centrifuge tube. NSC-34 cell culture (1 mL), which was exposed to UV irradiation, was introduced into each tube followed by centrifugation of the cell cultures at 280xg in a high-speed table microcentrifuge (Model TG16-WS, Shanghai Ronbio Scientific Co., Ltd., China) and extraction of the supernatant. Thereafter, the cells were re-suspended in PBS (pH 7.4). The nanosphere-cell culture samples were incubated for 30 mins at 37°C; thereafter the samples were viewed, and images acquired at successive time intervals for determination of nanosphere-cell interactions.

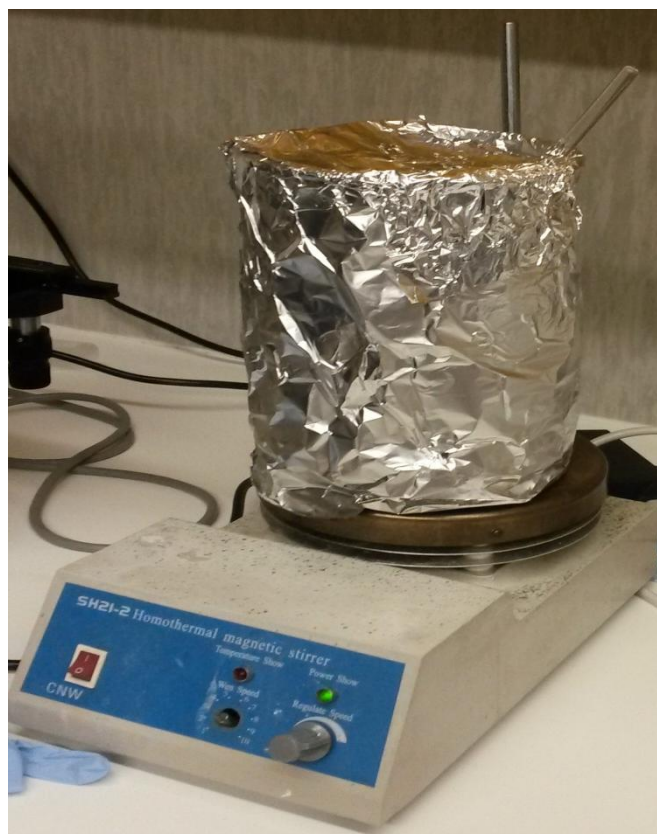


Figure 6.3: Conjugating the FITC to the nanospheres. The beaker had to be covered with foil to keep the solution in the dark

6.3. Results and discussion

6.3.1. Cell culturing

The NSC-34 cell line typically contains small cells derived from mouse motor neurons and large multinucleated cells derived from mouse neuroblastoma. When they differentiate, NSC-34 cells become small cells with extended neuronal processes. This was evident when culturing occurred in flasks. However, once the cells were grown on cover slips only the small cells were observed without the extended neuronal processes. This could be as a result of the cells not growing in conditions where their growth could thrive i.e. on cover slips. It is also possible that the cells required more time to reach the stage where the extended neuronal processes started being visible due to the different surface the cells were made to grow on.

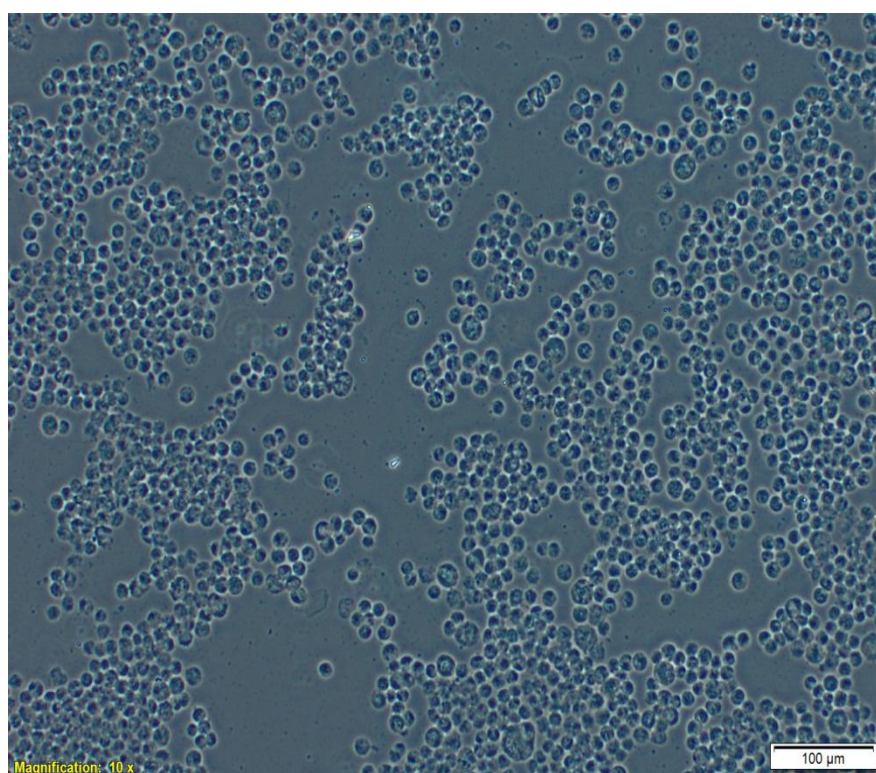


Figure 6.4: NSC-34 cells grown on cover slips. Small round cells were observed without the extended neuronal processes

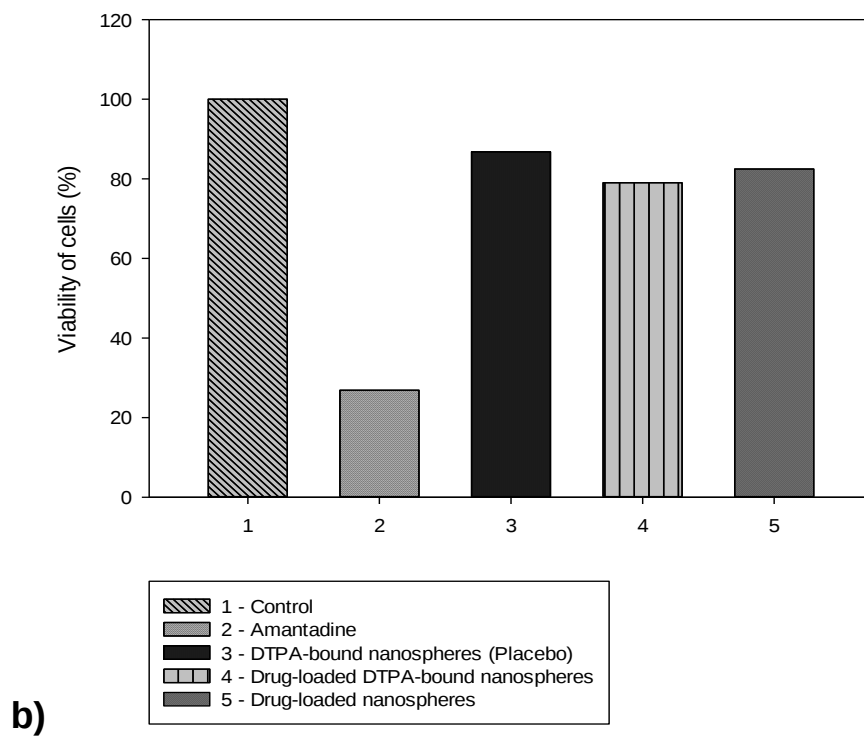
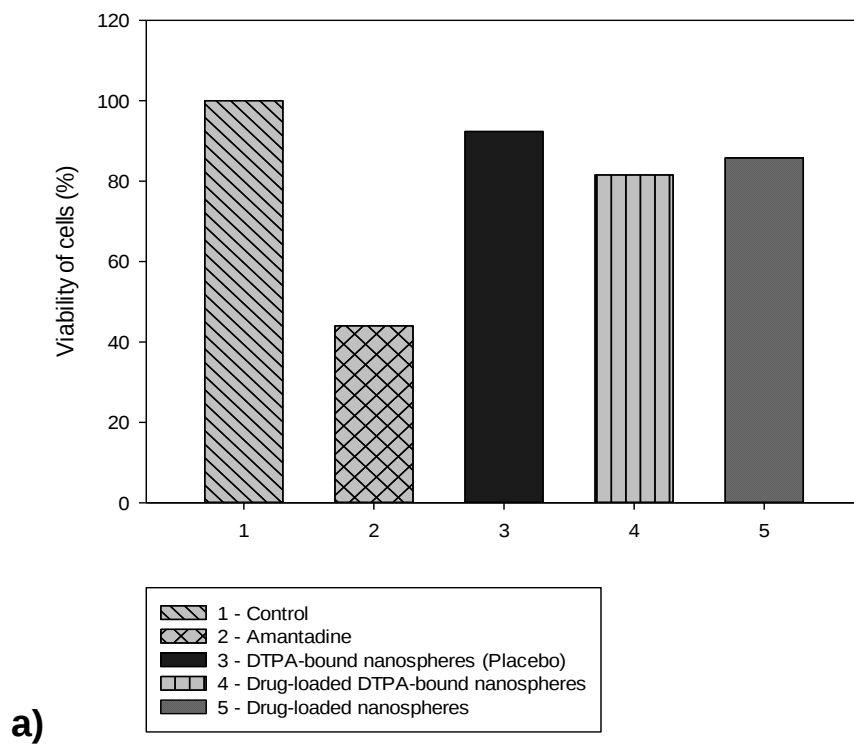
6.3.2. Cell viability

NSC-34 cell viability was determined after exposure to different concentrations of amantadine, drug-loaded DTPA-bound nanospheres, drug-loaded nanospheres (without DTPA), the placebo (DTPA-bound nanospheres without drug) through an MTT assay. The

thermosensitive hydrogel and the nanosphere-hydrogel composite were also tested using the MTT assay at various time intervals. In the concentration dependent cytotoxic experiment (Figure 6.5) the cell viability of NSC-34 decreased with increasing concentration of the all components of the nanospheres. This indicates that the cytotoxicity of the nanospheres was in a concentration dependent manner. Results also showed that amantadine was more toxic in its conventional form than when encapsulated in the nano-enabled drug delivery system with and without DTPA. This suggests that the nanospheres were able to expose the cells to less amantadine when compared to the directly added conventional drug and were able to release the amantadine slowly ensuring gradual exposure of the drug to the cells. The nano-enabled system without amantadine had no significant toxicity.

The polymers used to prepare the nanospheres as well as the chelating ligand were also individually tested for cytotoxicity (Figure 6.6) at different concentrations. The results show that there was also an overall decrease in cell viability with increasing concentration confirming the results obtained in Figure 6.5 that the cytotoxicity is reliant on concentration.

In the time dependent experiment to test the cytotoxicity of the nanospheres, 100µg/ml was selected as the concentration to investigate prolonged toxicity (Figure 6.7). Results show that there was a slight decrease in cell viability with increased incubation time. However, after 72 hours the cell viability almost levels out and stops decreasing. The hydrogel and the nanosphere-hydrogel composite were also tested for prolonged toxicity (Figure 6.7). They expressed similar results to the nanosphere formulation in terms of time dependent cytotoxicity. However, the hydrogel and the nanosphere-hydrogel composite showed less toxicity than the nanosphere formulation. There was insignificant difference in cytotoxicity between the hydrogel and the nanosphere-hydrogel composite.



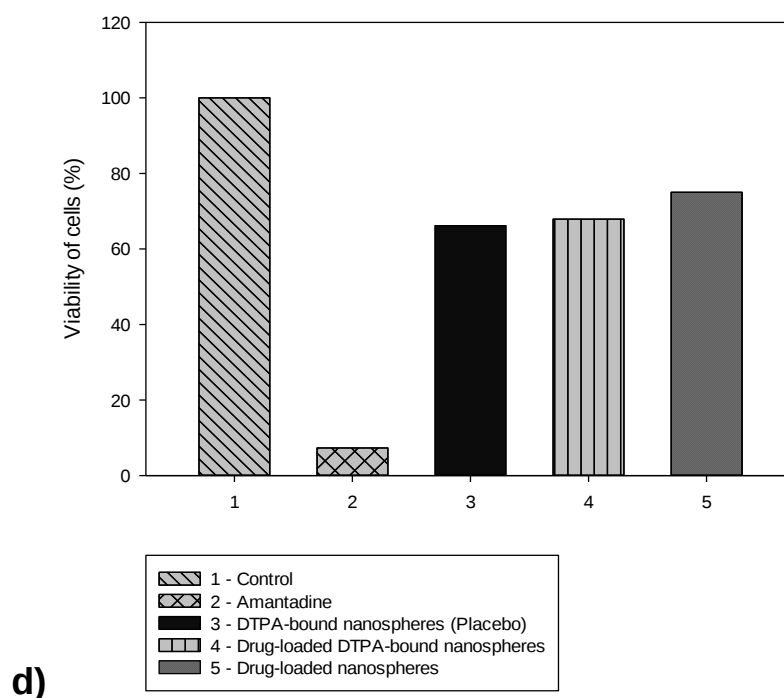
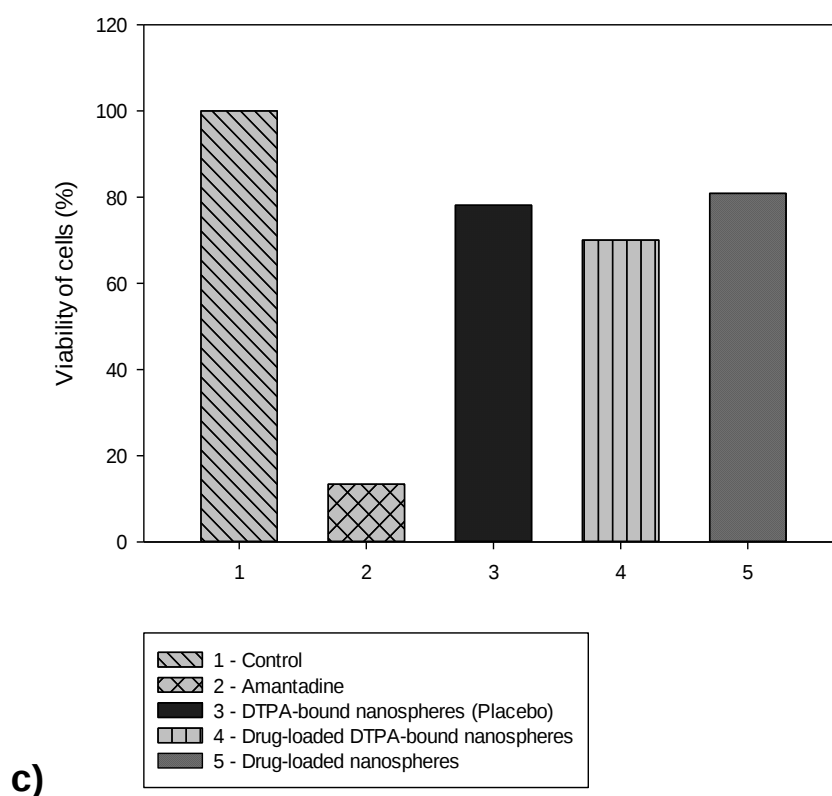


Figure 6.5: *In vitro* cytotoxicity of different concentrations of the various stages of the nanosphere formulation, including amantadine as the positive control and plain cells as negative control, after incubation for 48 hours; a) = 25µg/ml; b) = 50µg/ml; c) = 100µg/ml; d) = 200µg/ml

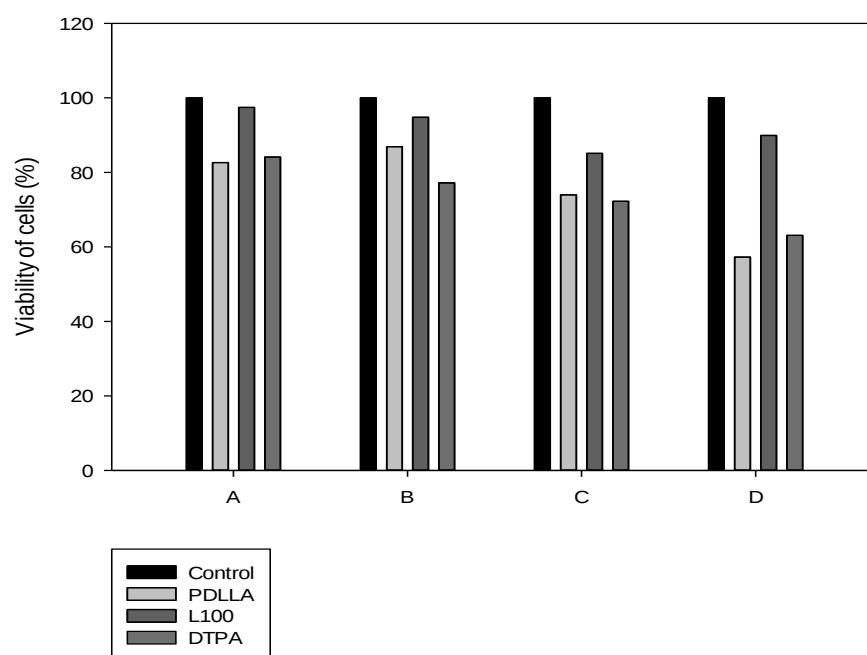


Figure 6.6 *In vitro* cytotoxicity of different concentrations of the polymers used for the nanosphere formulation, including DTPA, after incubation for 48 hours; A = 25 μ g/ml; B = 50 μ g/ml; C = 100 μ g/ml; D = 200 μ g/ml

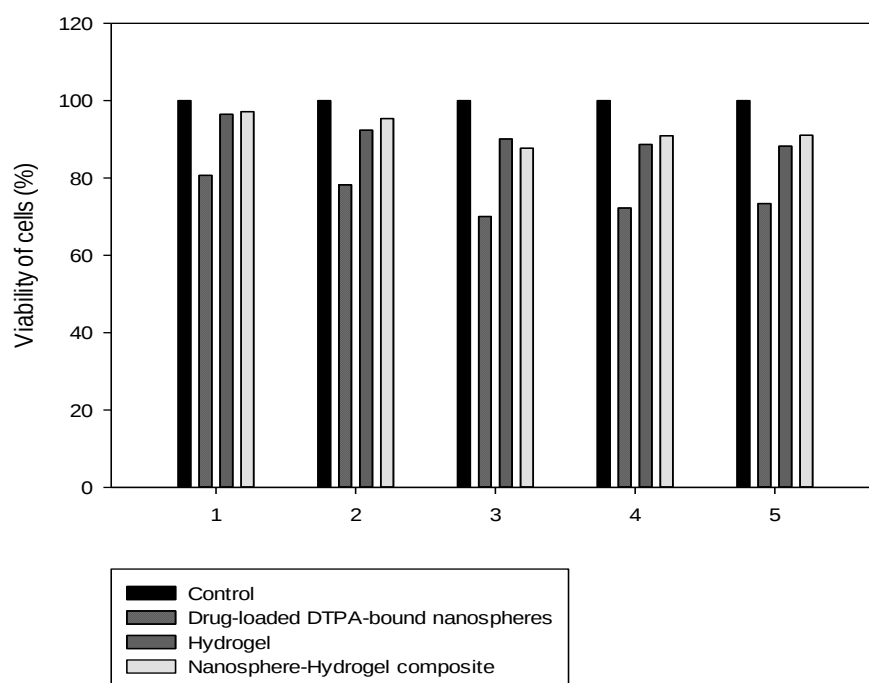


Figure 6.7: *In vitro* cytotoxicity of the complete nanospheres, the hydrogel and the nanosphere-hydrogel composite after incubation at different times; 1 = 12hrs; 2 = 24hrs; 3 = 48hrs; 4 = 72hrs; 5 = 96hrs

6.3.3. *Ex Vivo* Uptake of Modified DTPA-Bound Nanospheres

To investigate the effect of the DTPA on nanosphere uptake, *ex vivo* samples were characterised by fluorescence imaging. Results depicted high cellular uptake of the FITC-labelled DTPA-bound nanospheres (Figure 6.8). Unmodified nanospheres showed no significant fluorescence activity. As discussed in Chapter 4 Section 4.3.6, the DTPA-bound nanospheres had a slightly increased size as compared to the unmodified nanospheres. Small nanoparticles sediment less than larger nanoparticles and their contact with cells is determined by diffusion and convection forces. Conversely, larger nanoparticles settle more rapidly due to the additional influence of sedimentation forces. Well plates containing the DTPA-bound nanospheres were found to show slight sedimentation. It has been reported that nanoparticles with a fast sedimentation rate show higher cellular uptake (Lison and Huaux, 2011).

This study demonstrated that the modified DTPA-bound nanospheres had a greater cellular uptake. This result indicated that intracellular uptake of nanospheres was mediated by the DTPA conjugated onto the nanospheres.

To supplement the uptake studies, fluorescent nanospheres were viewed under the magnification of the CellVizio[®] imaging system from 30 minutes to an hour. Figure 6.9 shows that DTPA-bound nanospheres were most efficiently internalised by the NSC-34 cells in comparison to the unmodified nanospheres. The unmodified nanospheres showed insignificant fluorescence further confirming the results observed in Figure 6.8.

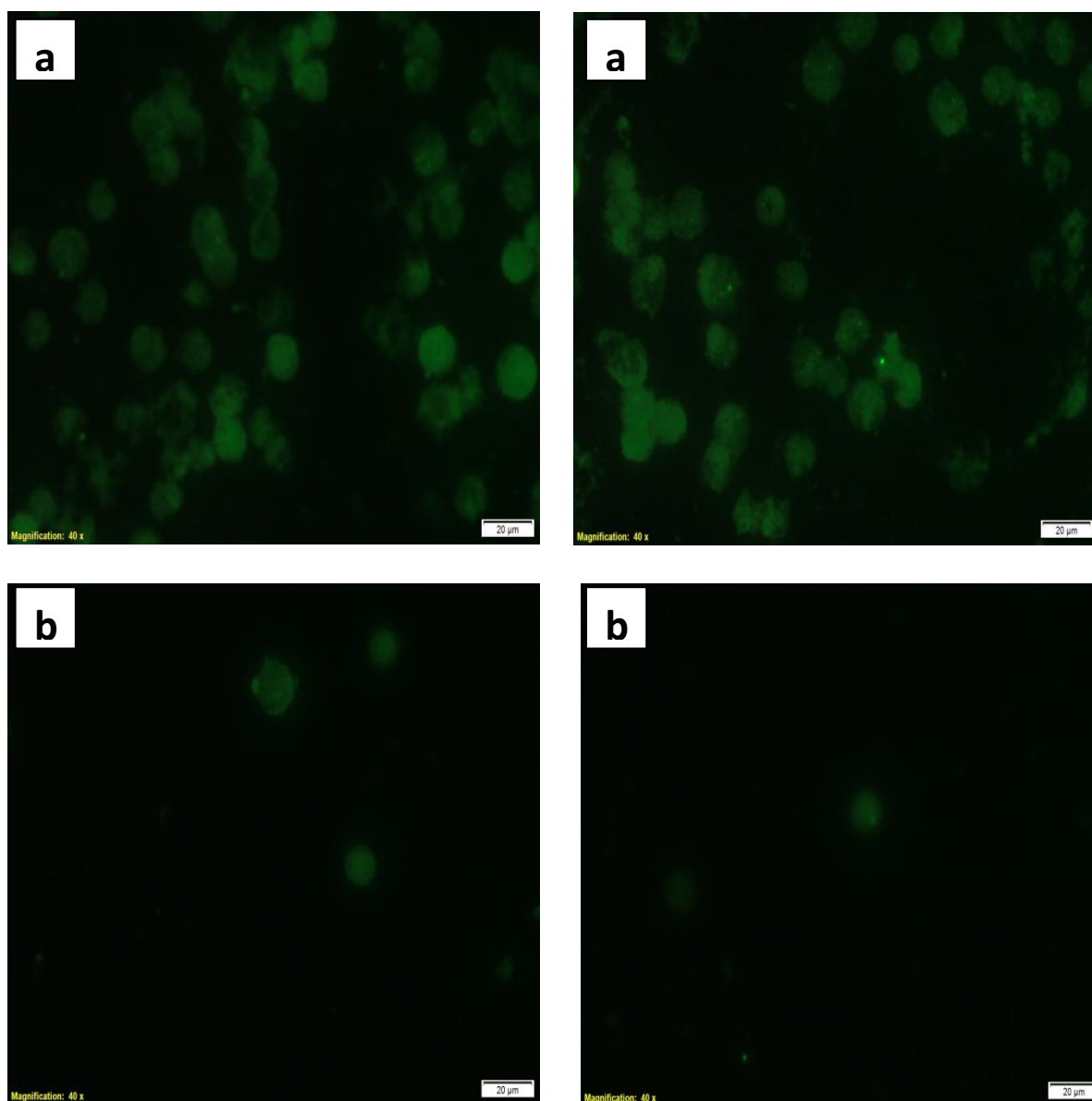


Figure 6.8: Light and microscopy analysis of NSC-34 cell line incubated with FITC labelled
a) DTPA-bound nanospheres; b) unmodified nanospheres

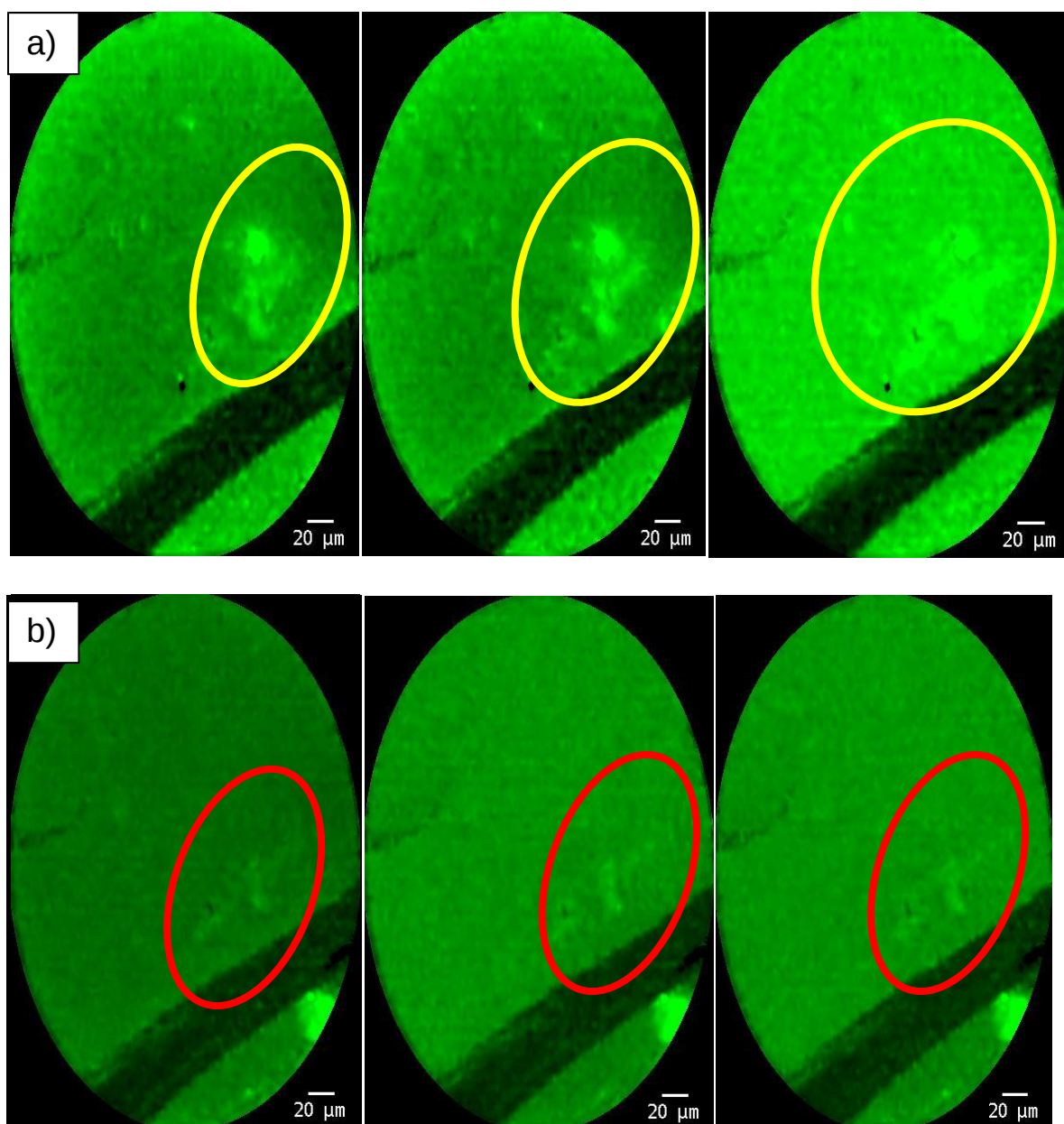


Figure 6.9: CellVizio® imaging of fluorescent nanospheres taken between 30 minutes and an hour; a) DTPA-bound nanospheres; b) unmodified nanospheres.

6.4. Concluding remarks

In conclusion, this study aimed to determine the effect of the components of the nanospheres as well as the elutes of the hydrogel and nanosphere-hydrogel composite on NSC-34 cells using an MTT assay. Concentration dependent studies as well as time dependent studies were performed and results showed that cytotoxicity relied both on concentration and time of incubation with the cells. It was also observed that the nanospheres were able to decrease toxicity of the study drug, amantadine, and the elutes of the hydrogel and nanosphere-hydrogel composite showed very little cytotoxicity towards the NSC-34 cell line.

Cellular uptake studies were also performed. The efficiency of DTPA in increasing the internalisation of the nanospheres by the NSC-34 cells was investigated using fluorescence microscopy and CellVizio[®] imaging. Results showed an increased uptake of the nanospheres that were modified with DTPA compared to unmodified nanospheres. DTPA therefore, mediated the cellular internalisation of the nanospheres.

The nanosphere-hydrogel composite thus presented promising results throughout the *in vitro* testing of the system.

CHAPTER SEVEN

CONCLUSIONS AND RECOMMENDATIONS

7.1. Conclusions

The challenges with managing neurodegenerative disorders due to, among other reasons, the BBB have brought forth the need for innovative development of a drug delivery system that will overcome this obstacle. ALS has a poor prognosis and numerous therapeutic agents have failed to produce much desired results. This study was aimed to design and develop a polymer based nano-enabled drug system for the treatment of ALS. In order to fabricate optimal nanospheres, preformulation studies were performed with poly (D,L) lactide and various polymethacrylates to determine the best combination. The anionic polymethacrylate, Eudragit® L100, was selected as it produced the best results overall, in combination with PDLLA. Amantadine, an antiviral drug commonly used to treat influenza A was chosen as the study drug because it has shown neuroprotective properties. The nanospheres were prepared using the double-emulsion solvent evaporation method. Statistical optimisation was performed employing the 3-factor Box Behnken design after variables (amount of L100, sonication time, solvent volume) were identified. The Box-Behnken design was used to determine the best nanosphere formulation in relation to particle size, zeta potential, drug entrapment efficiency and drug release. Dynamic light scattering was used to determine the particle size, zeta potential and polydispersity index of the nanosphere formulations. All experiments were investigated under simulated physiological conditions i.e. at pH 7.4. Constructed surface plots were used to elucidate the relationship between the variables. The physicochemical properties of the optimised nanospheres were investigated employing various experiments. The surface morphology of the nanospheres was characterized using TEM. The optimised formulation had an average zeta size of 80.41nm, zeta potential of -27.7mV, drug entrapment of 50.92% and a Mean Dissolution Time (MDT) of 19.3 hours. All these values were exceedingly close to the values predicted statistically.

The optimised nanospheres were conjugated to the chelating ligand, DTPA, to increase the uptake of the nanospheres by cells. The DTPA-bound nanospheres had a size of 105.6nm, a Pdl of 0.240 and zeta potential of -31.0mV. The conjugation efficiency was at 74%.

The nanospheres required a carrier in order to administer them to the brain. A thermosensitive hydrogel using ϵ -caprolactone and N-vinylcaprolactam was formulated employing solution polymerisation and investigated. The hydrogel was tested using oscillatory rheology and FTIR. The formulation displayed typical thermoresponsive behaviour with a Lower Critical Solution Temperature (LCST). FTIR spectra showed the blending of the two polymers to form the hydrogel. It was also revealed that there was a minor alteration in the bonds present in the hydrogel when it transitioned from liquid to sol state due to weak physical bonds. SEM showed that the hydrogel did not have a uniform morphology throughout its composition.

The hydrogel formulation was blended with the amantadine-loaded DTPA-bound nanospheres to form a homogenous solution. The nanosphere-hydrogel composite was tested for drug release. The hydrogel was able to greatly decrease the rate of release of amantadine and proved to be an efficient carrier of the amantadine-loaded nanospheres.

All the components of the drug delivery system were tested for possible cytotoxicity on NSC-34 cells. The system showed little cytotoxicity and demonstrated that the nano-enabled system decreased the toxicity of the conventional amantadine.

In conclusion, *in vitro* and *ex vivo* research showed promising results with highly stable nanospheres and an efficient thermosensitive hydrogel as a carrier.

7.2. Recommendations

There is currently not enough research into developing a nano-enabled system specific for ALS. It is recommended that more investigations into nanotechnology for the treatment of ALS should be done. Moreover, further research into amantadine should be considered as it has shown promising results in other neurodegenerative disorders.

In addition, more in depth studies (which would include various characterisation) on the difference between nanosphere preparation with cationic PMAs and anionic PMAs in combination with PDLLA would be useful for broader investigations. Further research can also be done to improve the drug entrapment efficiency as well as the drug release from the nanospheres. The drug release rate of amantadine from the nanosphere-hydrogel composite was however much lower. The gelation temperature of the hydrogel is recommended to be increased from 18.45°C to a temperature closer to body temperature.

Cell studies involving the investigation of the chelating efficiency of DTPA should also be performed because although it was proven that it increases the uptake of the nanospheres

its other function crucial to addressing ALS i.e. removing ions proposed to be involved in oxidative stress was not tested. It was just assumed that it would perform its chelating function from reading previous literature. Additionally, micrographs of NSC-34 cells in the presence of various nanospheres would be valuable for more expansive comparisons.

It would be highly beneficial to further investigate the nanosphere-hydrogel composite in *in vivo* studies. *In vivo* studies would provide additional pharmacokinetic data and determine the efficacy of the nanosphere-hydrogel composite in a suitable animal model. Such a study would also provide information on any unforeseen side effects which cannot be determined by *in vitro* studies alone. There are a few rodent models of ALS which include SOD1 rodent models, TDP-43 models, FUS models and VCP models that can be employed (McGoldrick *et al.*, 2013).

The nanosphere-hydrogel composite could be used to deliver other therapeutic agents for various other disorders that are difficult to treat due to the intricacy of the agents in reaching the target site. Cancer for example requires aggressive treatments which results in healthy tissue being exposed to chemotherapeutics. The drug delivery system designed and prepared for this study could be highly useful in delivering chemotherapeutics to cancerous cells, while avoiding contact with healthy cells. This could improve the efficiency of these therapeutics as well as patient compliance.

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APPENDICES

REVIEW

A Review of the Potential Role of Nano-Enabled Drug Delivery Technologies in Amyotrophic Lateral Sclerosis: Lessons Learned from Other Neurodegenerative Disorders

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ABSTRACT: Amyotrophic lateral sclerosis (ALS) has a multitude of factors implicated in its etiology. The complex neuro-etiology and the restrictive nature of the blood–brain barrier (BBB) have significantly hindered the drug therapy of ALS. Riluzole, a moderately performing drug, is the only agent approved for treating ALS. However, several promising nanocarrier approaches are surfacing that can provide more efficient drug delivery. In addition, biologicals such as stem cells are able to carry neurotrophic factors to their target site, providing motor neurons with the benefits of both, stem cells and neurotrophic factors. This review examines the current drug delivery strategies investigated for optimally treating ALS and related neurodegenerative disorders. Examples include cerium oxide nanoparticles in Alzheimer's disease, odonatalectin, and lactoferrin-coupled PEG–PLGA nanoparticles for urocortin transportation in Parkinson's disease that can also be employed in ALS to bypass the BBB and increase drug bioavailability. A concise incursion into the progress (and lack thereof) made in ALS clinical trials is also discussed. Nanocarriers can potentially eliminate the challenges of poor drug bioavailability in ALS as they have been proven to cross the BBB and reach target sites while minimizing systemic side-effects. Nanocarrier-based delivery of ALS drugs is an area that requires much needed investigation. © 2015 Wiley Periodicals, Inc. and the American Pharmacists Association *J Pharm Sci* 104:1213–1229, 2015

Keywords: amyotrophic lateral sclerosis; nanotechnology; drug delivery; neurotrophic factors; blood–brain barrier; clinical trials; neurodegenerative disorders; bioavailability

INTRODUCTION

An unrelenting predicament that has faced physicians and scientist alike is the failure to conquer the on-going complexity of treating central nervous system (CNS) disorders. Most detrimental of these are the neurodegenerative disorders that gradually lead to the loss of bodily functions and eventually death. Neurodegenerative disorders include, but are not limited to, Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD), and amyotrophic lateral sclerosis (ALS). Extensive research has been carried out in the development of diagnostic tools for early detection as well as successful treatment of these disorders. However, very modest advancement has been achieved. To date, the blood–brain barrier (BBB) remains one of the reasons for the lack of success in the development of treatments as it averts the penetration of therapeutic agents and diagnostic tools. There are a few approaches imminent in tackling the treatment of neurodegenerative disorders including the use of stem cells and antioxidants against mutant forms of the copper and zinc superoxide dismutase (SOD1),¹ as well as nanotechnology which require widespread innovation.

In this review, we look at the various theories for the pathophysiology of ALS and some of the ways these hypotheses led to the various clinical trials. Numerous ALS clinical trials have failed, and for the past decade, only one drug (riluzole) has been approved by the United States Food and Drug Administration (USFDA). The approval of only one drug over the years bears testimony to the very minimal progress that has been achieved in the treatment of ALS. We theorize that some of the unsuccessful therapeutic agents could have prospectively produced better results if firstly clinical trials had suitable, effective designs and secondly, if innovative drug delivery systems were employed to enhance the bioavailability of potential agents. We then look at studies and lessons from similar disorders that incorporate delivery systems to try overcoming the various barriers presented by neurological disorders.

The Hypotheses Surrounding the Cause of ALS

Motor neuron disease (MND) refers to a group of progressive neurodegenerative disorders that are distinguished by the deterioration of upper motor neurons and/or lower motor neurons.² Upper motor neurons have cell bodies located in the motor area of the cerebral cortex and have processes connecting with motor nuclei in the brainstem or the anterior horn of the spinal cord, whereas lower motor neurons have cell bodies located in

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APPENDIX B

Formulation, Optimisation and Characterisation of Drug-loaded Nanospheres for the Treatment of Motor Neuron Disease

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INTRODUCTION

Motor Neuron Disease (MND) is a detrimental neurodegenerative disorder with a poor prognosis. The intricate nature of MND and the blood-brain barrier (BBB) are a prominent reason for the lack of success in therapy advancement. The BBB averts the penetration of therapeutic agents and diagnostic tools, thus obstructing the progress that has been desired. There is therefore a need for a nano-scale drug delivery system that will overcome the BBB and enhance bioavailability of therapeutic agents at the targeted site. This study aimed to formulate, optimise and characterise drug-loaded nanospheres for enhanced treatment of MND.

EXPERIMENTAL METHODS

Nanospheres were prepared using the double-emulsion solvent evaporation technique. A combination of biocompatible and biodegradable polymers was used to formulate the nanospheres, evading the need for surgical removal once the drug-load has been exhausted. These polymers were dissolved together with the drug solution in a solvent mixture of dichloromethane and isopropyl alcohol. The resultant primary emulsion was added to the external aqueous phase to stabilise the formulations. This was followed by lyophilisation for 24 hours.

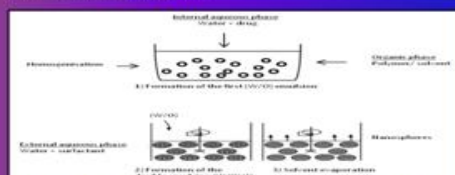


Figure 1: Double-emulsion solvent evaporation method

A 3-factor box-behnken design was employed to produce 15 experimental formulations. The 15 formulations were assessed for their drug entrapment potential. Dissolution studies were performed over 72 hours using a dialysis membrane and phosphate buffer saline (pH 7.4) as the dissolution media.

Characterisation:

The Zetasizer was used to measure zeta potential and to certify that nanosphere sizes were < 100nm. Transmission Electron Microscope (TEM) was used to determine the morphology of the nanospheres.

Optimisation:

From the experimental data, predicted data for the optimised formulation were generated. The optimised formulation was developed and characterised accordingly.

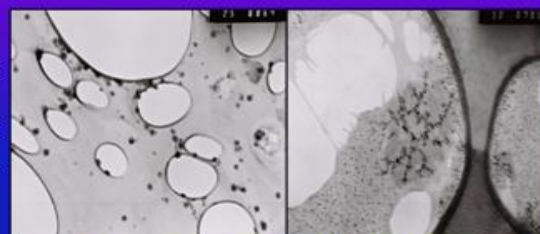


Figure 2: TEM images showing spherical drug-loaded nanospheres

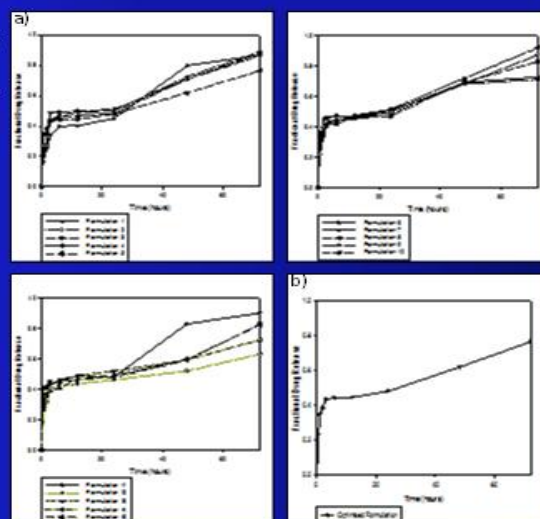


Figure 3: a) Drug release profiles of 15 experimental formulations; b) Drug release profile of optimised formulation

RESULTS

Based on the subsequent responses obtained for the 15 formulations, optimal size, drug entrapment efficiency and drug release were determined by the optimization process. The optimised formulation had an average zeta size of 80.43nm, zeta potential of -27.7mV, drug entrapment of 50.92% and a Mean Dissolution Time (MDT) of 19.3 hours. Distinctive TEM images show nano-sized and spherical particles were formed.

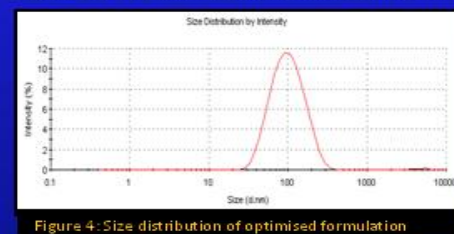


Figure 4: Size distribution of optimised formulation

DISCUSSION

TEM images confirmed the formation of nano-sized particles. Drug-loaded nanospheres were thus successfully prepared. The nanospheres will allow for effortless drug delivery across the BBB and furthermore, intracellular drug delivery. The high zeta potential of the nanospheres indicate that stable particles were formed. These nanospheres are proposed for employment in the sustained intracellular release of drug for the treatment of MND.

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