

A HYBRID THERMO-RESPONSIVE POLYMER SYSTEM FOR APPLICATION IN WOUND HEALING

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A dissertation submitted to the Faculty of Health Sciences,
University of the Witwatersrand,
in fulfilment of the requirements for the degree of
Master of Pharmacy

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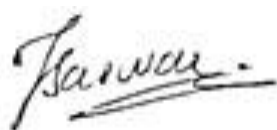
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Johannesburg, January 2022

DECLARATION

I, Taskeen Sarwan, declare that this dissertation is my own work. It is being submitted for the degree of Master of Pharmacy 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.....26.....day of January 2022

PUBLISHED OUTPUTS

Taskeen Sarwan, Pradeep Kumar, Yahya E. Choonara, & Viness Pillay. Hybrid Thermo-Responsive Polymer Systems and Their Biomedical Applications. *Frontiers in Materials*, 73, 2020. <https://doi.org/10.3389/FMATS.2020.00073>

RESEARCH PRESENTATIONS

Taskeen Sarwan, Pradeep Kumar, Yahya E. Choonara, & Viness Pillay. In-situ thermogelling systems for the treatment of wounds. BRICS Multilateral Joint Science and Technology Research Collaboration Seminar. 2021. eZone, University of the Witwatersrand, Johannesburg, 4 November 2021. International conference output.

Taskeen Sarwan, Pradeep Kumar, Yahya E. Choonara, & Viness Pillay. In-situ thermogelling systems for the treatment of wounds. Global Young Academy Young Scientist Ambassador Programme 2020. University of the Witwatersrand, Johannesburg, 17 November 2020. Virtual Presentation. International conference output.

ANIMAL ETHICS DECLARATION

I, Taskeen Sarwan, hereby confirm that the following study entitled “**Observing the effects of an injectable thermo-responsive polymer system on Sprague Dawley rat model with surface cut wound**” received the approval from the Animal Research Ethics Committee of the University of the Witwatersrand with ethics clearance number 2020/08/06/D (Appendix D).

ABSTRACT

The understanding of wound healing in the current decade has evolved from merely recognizing the three phases of the wound healing cascade, to having to take into consideration the complex cellular reactions and dynamic processes that promote the physiological wound healing process. Wounds are a global burden, and inadequate wound care has detrimental outcomes in terms of the patient's quality of life, increased hospitalizations due to infections, and added financial strain on the economy. Traditional wound management systems focus primarily on covering the wound and preventing infection. While this is important, these systems play a limited role in actively promoting the wound healing process. A better approach would be to develop a wound healing system that creates an optimal environment for the wound healing process to occur, and actively promotes this process. This approach has been adopted by researchers in the field of wound management. As such, hydrogels have been deemed a favourable option in dealing with wounds as they provide the optimal environment for rehydration, gaseous exchange, angiogenesis, proliferation of connective tissue and re-epithelialization. In recent years, advancements in the field of pharmaceuticals have seen the development of in-situ hydrogel forming systems with the use of "smart" stimuli responsive materials.

Targeted and controlled drug delivery employing "smart materials" is a widely investigated field, within which stimuli-responsive polymers, particularly those which are thermo-responsive, have received considerable attention. Thermo-responsive polymers have facilitated the formulation of in situ gel forming systems which undergo a sol-gel transition at physiological body temperature, and have revolutionized the fields of tissue engineering, cell encapsulation, and controlled, sustained delivery of both drugs and genes. However, the use of single thermo-responsive polymers in the creation of these systems has posed numerous problems in terms of physico-mechanical properties, such as poor mechanical strength, high critical gelation concentrations (CGC) resulting in increased production costs and solutions that are too viscous, toxicity, as well as gelation temperatures that are incompatible with physiological body temperatures. Hybridization of these thermo-responsive polymers with other polymers has therefore been employed, resulting in the creation of tailor-made drug delivery systems that have optimal gelation temperatures and concentrations, ideal viscosities, and improved gel strengths.

Thus, the aim of this research was to create a hybrid thermo-responsive polymer system that actively encourages and facilitates the wound healing process. This was achieved with use of polymers such as chitosan and cellulose that are thermo-responsive but are also known to have positive effects in terms of promoting wound healing. Hybridizing cellulose with chitosan allowed for the development of a thermo-responsive polymer system that displayed gelation characteristics suitable for in-situ gelation, adequate gel strength, was bio-erodible and bio-compatible.

Rheological analysis was conducted to determine the gelation kinetics of the formulation, as well as its suitability in terms of mechanical strength, structural behaviour in response to temperature and viscosity for ease of application. Analytical methods such as Fourier Transform Infrared Spectroscopy (FTIR), Differential Scanning Calorimetry (DSC), Thermogravimetric Analysis (TGA) and morphological analysis were conducted to determine the physico-chemical nature of the system. The swelling and erosion behaviour, as well as the release kinetics of the system were also evaluated. Finally, in-vitro cytotoxicity analysis, migration studies and in-vivo animal studies were conducted to determine the efficacy of the system in healing wounds.

The chitosan-cellulose hybrid thermo-responsive system was able to form a gel at physiological temperature within twelve minutes, while remaining in a sol state at room temperature. The low viscosity of the system at room temperature enables ease of application, while the increase in viscosity and mechanical strength of the system at 37°C will enable the system to withstand any physiological stressors at the wound site. The system proved to be bio-erodible and was also able to release the incorporated trans ferulic acid during the initial stages of wound healing, when it is needed. The chitosan—cellulose hybrid system proved to not only be non-cytotoxic, but stimulated cell proliferation as well. Favourable results in terms of efficacy of the system were seen both in-vitro and in-vivo. In-vivo analysis confirmed the wound healing potential of the system, in that it actively encouraged the wound healing process, displayed inherent bioactivity, accelerated the rate of wound closure, and promoted tissue regeneration as opposed to healing with mere tissue repair and the formation of scar tissue. The greatest percentage closure at each time point was exhibited by the pristine chitosan-cellulose based gel treatment group, with a 61% closure on day three of healing, 88% closure on day seven of healing, and a 98% closure rate at the end of fourteen days, with a notable normal epidermis displayed at the end of the wound healing period.

ACKNOWLEDGEMENTS

First and foremost, all praise and gratitude is due to the Almighty, as this journey was only possible through His guidance, mercy and divine help.

I am indebted to numerous individuals for their invaluable time, wisdom, guidance, and support. I would like to thank Professor Yahya Choonara for his valuable input in this study. His critical analysis and vast knowledge of Pharmaceutical Sciences and drug delivery were of great benefit in the undertaking of this research. The great passion and enthusiasm for research and the pharmaceutical sciences shown by Professor Choonara and Professor Viness Pillay play a huge role in making the Wits Advanced drug Delivery Platform what it is and is an inspiration to us all. I am extremely grateful to have been part of such a prestigious research group, and one that strives to break through the barriers of science.

I would like to express my sincere appreciation and gratitude to my supervisor Professor Pradeep Kumar, for encouraging me to undertake this journey and for the opportunity to complete my masters under his exemplary mentorship. I am extremely lucky to have been able to take benefit from his years of expertise and experienced guidance and would like to thank him for imparting upon me invaluable knowledge, skills, and life lessons.

My sincere gratitude goes to the excellent staff and lab technicians in the Department, Mr Kleinbooi Mohlabi, Mr Bafana Temba, Ms. Tshidi Mosete, Ms. Nompumelelo Damane, Mr Sello Ramarumo and Dr. Hillary Mndlovu for their immense help.

A special thanks goes to Dr. Philemon Ubanako for his guidance, expertise, time, and encouragement. His positive attitude and patience made the daunting task of cell work much lighter.

To Dr. Farzana Mahomed and Dr.Zvifadzo Matsena-Zingoni for selflessly volunteering their time and expertise and assisting me, thank you very much.

To the team at the Central Animal Services at the University of the Witwatersrand not only for making the in-vivo analysis of this research possible, but for making it a positive

experience as well. I would like to express my appreciation to Henna Cassimjee and Michelle Lategan for their time and assistance in carrying out animal studies.

I would like to express my gratitude to the National Research Foundation of South Africa for their financial assistance in undertaking this research.

I am forever indebted to my wonderful parents, Sabier and Shanaz Sarwan. Thank you for all your sacrifices and hard work in providing me with the opportunities that have allowed me to be where I am today. It is only through your unconditional love and support, encouragement, guidance, and countless prayers that I have come thus far in life. Thank you for always supporting me in my ventures and encouraging me to reach for the stars. I am truly honoured to share this accomplishment with you, and I hope that I have made you proud. I can never thank you enough for all that you are and all that you have done for me, I love you both with all my heart.

To my very best friend, Talha Nanabhay, I am eternally grateful. Thank you for being the most wonderful, caring, and loving husband. Thank you for your patience, encouragement, and unwavering support, always. Thank you for standing by my side, for being my shoulder to cry on, for picking me up when I am down, and believing in me even when I have little faith in myself. This journey has not been easy, and I truly appreciate the countless hours that you have put in with me. You make every day better, and I could not have done this without you.

To my amazing brother Muhammad Faheem, you are the epitome of a bigger brother. Thank you for always being so caring, and for all your advice and encouragement throughout the years. To my sister-in-law Aadilah, thank-you for your kind words, encouragement, and unfailing support. I love you both and appreciate you more than you know.

Finally, to my parents-in-law, Imtiaz and Haseena Nanabhay, and brothers-in-law, Uwaiz and Ahmed, your support and prayers played a vital role in the achievement of this degree, and for that I am truly grateful.

DEDICATION

This work is dedicated to my beloved parents, Sabier and Shanaz Sarwan. I would not be where I am without your love, support, guidance, and prayers. I am, because of you.

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CHAPTER ONE

INTRODUCTION

1.1 Background to this Study

Wounds have been termed “The Silent Epidemic”. In the past decade, an estimated 27-50% of hospital beds were occupied by patients requiring wound management (Lindholm and Searle, 2016). The burden of wound management is global, and to adequately treat wounds, a thorough understanding of the functions and anatomy of the skin is needed (Hess, 2011). Loss of integrity of large portions of the skin through injury and wounds not only affects normal bodily functions, but also results in a breach in the body’s first line of defence (Zhong et al., 2010).

A wound is a tear in the skin resulting in the integrity of the skin being compromised and a decline or loss of function of the skin (Boateng et al., 2008). Wounds may progress into ulcers and tumours should they not heal adequately (Shanmagasundaram, 2005). Infection of the wound is a major concern as this in turn delays the healing time, complicates the treatment process, and may even threaten loss of the limb and systemic spread of the infection. Therefore, optimal wound care is imperative. The goal of wound healing systems is to heal the wound in the shortest period of time with minimal scarring, and the prevention of infection by preventing microbial invasion of the wound.

Wound care is one of the most challenging aspects of medicine (Garwood and Steinberg, 2016). The wound healing cascade is traditionally divided into three overlapping phases, namely: inflammation, proliferation, and remodelling (Li et al., 2012). These complex reactions between cells and mediators result in a specific environment being created to facilitate the wound healing process. Therefore, an advanced wound care system is needed that actively promotes and facilitates this optimal environment to enhance the wound healing process. Numerous wound healing systems have been marketed in the past, however many of these systems lack the required characteristics that are needed to facilitate adequate wound healing and promote an optimal wound environment (Archana, Dutta and Dutta, 2013).

The ideal wound healing system should provide a moist wound environment that facilitates the rehydration of desiccated tissue thereby promoting angiogenesis and proliferation of connective tissue (Halim, Khoo and Mat Saad, 2012). It should also allow for the debridement of necrotic tissue and foreign bodies, as well as promote the absorption of excess exudates that would hinder cell proliferation and activity. Gaseous exchange should be facilitated to stimulate the activity of fibroblasts and promote angiogenesis (Mayet et al., 2014). The system should also be easily removed or allow for biocompatible disintegration to avoid causing pain to the patient or further tissue damage when removing, reapplying, or changing the dressing. A low frequency of reapplying or dressing changes to promote patient compliance and cost efficiency would be practical, therefore adequate tensile strength and durability are also needed (Boateng et al., 2008). Lastly, the promotion of collagen synthesis and tissue repair and proliferation, as well as the prevention of microbial invasion which often results in complications such as wound infection, tissue damage, and delays in the wound healing process, are important factors to be considered when formulating the ideal wound healing system.

1.2 Rationale and motivation for this Study

Traditional wound dressings such as gauze play a limited role in actively encouraging the wound healing process (Mayet et al., 2014). Hydrogels do not merely play a protective role by covering the wound, but actively promote the wound healing process by creating an environment that facilitates wound healing. A moist environment needs to be created to provide for rehydration, angiogenesis, and proliferation of connective tissue (Mayet et al., 2014). In addition, the system needs to absorb excess exudates that may hinder cell production and produce an odour (Mayet et al., 2014). Gaseous exchange is essential to facilitate the production of granulation tissue, the epithelialization process, and the contraction of the wound so that secondary wound closure may occur (Mayet et al., 2014). Hydrogels consist of a liquid phase entrapped within a crosslinked polymer network (Kamoun et al., 2017). Due to the many hydrophilic groups present within hydrogels, they are capable of retaining a large amount of water or bodily fluids. Therefore, hydrogels have been deemed ideal in wound healing applications as they have the ability to maintain a moist wound environment, remove excess wound exudate and allow for gaseous exchange. In addition, their ease of application, low frequency of change, cost effectiveness and

non-irritant nature all play a role in promoting patient compliance, further advocating their use.

Recent advancements in the use of hydrogels for biomedical applications has seen the development of in-situ hydrogel forming systems (Klouda, 2015) which are in a sol state prior to administration, but undergo gelation once exposed to physiological conditions. One such mechanism by which this has been achieved is through temperature modulation (Ahmed, 2015), resulting in thermo-responsive hydrogel systems for drug delivery. This study will involve the use of thermo-responsive hydrogels in the creation of an ideal wound healing system for optimal wound management. The advantage of thermo-responsive hydrogels over conventional wound healing systems is that they are able to fill any wound size, shape and depth, resulting in homogenous distribution of the hydrogel within the wound area.

Furthermore, the potential impact of the study are as follows:

- The concept of thermo-responsive hybrid polymer systems for wound management is a relatively novel research area, thereby serving as a gateway with opportunities for future expansion and knowledge generation. This is in keeping with the United Nations Sustainable Development Goals (UN SDG) for good quality education.
- The ninth UN SDG for innovation and infrastructure is also contributed to by this research. Should the proposed research yield positive results, it will be a huge stride toward the development of smart biopolymer hybrid systems for drug delivery, as well as an improvement in the current approach to treating wounds. A successful wound healing product will be launched on the market with better treatment outcomes and quicker healing times.
- Financial strain on the economy from the burden of wounds will be relieved. When wounds are not adequately treated, they become debilitating, as not only are patients unable to work and contribute to the economy, but disabilities resulting from wounds also lead to more people becoming reliant on social welfare and disability grants. This success of this research will aid in alleviating this burden, thereby aligning with the UN SDG for decent work and economic growth.
- Infected wounds result in an increased hospital stay at a cost to both the patient and the healthcare system, as well as an increase in the need for antibiotic use,

thereby contributing to antimicrobial resistance. The proposed project is a revolutionary approach to wound treatment, aiming to meet all requirements of an ideal wound healing system to promote quick and effective wound healing, thereby preventing secondary infections and mitigating the need for antibiotic use, aligning with the fourth UN SDG to promote good health and wellbeing, as well as reduce economic strain in an effort to alleviate poverty, which is the first goal of the UN.

- The cosmetic implication of wounds is yet another aspect in which the proposed system aims to produce better outcomes by reducing the residual scar tissue, thereby improving both the quality and quantity of life of the patient.
- Patient compliance and ultimately treatment outcomes will be improved, as the system aims to be convenient and non-irritant, with a reduced need to change the wound dressing often.

1.3 Aim and objectives of this Study

The aim of this study is to develop a hybrid thermo-responsive sol-gel system for optimal wound healing. The key objectives identified to achieve this aim are as follows:

1. To select two compatible, biodegradable, thermo-responsive polymers in the creation of a hybrid thermo-responsive polymer system that is capable of forming a gel at the physiological temperature of 37°C.
2. To determine the thermo-responsive gelling properties (gelling temperature) of the hydrogel by conducting temperature dependant rheological and viscoelastic analysis. The first and second objectives are outlined in chapter three of this dissertation.
3. To determine the physiochemical and mechanical properties of the hybrid system via appropriate analytical studies, so that the molecular, thermal and phase transitions, water absorption, structural changes and degradation of the system can be seen.
4. To incorporate a bioactive with wound healing properties and determine the release of the bioactive from the system, to confirm the efficacy of the drug delivery system. The third and fourth objectives will be outlined in chapter four of this dissertation.

5. To assess the wound healing potential of the system by conducting in vivo animal studies. The final objective is outlined in chapter five of this dissertation.

1.4 Novelty of this Study

The design of a novel system for wound healing is achieved by the formulation of a hybrid thermo-responsive in-situ sol-gel system, capable of filling irregularly shaped wounds, resulting in homogenous distribution of the system and the incorporated bioactive. The system is biocompatible and novel in that it is inherently bioactive, and promotes an optimal environment needed to facilitate and actively enhance the wound healing process, with or without the incorporation of a bioactive.

1.5 An Injectable Thermo-Responsive System for Wound Healing: Concept and Outline

The wound healing cascade consists of three phases, namely: inflammation, proliferation, and regeneration. The initial phase, inflammation, is characterized by localized swelling, erythema, and heat. Studies have shown that the average temperature of a wound during inflammation is approximately 38-39°C (Chanmugam et al., 2017). By using thermo-responsive polymers, this project aims to create a thermo-responsive polymer sol-gel system that is capable of forming a gel within a temperature range of 37-39°C. The system will be in a sol state at room temperature and will thus be applied via syringe into the wound area. The solution will then settle into the depth and shape of the wound, forming a gel once in contact with the wound area and exposed to the increased wound temperature. Sterile gauze can thereafter be applied to the wound as a secondary dressing.

Setbacks that have been identified in terms of single thermo-responsive systems include low mechanical strength when they are composed of only a single polymer (Kamoun et al., 2017), as well as unsuitable gelation temperatures and concentrations. For this reason, the project aim is to combine two thermo-responsive polymers to create a hybrid thermo-responsive hydrogel, with improved mechanical strength and performance. By hybridizing thermo-responsive polymers, the ideal gelation temperature and time can also be achieved. Hybridizing polymers leads to novel polymer combinations with combined characteristics that are more favourable than

each individual polymer in terms of mechanical, chemical, and biological properties (Mayet et al., 2014). Hybridizing polymers to create a hybrid thermo-responsive polymer system can also be achieved by combining a thermo-responsive polymer with polymers that are not thermo-responsive.

Thermo-responsive polymers are capable of undergoing a phase transition in-situ (Klouda and Mikos, 2008). Among the thermo-responsive polymers that will be investigated for use in this study are polymers which have displayed favourable properties for wound healing, to create a system with inherent bioactivity. Chitosan- β -Glycerophosphate is an example of such a polymer system. Due to its non-toxic, biocompatible, biodegradable (Patois et al., 2008), haemostatic (Janvikul et al., 2006), antimicrobial (Agnihotri et al., 2004), and anti-inflammatory nature, Chitosan is an attractive biomaterial in wound healing. Its monomer, N-acetylglucosamine, is found in an extracellular macromolecule which is essential for wound healing. Chitosan plays an active role in all stages of wound healing. It stimulates haemostasis during the initial phase of wound healing, assists with differentiation, re-epithelialisation, and fibroplasia, and accelerates tissue regeneration.

Other examples include Pluronic F127 and methylcellulose, with favourable properties such as water solubility, film-forming capabilities, thermo-intelligence, cost effectiveness and lipid barrier functionality (Chatterjee et al., 2018). These thermo-responsive polymers and their role in stimuli-responsive drug delivery systems will be further discussed in the second chapter of this dissertation.

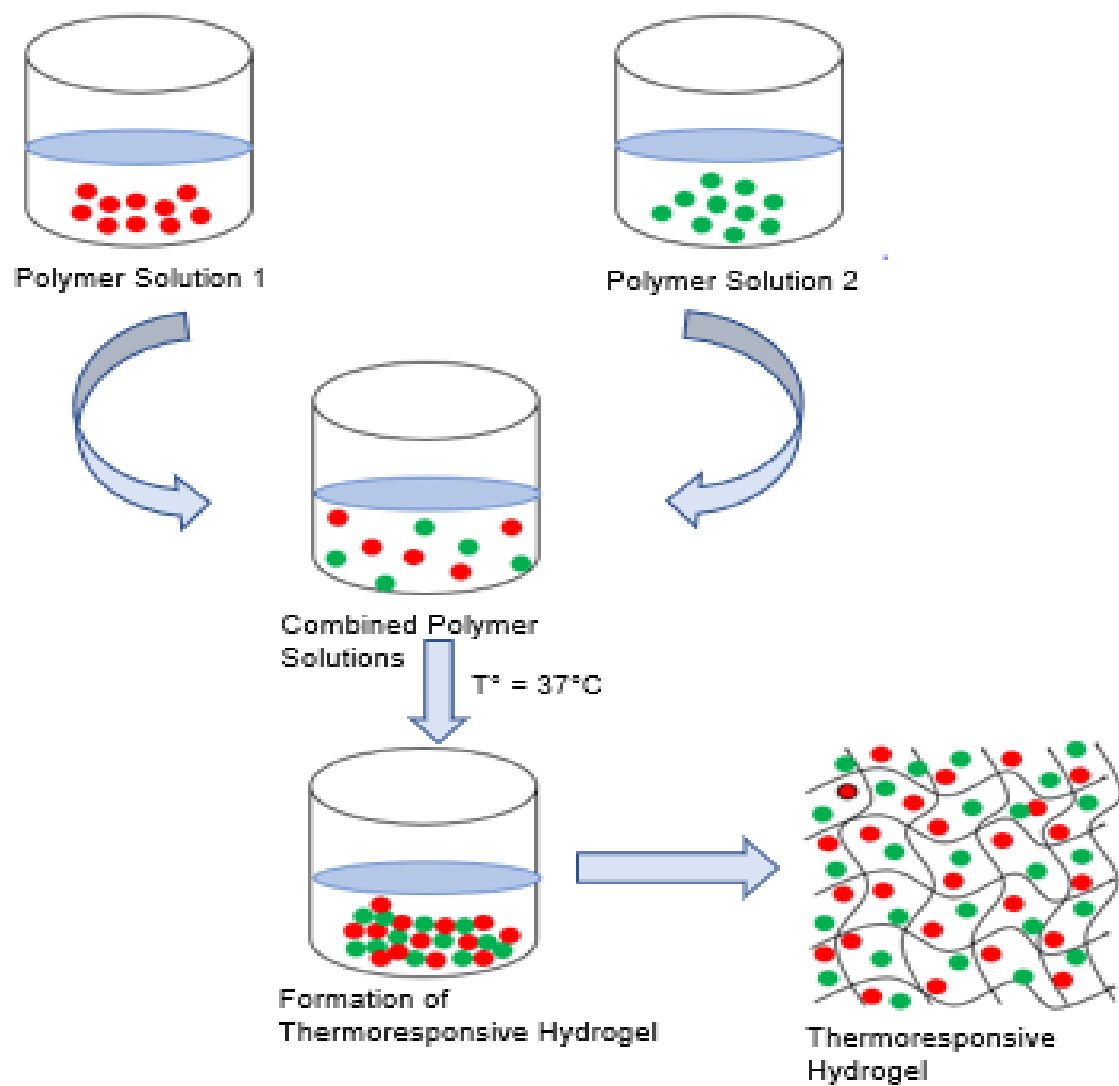


Figure 1.1: Schematic of the proposed thermo-responsive hybrid polymer hydrogel system.

1.6 Overview of this dissertation

Chapter One of this dissertation provides a brief background to the study regarding the burden of wounds and factors affecting wound management. A rationale for the proposed system to address the wound management problem is then given. An overview of the proposed system, the novelty of the study, as well as the aims and objectives are listed.

Chapter Two of this dissertation discusses various thermo-responsive polymers, including those which are employed in this study. The chemical properties, as well as the mechanisms of gelation of these thermo-responsive polymers, are detailed. This

chapter also highlights the hybridization of these polymers in the creation of novel tailor-made “smart” responsive systems. Applications of these combinations in the biomedical field are reported.

Chapter Three of this dissertation focuses on the synthesis of an in-situ sol-gel system by employing hybridization of a natural thermo-responsive polymer, cellulose, with the semisynthetic thermo-responsive polymer chitosan- β -glycerophosphate, and the synthetic Pluronic F127 respectively. The gelation properties of these systems including the gel strength, viscosity, and rheological behaviour with temperature variations are discussed in this chapter.

Chapter Four of this dissertation provides insight into the physiochemical properties of the hybrid system. The molecular, thermal and phase transitions, morphological properties and swelling and erosion behaviour of the system are reported. Incorporation of a bioactive into the system and release kinetics of the system are discussed in this chapter as well.

Chapter Five of this dissertation focuses on the safety and efficacy of the final hybrid thermo-responsive polymer system, employing cellulose and chitosan- β -glycerophosphate. The in-vitro and in-vivo toxicity, together with the potential for wound healing are investigated and discussed.

Chapter Six summarizes and concludes the study and makes recommendations for future works and developments.

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Hybrid Thermo-Responsive Polymer Systems and Their Biomedical Applications

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2.1 Introduction

Stimuli responsive drug delivery systems have been widely investigated due to their role in facilitating targeted and controlled drug delivery (Thambi and Lee, 2019). Among the stimuli responsive systems, thermo-responsive drug delivery systems have received considerable attention, and will thus be the focal point of this chapter (Mura et al., 2013; Huang et al., 2019). Thermogels are gels that undergo an in-situ sol-gel transition by virtue of the difference in their ambient storage temperature and the physiological temperature of the body (Kumar et al., 2018; Figure 2.1). Ideally, thermogels should be free flowing solutions at room temperature, and only form gels once administered into the body (Ruel-Gariépy and Leroux, 2004). This thermo-responsive behaviour is attributed to a change in the hydrophilic and hydrophobic interactions among the polymer molecules and water molecules that make up the solution (Bajpai et al., 2008; Shah et al., 2018).

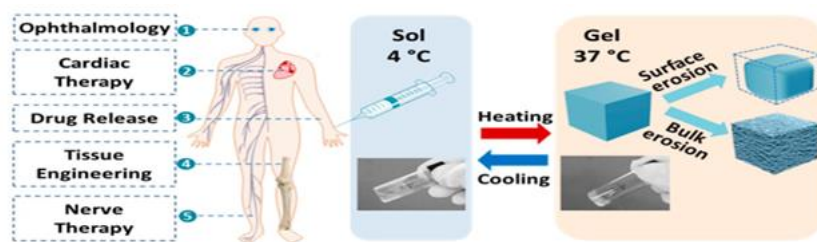


Figure 2.1: A graphical abstract of thermo-gelling systems and their biomedical applications. Reproduced with permission from (Liow et al., 2016). Copyright 2016 American Chemical Society.

Among the decisive properties associated with thermo- responsive gels are the critical gelation concentration (CGC) and critical solution temperature (CST). The CGC is defined as the concentration of a material that results in thermo- gelation (Matanovic' et al., 2014). This is a crucial consideration in the formulation of thermogels (Bromberg and Ron, 1998), as it in turn affects the viscosity, gelation temperature and practicality of the formulation in terms of both usability and production costs. The CST is the temperature that is noted when phase separation of the solution occurs (Saeki et al., 1976). When polymers gel upon heating, they have a lower critical solution temperature, below which they are soluble and above which they become insoluble and hydrophobic (Matanovic et al., 2014). Conversely, when polymers gel upon cooling, they display an upper critical solution temperature (Peppas et al., 2000). Hybridization of thermo-responsive polymers with other materials has allowed researchers to formulate ideal thermogels to suit their requirements, thereby mitigating issues that arise when the gelation concentrations of thermo-responsive polymers are too high, or gelation temperatures are not feasible for in situ gelation.

Properties of thermogels that make them advantageous for biomedical applications as opposed to existing, conventional drug delivery systems are as follows: (1) Thermogels mitigate the need for invasive surgical procedures and have a faster onset of action and better absorption as they by-pass first pass metabolism. Their sol-phase at ambient temperatures allows them to be injected at the site of action either intravenously or subcutaneously and take the shape of the area that they are injected into, where possible (Jeznach et al., 2018; Loh et al., 2018). (2) Thermo-gelling systems serve as a depot once injected into the body, mitigating the need for multiple daily dosing, thereby improving patient compliance and resulting in better therapeutic outcomes (Loh et al., 2007). Sustained release of a constant amount of drug is

possible with thermogels, making it ideal in the treatment of chronic conditions (Liow et al., 2016). (3) Drug levels can be controlled, thus promoting safety of the patient. The need to administer a single dosage of drug at a high concentration is mitigated, thereby reducing the risk of systemic toxicity and unwanted adverse effects due to drug levels fluctuating or rising above safe concentrations. (4) The water content within the matrix of thermogels promotes their biocompatibility and enables them to serve as a system for drug delivery and lubrication in sensitive areas of the body (Caramella et al., 2015). (5) Most thermogels are easy to manufacture with the use of aqueous solvents, and are physically crosslinked in the presence of heat, thereby mitigating the need for organic solvents or crosslinking agents (Yan et al., 2015). This facilitates the delivery of proteins, peptides and genes via thermogels, by decreasing the risk of denaturation (Packhaeuser et al., 2004).

Thus, thermo-responsive systems have revolutionized the field of drug delivery. However, the use of single thermo-responsive polymers in drug delivery applications does have many setbacks which need to be overcome. These include: (1) high gelation concentrations which affects both manufacturing costs and convenience of application, (2) poor gel strengths resulting in very low viscosity gels which are unsuitable for certain applications, (3) high gelation temperatures that do not correlate with physiological body temperature, thereby not facilitating in situ gelation, and occasionally, (4) cytotoxicity. Hybridization of these single thermo-responsive polymers has allowed for the creation of tailor-made, targeted delivery systems with physico-mechanical properties precisely suited to the needs of the system (Mayet et al., 2014; Zarrintaj et al., 2019). These in situ gel forming systems have been shown to serve as excellent matrices for cell encapsulation, tissue engineering, and targeted, prolonged delivery of both drugs and genes. Their sol phase at ambient temperatures enables ease of application by injection at the desired site of action, and their ability to release drugs and genes in a sustained manner allows for less frequent dosing and administration, thereby contributing toward improved patient compliance and treatment efficacy.

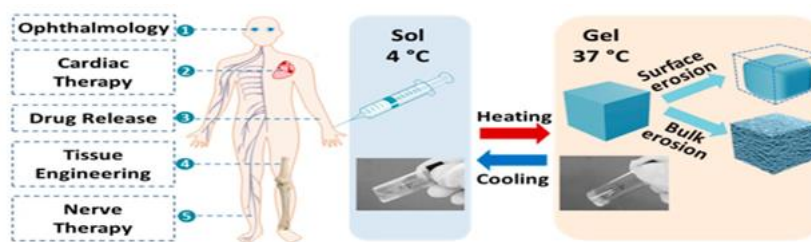


Figure 2.1: A graphical abstract of thermo gelling systems and their biomedical applications. Reproduced with permission from

This chapter is a review of key thermo-responsive polymers that have been used to form in situ gelling systems, namely: chitosan- β -glycerophosphate, poly (N-Isopropylacrylamide) (pNIPAAm), pluronic F127, methylcellulose and PEG- PCL. Special focus is given to the hybrid thermogels that have been formulated from each polymer, the advantages or reasons for hybridization and their applications in the biomedical field. This chapter serves as the foundation of our understanding of various thermo-responsive polymers, their mechanisms of gelation, and the hybridization of these polymers to suit our requirements in the formulation of our in situ thermo-gelling system for wound healing.

2.2 Hybrid Thermo-responsive Polymer Systems

2.2.1.1. Chitosan Based Thermogels

Chitosan is a biodegradable, biocompatible polysaccharide that has been widely used in drug delivery applications. Consisting of N-acetyl-D-glucosamine and b-(1,4)-linked D-glucosamine that is randomly arranged, chitosan is a cationic polymer that is hydrophilic and has gel forming ability (Kristl et al., 1993). When the pH of a chitosan solution is increased to 7.2, chitosan can gel at body temperature. The first chitosan-based thermogel was created by Chenite et al. (2000). Chenite et al. (2000) developed a thermo-responsive gelling system by the addition of β -glycerophosphate, a polyol salt, to pH responsive chitosan solution. The β -glycerophosphate serves to increase the pH of the acidic chitosan to 7–7.4 by acting as a weakly basic salt, allowing a gel to form in a controlled manner at 37°C, while maintaining its liquid state at 10°C (Chenite et al., 2000, 2001; Ruel-Gariépy et al., 2000). The resulting solution was a clear liquid at room temperature but gelled once at physiological body temperature. This thermo-gelling system was created without the use of any detergents, crosslinking agents or organic solvents. As the degree of deacetylation of chitosan increased, the

sol stability at room temperature and the gelation time decreased (Ruel-Gariépy et al., 2000).

The rationale behind the thermo-responsive gelation of chitosan- β -glycerophosphate is as follows (Figure 2.2): (1) A reduction in the polarity of chitosan, resulting in an increase in the hydrophobicity of the matrix, (2) dehydration of the chitosan chain, (3) opportunities for hydrogen bonding together with hydrophobic interactions created by a shift in the charge density and attraction between chitosan and β -glycerophosphate due to the transfer of protons from chitosan to β -glycerophosphate (Ruel-Gariépy et al., 2000; Chenite et al., 2001). The above phenomena are achieved when an ideal balance between the following forces is achieved: (1) intramolecular electrostatic repulsive forces within chitosan, (2) attractive forces between the positively charged amine groups within chitosan and the negatively charged phosphate group within β -glycerophosphate, (3) hydrogen bonding and hydrophobic attractions that exist between the chitosan fragments, and (4) the ability of glycerol moieties to restructure water molecules (Chenite et al., 2001).

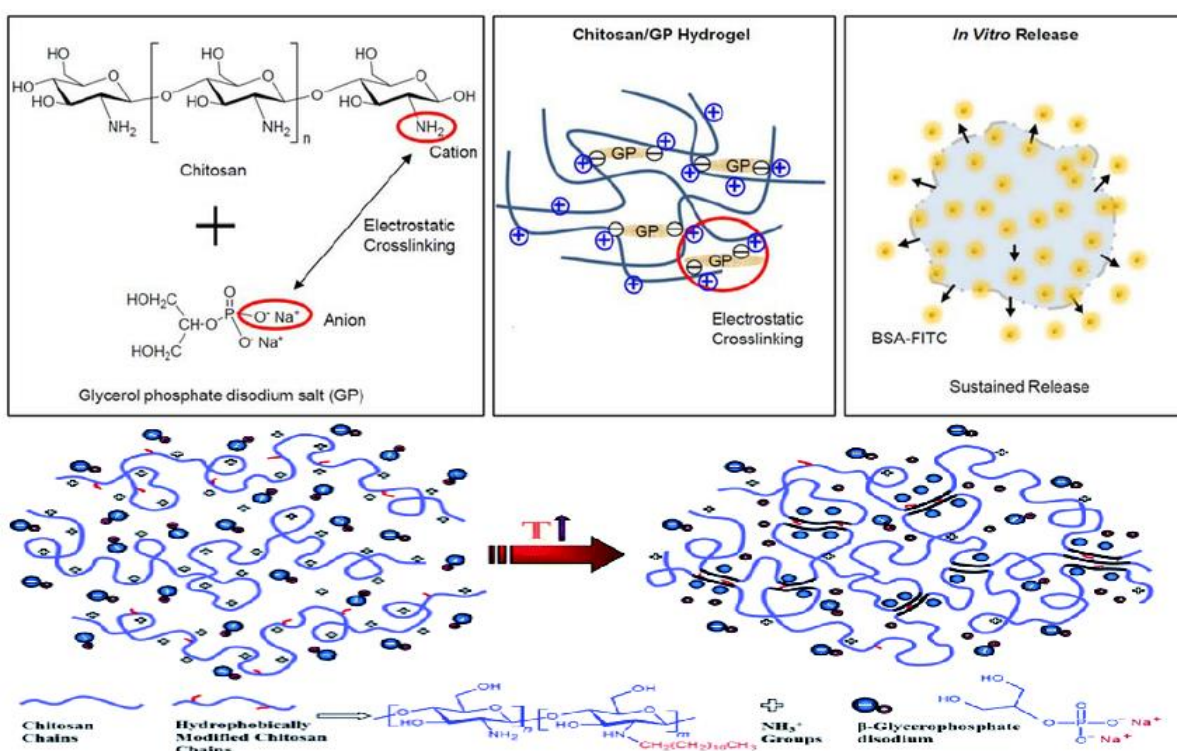


Figure 2.2: A. Schematic image of chitosan/GP hydrogel formation via electrostatic crosslinking between amine cationic groups of the chitosan chains and the phosphate anionic groups of the GP, and in vitro release of BSA-FITC from BSA-FITC-loaded chitosan/GP hydrogels (Kim et al., 2012). Licensed under Creative Commons: Kim,

Gaon & Kim, Nawoo & Kim, Dayeon & Kwon, Jin & Min, Byoung-Hyun. (2012). An Electrostatically Crosslinked Chitosan Hydrogel as a Drug Carrier. *Molecules* (Basel, Switzerland). 17. 13704-11. 10.3390/molecules171213704. **B.** Illustration of the molecular interactions involve in the thermo-gelation process of chitosan- β -glycerophosphate. Licensed under Creative Commons: Dashtimoghadam, E., Mirzadeh, H., Taroni, F. A., and Nyström, B. (2014). Thermoresponsive biopolymer hydrogels with tunable gel characteristics. *RSC Adv.* 4, 39386–39393. doi:10.1039/c4ra05246c.

Furthermore, Cho et al. (2005) attributed the thermo-gelation of chitosan- β -glycerophosphate to a decrease in hydrogen bonding within the chains, stating that as the temperature increases, vibrational and rotational energy transitions are experienced by the hydrated structures that surround the chitosan fragments, subsequently resulting in disorientation and dehydration. The now hydrophobic structures experience a change in conformation from a coiled, compact physical gel junction with greater intramolecular hydrogen bonding at lower temperatures, to an unfolded three-dimensional structure, with fewer intramolecular hydrogen bonds but more intermolecular hydrogen bonds, that is capable of forming contacts between zone junctions, thereby forming a gel at physiological body temperature (Kim et al., 2010).

2.2.1.2. Hybridization of Chitosan Based Thermogels

Many attempts have been made in the hybridization of chitosan- β -glycerophosphate, particularly in the field of tissue engineering. It was found that hybridization of chitosan-glycerophosphate with collagen produced significantly better stem cell proliferation due to a reduction in toxicity of the thermogel as opposed to pure chitosan-glycerophosphate thermogels. This was depicted in a study conducted by Wang and co-workers, where chitosan-glycerophosphate was combined with type 1 collagen to form an in-situ gel forming system capable of functioning as a matrix to encapsulate and deliver DNA and stem cells for tissue repair. Over a 12-day period, the content of DNA within the pure chitosan-glycerophosphate thermogel decreased by fifty percent, while a seventy percent increase in the hybridized thermogel was shown. The collagen containing composites promoted remodeling of the gel as well as survival of the cells when combined with the chitosan, which had an osteogenic effect (Wang and Stegemann, 2010). Furthermore, greater gel stiffness and gel compaction was

observed. More recently, Song et al. (2017) depicted similar favorable results in terms of hybridization with collagen with an optical density that increased right up till day seven of their study, indicating cell growth, whereas the chitosan-glycerophosphate hydrogel devoid of collagen depicted an increase in optical density only until day three. The formulation gelled within 12 min at a temperature of 37°C, showed good mechanical strength and was able to maintain its integrity within the cell culture media for the entire duration of 4 weeks. In addition, in vivo studies depicted more favorable results for the hybridized thermogel than the pure chitosan-glycerophosphate thermogel, with lower immune resistance, better biocompatibility and approximately half of the gel biodegraded within 4 weeks post administration (Song et al., 2017).

Furthermore, the current research shows that hybridization with NIPAAm also significantly reduces the toxicity of chitosan based thermogels, with an increase in cell proliferation correlating with an increase in the NIPAAm content within the hybridized thermogels. The addition of NIPAAm was also said to improve the solubility of the gels. A chitosan-NIPAAm thermogel formulated by Luo et al. (2020) was successful in the treatment of oral mucosal ulcers, with thermo-gelling temperatures of between 30.1 and 31.8°C thereby facilitating ease of application by injection at ulcer sites within the oral cavity. Chitosan was also shown to have antimicrobial activity in this study, and was able to inhibit both gram positive and gram negative bacterial growth, as well as promote gingival fibroblast proliferation and improve blood clotting, ultimately promoting the healing of ulcers and resulting in reduced ulcer healing times (Luo et al., 2020).

Interestingly, the release profile of chitosan based thermogels was optimized by first employing thiolation to form chitosan- 4-thio-butylamide (CS-TBA), and then combining it with β -glycerophosphate and hydroxyapatite. The formulation gelled at a temperature of 37°C within 10 min and depicted good mucoadhesive properties, as well as good water solubility at a neutral pH. In vitro release studies showed an initial burst release of bovine serum albumin, followed by sustained release until day twenty of the study, with a total release of 98.8%. This release profile was attributed to the thiol groups and disulfide bonds which resulted in the formation of disulfide- linked covalent aggregates between bovine serum albumin and chitosan-4-thio-butylamide. The formulation showed potential in drug delivery and tissue engineering applications due to its low cytotoxicity, porous structure, favorable degradation rate, and uniform nano-

hydroxyapatite distribution. Furthermore, the enhanced mucoadhesive properties of the system makes this system ideal for transmucosal drug delivery via oral, nasal, rectal, vaginal and ocular routes (Liu et al., 2014). In another study, hybridization with alginate was employed to improve the release profile of an mRNA loaded chitosan-glycerophosphate thermogel. The resulting system was able to gel at 37°C and showed a total of seventy percent cumulative release of the mRNA over a 3-week period. When chitosan was used alone, a slow release was depicted, and this was attributed to the positively charged chitosan interacting with the negatively charged mRNA, thereby retaining it. When alginate was used on its own, a fast release was shown, and this was attributed to electrostatic repulsive forces between the negatively charged alginate and the negatively charged synthetic mRNA. Thus, combining the negatively charged alginate with the positively charged chitosan-glycerophosphate resulted in a hybrid thermogel capable of sustained release of synthetic mRNA, at an optimal rate, faster than that of chitosan alone, but slower than that of alginate alone (Steinle et al., 2018).

Lastly, the gelation time as well as the gel strength of chitosan based thermogels was optimized in two ways. Shirotsaki et al. (2015) developed a chitosan-glycerophosphate- γ glycidoxypropyltrimethoxysilane (GPTMS) thermogel for use as an injectable biomaterial in clinical applications. The addition of the γ -glycidoxypropyltrimethoxysilane decreased the gelation time of the chitosan-glycerophosphate hydrogel significantly, and increased the gel strength, in a proportionate manner. Results showed that the chitosan- γ -glycidoxypropyltrimethoxysilane gels did not degrade when exposed to phosphate buffer solution, and the rate of mass loss when the gel was exposed to lysozyme solution was inversely proportionate to the amount of γ -glycidoxypropyltrimethoxysilane added. The formulation formed an opaque gel at 36.5–37°C and showed excellent cytocompatibility with human osteoblasts and MG63 osteosarcoma cells. As the amount of γ -glycidoxypropyltrimethoxysilane was increased, the surface stiffness of the gel increased as well, resulting in improved cell attachment. The most favorable results in terms of cell proliferation was achieved when a molar ratio of 1:0.5 chitosan: γ -glycidoxypropyltrimethoxysilane was used (Shirotsaki et al., 2015). Furthermore, manipulation of the gelation time and gel strength was also achieved when the effects of nanostructured chitosan/gelatin/bioactive glass thermogels in bone tissue engineering was investigated by Moreira et al. (2018). Increasing the content of gelatin and bioactive glass within the thermogel resulted in a

decrease in the gelation time and temperature, as well as improved mechanical properties. Rheological studies depicted an increase in the elastic modulus (G') of the gels from 5.4 Pa in pure chitosan gels to 12.4 Pa in the composite chitosan gels containing gelatin and bioactive glass. The live cell-viability of the osteosarcoma cell line was indicative of the cytocompatibility of this thermogel with human cells. The resulting formulation gelled at 37°C and was cationic, enabling it to interact with molecules that were negatively charged within the native extracellular matrix (Moreira et al., 2018).

Overall, hybrid chitosan based thermogels show promising results in terms of their potential in tissue engineering applications due to the reduction in toxicity and improvement in gel strength provided by hybridization (Zarrintaj et al., 2020). Hybridization of chitosan- β -glycerophosphate is possible with a range of different polymers to optimize the gel strength, gelation time, release profile and cytocompatibility of the gel.

2.2.2.1 Poly (N-Isopropylacrylamide) Based Thermogels

Poly (N-Isopropylacrylamide) (pNIPAAm) is synthetic thermo-responsive polymer derived from an acrylamide monomer, N-isopropylacrylamide (NIPAAm), consisting of amide and propyl moieties. pNIPAAm is an attractive prospect for thermo-responsive drug delivery applications due to its lower critical solution temperature (LCST) of approximately 32°C, close to the physiological human body temperature. Below the lower critical solution temperature, water molecules solvate the hydrophilic amide groups, pNIPAAm has good water solubility and exists in a flexible, coiled form (Doorty et al., 2003; Pei et al., 2004) with a hydration shell that is well structured. Above this temperature, pNIPAAm becomes insoluble in water and exists in globular form (Zhang et al., 2001) as interactions between hydrophobic propyl groups within the molecule become stronger and the hydrogen bonds weaken (Almeida et al., 2012). The chains within the polymer structure collapse and there is a loss of water from the structure, this volume phase transition (VPT) occurs at approximately 34°C (Figure 2.3) (Schild, 1992).

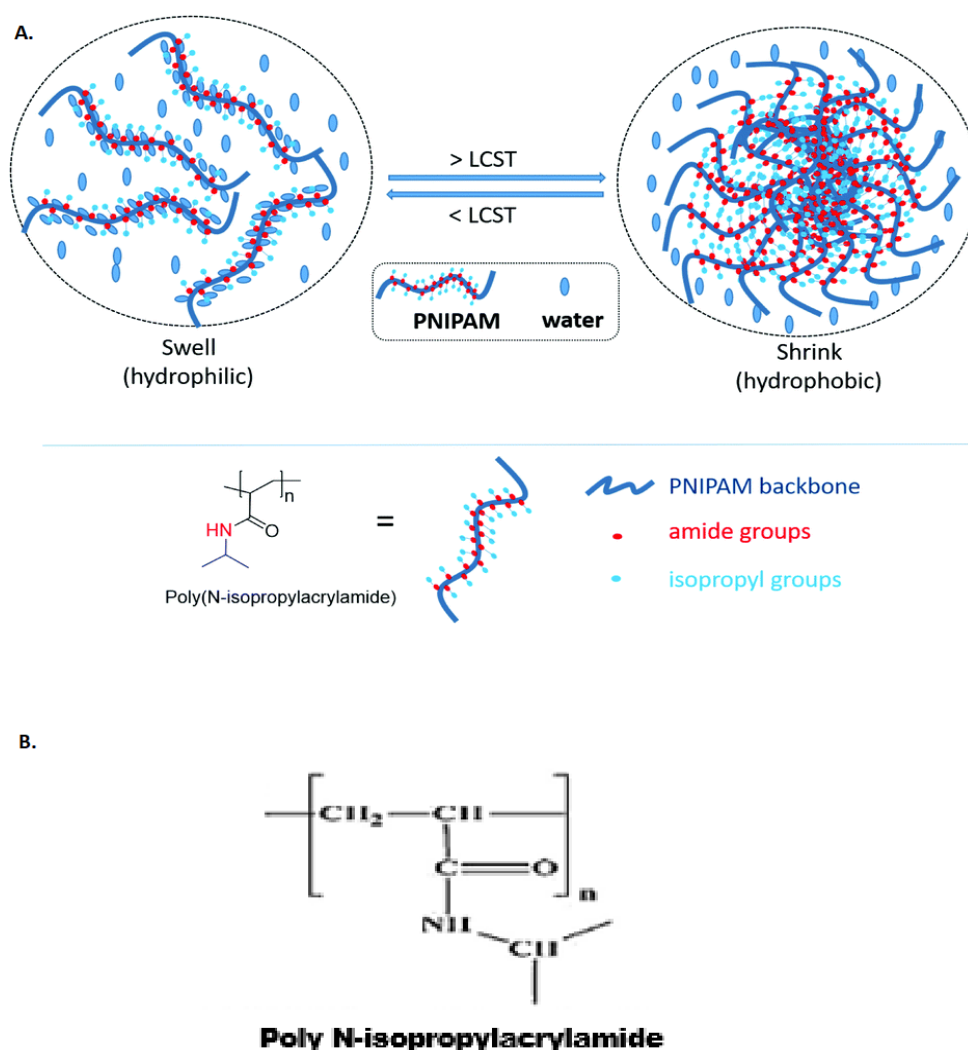


Figure 2.3: Schematic illustration of the chemical structure and thermo-gelling LCST behaviour of PNIPAAm. Reproduced with permission from the Royal Society of Chemistry, Doberenz, F., Zeng, K., Willems, C., Zhang, K., and Groth, T. (2020). Thermoresponsive polymers and their biomedical application in tissue engineering - a review. J. Mater. Chem. B 8, 607–628. doi:10.1039/c9tb02052g.

In the formulation of thermogels however, the use of pNIPAAm alone is limited, due to its poor mechanical strength (Zhang et al., 2016). This has resulted in copolymerization of NIPAAm with various other polymers such as poly (ethylene glycol) (PEG), acrylic acid (AA) and cellulose, to name a few. Copolymerization with polymers that are hydrophobic in nature results in a decrease in the lower critical solution temperature of pNIPAAm, while the opposite is true for copolymerization of NIPAAm with hydrophilic polymers (Kim et al., 2009). The increase in LCST when copolymerization with hydrophilic polymers occurs is attributed to an overall increase in hydrophilicity of the

molecule, and hence an increase the hydrogen bonding interactions between the polymer and water molecules (Feil et al., 1993).

2.2.2.2. Hybridization of Poly (N-Isopropylacrylamide) Based Thermogels

When NIPAAm was copolymerized with cellulose, the LCST was increased when the ratio of methylcellulose within the copolymer was greater than that of NIPAAm, whereas when the percentage of methylcellulose was less than that of NIPAAm within the copolymer, the LCST was decreased. Furthermore, an improvement in mechanical strength of the gel was seen, with increasing elastic moduli values at 36°C depicted as the cellulose content within the copolymer was increased. The resulting thermogel was shown to have promising application in blood vessel repair (Liu et al., 2004).

Interestingly, unusual thermo-responsive behavior was seen when a poly(NIPAAm-co-AM)/PEG/PTA composite gel was developed by Guo et al. (2015). The formulation showed an upper critical solution temperature of 59.6°C, with no lower critical solution temperature seen within a 25–70°C temperature range. The upper critical solution temperature behavior was attributed to competitive behavior between PEG, p(NIPAAm- co-Acrylamide) and phosphoric tungstic acid (PTA). It was found that the upper critical solution temperature varied with the concentration of PEG. This thermo-responsive system has potential for application in catalysis and other separation systems (Guo et al., 2015).

Various studies have been conducted to improve the mechanical properties of NIPAAm by hybridization with other materials, as discussed below. Barnes et al. (2016) established that the addition of collagen to a poly(N-isopropylacrylamide) based thermogel resulted in a thermo-gelling system with improved mechanical strength, without compromising the cytocompatibility of the gel. It was found that with varying concentrations of the gels, the resulting formulations had shear moduli comparable with that of the soft tissues found within the human body, ranging from 105 Pa to less than 102 Pa. The pores and micro-structures within these gels also allowed for the encapsulated stromal cells to develop different morphologies, thus showing potential for directing the course of cell fate (Barnes et al., 2016).

Hybridization with mesoporous silica (KIT-6) also proved to be an effective strategy in improving the mechanical strength of poly(N-isopropylacrylamide) based thermogels. The formulation, developed by Kamachi et al. (2016), had a volume phase transition at 34°C with the improved mechanical strength attributed to the formation of both topological and rigid crosslinks upon the addition of mesoporous silica. In addition, the employment of the mesoporous silica resulted in an increase in the adsorption capacity of the gel, with almost all the space offered by the mesopores available for drug or compound entrapment even after gelation, thereby offering a higher loading capacity for drugs or compounds (Kamachi et al., 2016).

Zhang et al. (2016) successfully manipulated the mechanical properties of poly(N-isopropylacrylamide) via hybridization with acrylic acid, resulting in a dually crosslinked thermogel comprising of a two-level network structure. The first of which being the covalently crosslinked poly (N-isopropylacrylamide-co-acrylic acid) by the addition of a crosslinking agent, and the second being the multivalent ion pairing between acrylic acid groups in the poly (N-isopropylacrylamide-co-acrylic acid) and Fe (III). The resulting thermogel had a lower critical solution temperature of 32°C, with mechanical properties that varied per the concentration of acrylic acid within the hybrid gel. The mechanical strength of the gel was greatly improved, with results depicting strengths of up to 6 MPa tensile strength and 69.8 MPa compressive strength, with potential for application as tissues, soft robotics, sensors and manipulators (Zhang et al., 2016).

Lastly, yet another strategy to improve the elasticity and strength of poly(N-isopropylacrylamide) based thermogels was developed by Yuan et al. (2018), by the reversible addition-fragmentation chain-transfer polymerization (RAFT synthesis) with ABA triblock copolymer flower-like nanogels. Apart from enhanced mechanical properties, the resulting formulation showed a phase transition temperature of 32°C with a rapid response and large swelling ratio. These properties were attributed to the small uniform pores within the gel structure, as well as the entanglement and crosslinking present between the gel matrix and the PDMA loops of the flower-like nanogel (Yuan et al., 2018).

Furthermore, the release profile of NIPAAm based thermogels can also be optimized by hybridization. A group of researchers formulated an injectable protein-reactive thermogel for the extended and sequential delivery of proteins, by first hybridizing

NIPAAm with 2-Hydroxyethyl methacrylate (HEMA) and methacrylate polylactide (MAPLA) via free radical polymerization, and then incorporating protein loaded microspheres. The formulated copolymers were able to gel below 37°C and released protein for over 3 months. The injectability, biodegradability, and the extended and simultaneous sequential release characteristics of this system make it an attractive prospect in tissue engineering applications, particularly, intramyocardial injections following ischemic injury (Nelson et al., 2012).

In another study by Lee et al. (2018), conjugation of NIPAAm with sulfonated poly(serinol hexamethylene urea) (SPSHU) was undertaken to formulate a thermogel for the controlled delivery of vascular endothelial growth factor (VEGF) in the promotion of therapeutic angiogenesis. The sulfonate groups within the gel created an electrostatic binding affinity to vascular endothelial growth factor (VEGF), thereby allowing the gel to bind to VEGF and localize it, facilitating its spatiotemporal control. Additionally, a decrease in the initial burst release was seen, together with a reduction in the sustained rate of release, showing the system's potential for the delivery angiogenic factors (Lee et al., 2018).

NIPAAm has also shown promising results in the formulation of thermo-responsive, shape adaptive hydrogel actuators when hybridized with less- or non-responsive polymers, due to the differences in the thermo-responsive swelling and shrinking properties of the polymers (Zhang et al., 2019). Successful manipulation of both the lower critical solution temperature and shape adaptive behavior was achieved via copolymerization of NIPAAm with N-[3-(Dimethylamino)propyl]methacrylamide (DMAPMA) and acrylamide (AM) respectively. By combining pNIPAAm with polymers that are responsive to different stimuli, hydrogels with distinct swelling characteristics in each layer were formulated, such as a dually thermal and pH responsive pNIPAAm-PVA/PDMAEMA-PSS hydrogel, and the thermal and c(H') responsive pNIPAAm-PDMAEMA. Further, to achieve faster, reversible, bidirectional bending in both hot and cold temperature conditions, NIPAAm, which possess a LCST, was copolymerized with poly(acrylic acid-co-acrylamide), which has an opposing UCST.

As it can be seen, hybridization of poly(N- isopropylacrylamide) in the formulation of thermogels not only results in an improvement in the mechanical strength and release profiles of the gel, but depending on the polymers used and the interactions between

thermo-responsive NIPAAm and other less- or differently-responsive polymers, may display other favorable properties that may be exploited as well.

2.2.3.1 Pluronic Based Thermogels

Pluronic (commonly known as poloxamer) is a triblock co- polymer consisting of hydrophobic poly-propylene-oxide (PPO) at the center with hydrophilic poly-ethylene-oxide (PEO) on either side (Kabanov et al., 2002). The precise mechanism of gelation of pluronic has not been confirmed, but the following mechanisms have been proposed (Figure 2.4): (1) With an increase in temperature, there is a decrease in the micellar concentration due to changes of the properties of micelles with regards to aggregation and symmetry (Rassing and Attwood, 1982; Abou-Shamat et al., 2019); (2) The forces of friction between the copolymer micelles cease to exist with the dehydration of poly-propylene-oxide, consequently, there an increase in the viscosity of the system with gel formation (Rassing et al., 1984); (3) Water molecules around the core undergo re-organization, resulting in a change in entropy for (O–C–O) groups from a polar to a non-polar state, thereby propelling the sol-gel transition (Vadnere et al., 1984); (4) Hard-sphere crystallization occurs whereby above the critical micelle concentration, there is an equilibrium between the copolymer unimers and micelles at lower temperatures, with an increase in micelle formation and volume fraction in comparison to the unimers corresponding with an increase in temperature (Abdi et al., 2012).

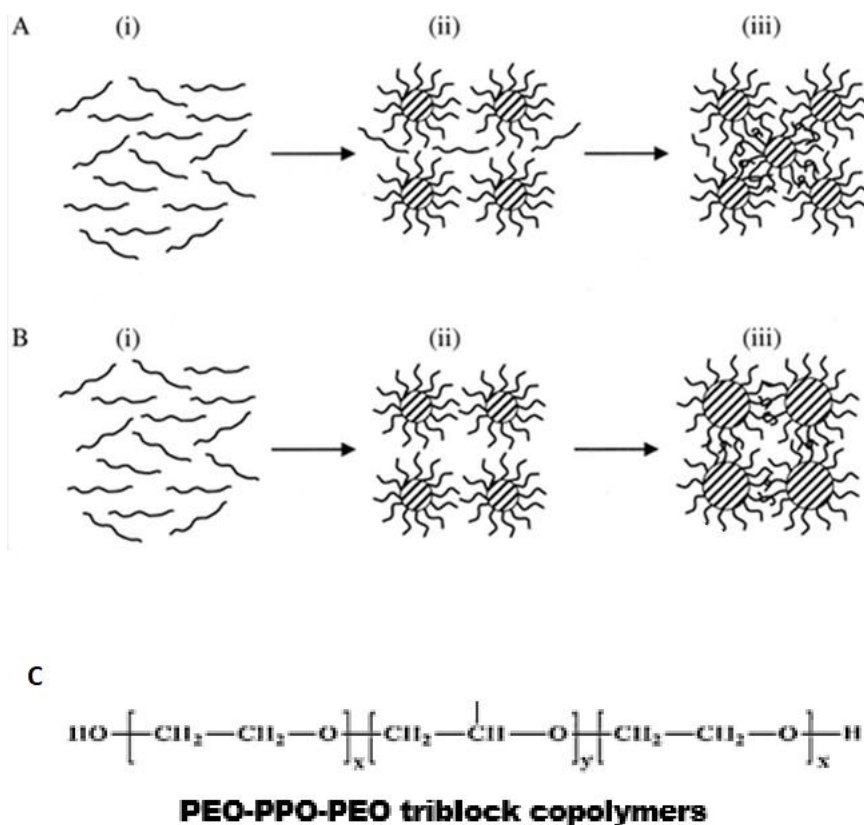


Figure 2.4: Schematic diagram for two mechanisms (**A and B**) for the gelation of F108 through micellization. From stage i to stage iii, the arrows indicate the direction of increasing temperature. F108 molecules exist as unimers at stage i, and the micelles are formed during stage ii. In mechanism A, the micellar sizes remain the same, but new micelles are formed at stage iii so that the intermicellar distance is shortened and gelation through entanglements is allowed. In mechanism B, the number of micelles remains unchanged during stage iii, but their size is increased with temperature so that the intermicellar distance is shortened and gelation through entanglements is allowed. Reproduced with permission from John Wiley and sons. Journal of Polymer Science Part B: Polymer Physics, Volume: 42, Issue: 10, Pages: 2014-2025, First published: 14 April 2004, DOI: (10.1002/polb.20105). **C.** Chemical structure of PEO-PPO-PEO triblock co-polymers.

Pluronic F127 (also known as poloxamer 407) is a commonly used (PEO-PPO-PEO) triblock copolymer with a PEO: PPO ratio of 2:1 (Lin and Sung, 2000; Escobar-Chávez et al., 2006), and a gelation concentration greater than 20% at 25°C. At concentrations between 20 and 30%, the solution exists in the sol phase below 25°C and forms a thermo-reversible gel at body temperature (Miyazaki et al., 1984; Lenaerts et al., 1987). However, this high CGC is not ideal, resulting in the formation of gels with high

viscosity (El-Kamel, 2002), gelation at ambient temperatures, and potential for cytotoxicity. In addition, the delicate structure of pluronic affects the stability of pluronic based thermogels in vitro in certain applications (Rangabhatla et al., 2016). To mitigate these problems, hybridization of pluronic has been used.

2.2.3.2. Hybridization of Pluronic Based Thermogels

To combat the issue of high gelation concentrations, it was found that when hybridization with methylcellulose was employed, thermo-gelation was possible at concentrations of pluronic as low as 12% (Rangabhatla et al., 2016). In a study conducted by Rangabhatla et al. (2016), hybrid thermogels in ratios of 12 pluronic: methylcellulose and 14 pluronic: methylcellulose showed thermo-gelation at 37°C, while existing in the sol phase at room temperature. Furthermore, at room temperature, the gels exhibited low viscosities, thereby facilitating ease of injection into the body. Cell studies using these formulations depicted that the viability of the cells was increased upon the addition of methylcellulose to the pluronic based thermogel. The resulting formulation showed potential for application in bone remodeling as sustained release over a 28-day period was shown when etidronate disodium was incorporated (Rangabhatla et al., 2016).

To increase the strength of pluronic-based thermogels and improve the cell viability within pluronic-based thermogels, Abdi et al. (2012) proposed hybridization with alginate and hyaluronic acid. A blended hydrogel was formulated for cell injection applications. Due to the high concentrations of pluronic that is used in the formation of thermogels, toxicity is often problematic, resulting in poor cell viability. The formation of a pluronic-alginate blend increased the cell viability up to 80%. Furthermore, the incorporation of small amounts of hyaluronic acid (0.1%) to the blend showed even better cytocompatibility results, since hyaluronic acid is a component of the natural extracellular matrix, thereby facilitating the growth and survival of cells. The formulation also showed that as the concentration of alginate within the blend increased, the strength of the gel that was formed at physiological body temperature of 37°C was increased as well. When 3% of alginate was used, a strong gel capable of sustaining cells at physiological temperature and pH was achieved (Abdi et al., 2012). More recently, in a similar study, the efficacy of hybridization of pluronic with alginate in the formation of thermo-gelling systems was once again demonstrated by Quah et al.

(2018). An increase of approximately 100-fold was also seen in the elastic moduli of the formulated thermo-gelling system as the temperature increased from 15 to 40°C, indicating an increase in the stiffness of the gel, showing potential for application in soft tissue engineering, dental applications, wound healing and as an injectable drug delivery system. Interestingly, it was also found that while increasing the concentration of pluronic within the thermogels decreased the gelation temperature, increasing the concentration of alginate increased both the upper and lower transition temperatures (Quah et al., 2018).

To modify the gelation temperature of Pluronic F127 based thermogels such that they would only gel in situ, a group of researchers proposed hybridization with methacryloisobutyl- polyhedral oligosilsesquioxane (MA-POSS). The addition of MA-POSS to pluronic F127 via atom transfer radical polymerization resulted in the formation of a thermogel with a gelation temperature of 33.5°C, ten degrees higher than that of the pristine pluronic F127 gel, which displayed a gelation temperature of 23.5°C. This was attributed to the hinderance from the association of the hydrophobic poly-(propylene glycol) segments within pluronic F127 which induces gelation, by the rigid nano-block structure of POSS. Furthermore, the degradation temperature of the resulting hybrid thermogel was increased by 15°C as compared to that of the pristine pluronic F127 thermogel. In addition, rheological studies showed that the modulus of the gel was maintained upon the incorporation of MA-POSS, thus the strength of the gel was not compromised by the addition of POSS (Dou et al., 2016).

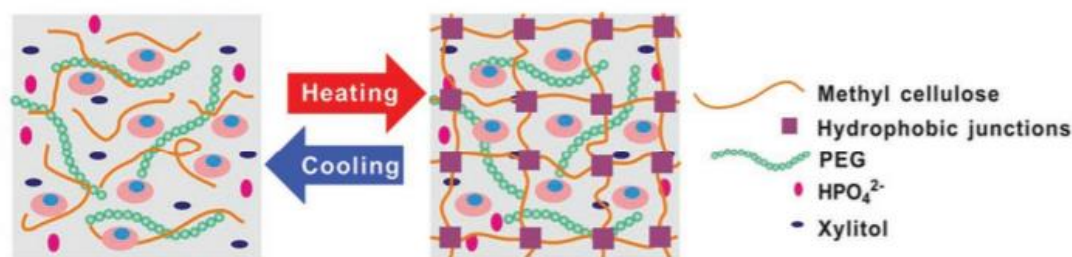
Similarly, when pluronic F127 was hybridized with carboxymethyl chitosan (CMCS) for the localized and systemic delivery of chemotherapeutic agents, an increase both the gelation temperature and time was seen. This was attributed to the increase in hydrophilic groups upon the addition of the hydrophilic CMCS, and consequently a reduction in hydrophobic interactions. The incorporation of the chemotherapeutic 5-Flourouracil also resulted in an increase in the gelation time and temperature, by virtue of an increase in the hydrophilicity once again. Further, the increase in the hydrophilic carboxyl groups present when increasing the contents of CMCS within the formulation also resulted in an increase in drug release, particularly at pH7.4, due to a resulting increase in water uptake and subsequently drug diffusion (Khan et al., 2018).

Thus, it can be seen that pluronic, while quite difficult to work within its pure form, can be copolymerized and hybridized with a range of different, easily available polymers to formulate ideal thermo-gelling systems that are suitable for use in a wide range of biomedical applications. Hybridization of pluronic in the formation of thermogels not only facilitates the use of lower concentrations of the polymer, but also results in improved strength and mechanical properties, and reduced toxicity.

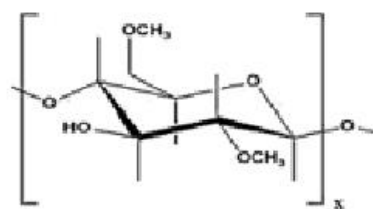
2.2.4.1 Cellulose Based Thermogels

Methylcellulose and hydroxypropyl methylcellulose are hydrophobically substituted water soluble cellulose derivatives that are thermo-responsive (Barros et al., 2014). At concentrations between one and ten percent, aqueous solutions of these polymers exist in solution form at lower temperatures, but form gels when the temperature is increased beyond the lower critical solution temperature. The sol-gel phase transition of methylcellulose occurs between 40 and 50°C, whereas that of hydroxypropyl methylcellulose occurs between 75 and 90°C (Sarkar, 1979; Kim J. K. et al., 2012). Several mechanisms of thermo-reversible gelation of methylcellulose have been proposed (Figure 2.5), including:

- (1) Crosslinking loci formed by crystalline structures of trimethyl glucose create a gel network that has hydrogen bonds, hydrophobic interactions and dipolar interactions which support it (Kato et al., 1978);
- (2) At low temperatures, hydrophobic clustering occurs, resulting in unsubstituted chains aggregating and forming bundles, which then open at moderate temperatures, exposing the methyl groups to the aqueous solution thereby resulting in an increase in the volume of the solution. At high temperatures, hydrophobically crosslinked networks that are highly stable are formed as a result of loss of constituent water from the methyl side chains, depicting thermal hysteresis (Haque and Morris, 1993);
- (3) A cluster is formed due to the hydrophobic interactions between the methyl groups. Thereafter, at high temperatures, lattice restructuring occurs due to the imbalance between the polymer-polymer and polymer-water hydrogen bonding interactions, resulting in thermogelation induced by a phase-separation (Kobayashi et al., 1999; Bodvik et al., 2010);
- (4) Polymer chains aggregate and orient themselves parallel to each other, thereby reducing the interactions between water molecules and the hydrophobic groups, forming fibrillar structures (Jain et al., 2013).



A.



Methylcellulose

B.

Figure 2.5: A. Schematic illustrating the thermogelation of methylcellulose, as adapted from Wang, R., Yao, X., Li, T., Li, X., Jin, M., Ni, Y., et al. (2019). Reversible Thermoresponsive Hydrogel Fabricated from Natural Biopolymer for the Improvement of Critical Limb Ischemia by Controlling Release of Stem Cells. *Adv. Healthc. Mater.* 8, 1900967. doi:10.1002/adhm.201900967. Reproduced with permission from John Wiley and sons. **B.** Chemical structure of methylcellulose.

Recently, a group of researchers investigated the crosslinking kinetics of varying concentrations of methylcellulose in terms of heat exchange (Figure 2.6), onset temperatures (Figure 2.7) and reaction rates (Figure 2.8). It was found that methylcellulose displayed endothermic behavior during heating, with an exothermic effect preceding this in concentrations of methylcellulose below 2%. Furthermore, it was seen that exothermic heat was strongly dependent on the concentration of methylcellulose below 1%, and weakly dependent on the concentration methylcellulose above 1%.

This phenomenon explained as follows: (1) At ambient temperature, hydrophilic interactions among the water molecules themselves and the between water molecules and methylcellulose are dominant. (2) There is a release of heat accompanied by the formation of methoxy groups and “water cages,” with water molecules surrounding the

hydrophobic areas of methylcellulose in an orderly manner. (3) Intramolecular interactions occur between the polymer chains. (4) Upon heating the system, energy is consumed resulting in the release of methoxy groups and weakening of the “water cages.” This is known as the low temperature endothermic effect. (5) Intra- and intermolecular hydrophobic interactions result in the crosslinking of the methoxy group within the methylcellulose chains. This is known as the high temperature endothermic effect. In addition, at lower concentrations of methylcellulose, the destruction of the water cages (low temperature endothermic effect) occurred at a slower rate. In contrast, the rate of formation of the water cages increased as the concentration of methylcellulose increased. Lastly, the rate of crosslinking decreased with higher concentrations of methylcellulose (Niemczyk-Soczynska et al., 2019).

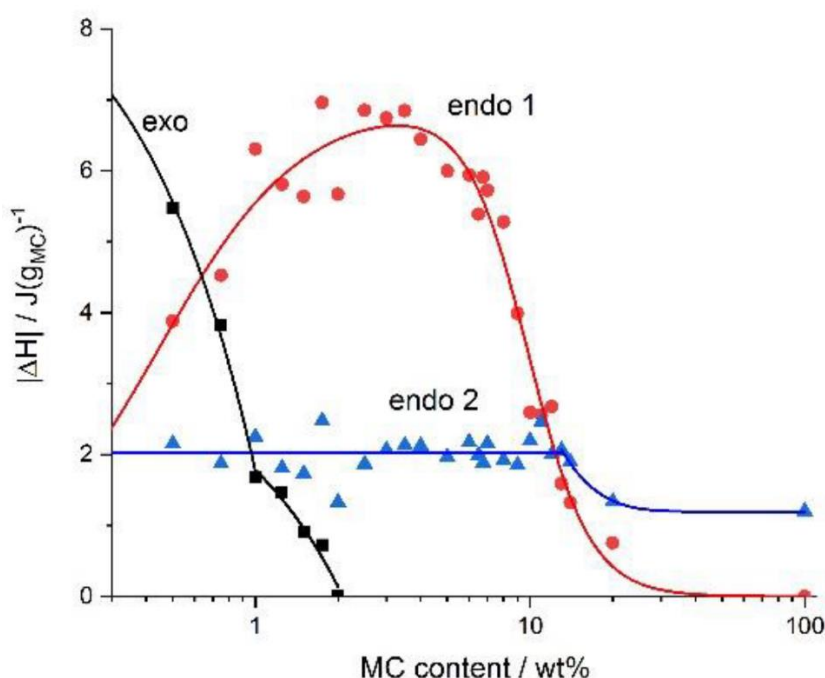


Figure 2.6. The absolute value of the heat exchanged upon heating during the exothermic, the low-temperature (endo 1) and the high-temperature (endo 2) endothermic effects versus the MC content. Reproduced with permission from (Niemczyk-Soczynska et al., 2019). Copyright 2019 Polymers.

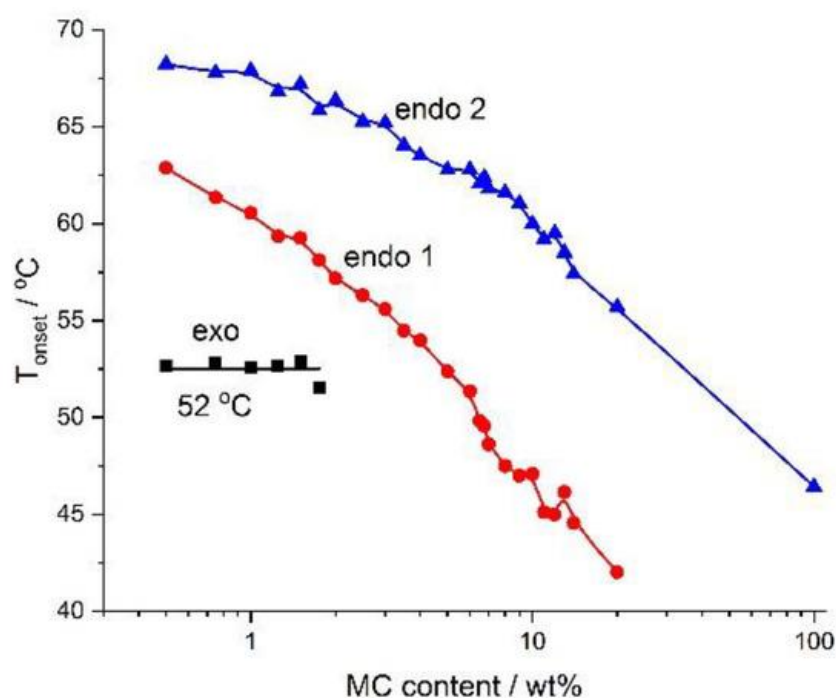


Figure 2.7. Phase diagram for various concentrations of MC constructed using the onset temperature. Reproduced with permission from (Niemczyk-Soczynska et al., 2019). Copyright 2019 Polymers.

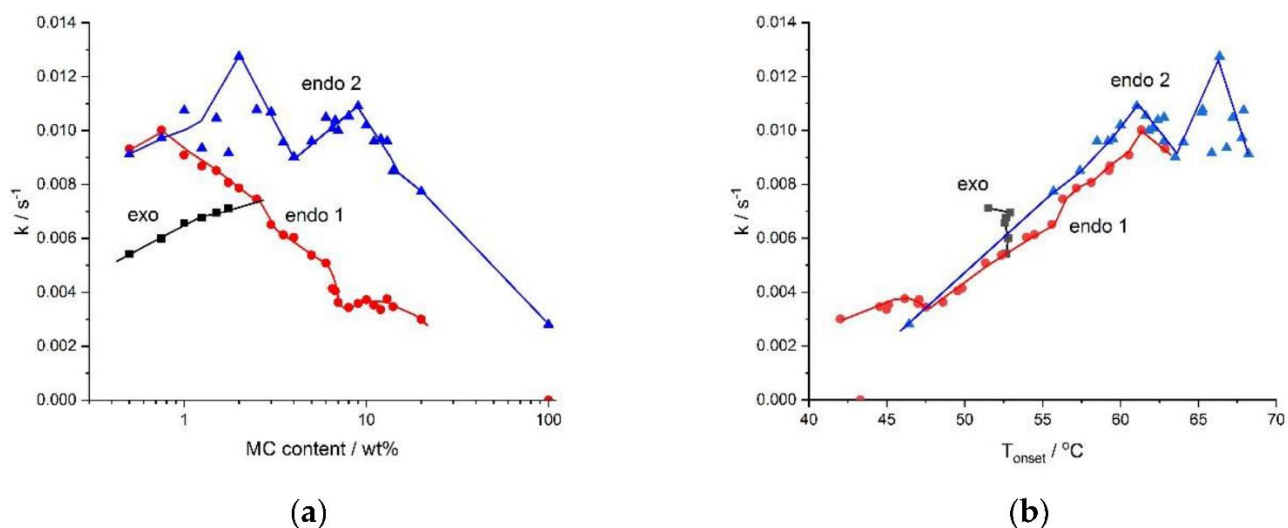


Figure 2.8. The rates, k , of water cages formation (exo), their destruction (endo 1), and of crosslinking (endo 2), as a function of—(a) the MC content and (b) the temperature, T_{onset} . Reproduced with permission from (Niemczyk-Soczynska et al., 2019). Copyright 2019 Polymers.

2.2.4.2. Hybridization of Cellulose Based Thermogels

Due to the high gelation temperature of cellulose based thermogels, hybridization of these polymers has been employed to formulate thermogels with gelation temperatures that are compatible with physiological body temperature. Nowald et al. (2016) developed a thermogel for wound healing applications by the addition of methylcellulose as a mechanical adjuvant to mucin. To combat this problem and induce gelation at a temperature of 37°C, glycerol was used. It was also shown that the higher the molecular weight of the methylcellulose used, the longer the gelation time, with a gelation time of approximately an hour for an 80 kDa methylcellulose gel compared to gelation within a mere few minutes when a 17 kDa methylcellulose was used. The only shortfall when using a 17 kDa methylcellulose, however, was the reduction in the stiffness and overall strength of the gel. To combat this problem, the concentration of methylcellulose that was used was increased simultaneously, yielding positive results. Furthermore, the substitution of NaCl with a kosmotropic ion KCl in an effort to speed up the gelation process in this study did not show any effect on the gelation kinetics of the gel (Nowald et al., 2016).

Promising results in reducing the gelation temperature while simultaneously speeding up the gelation process were shown in another study, however, where xylitol was used in addition to sodium phosphate dibasic to reduce the gelation temperature of a methylcellulose based thermogel to 32°C, below the physiological body temperature, to facilitate the delivery of placental mesenchymal stem cells (PMSC) at lower body temperatures for the treatment of ischemia within limbs. The addition of polyethylene glycol to this system resulted in an increase the speed of gelation from 20 min to a mere 150 s. It is worth noting that the sodium phosphate dibasic and xylitol worked synergistically to reduce the gelation temperature to 32°C, as when the sodium phosphate dibasic was used alone, the gelation temperature was only reduced to 36°C, and when the xylitol was used in isolation, a gelation temperature of 40°C was noted. The successful delivery of this thermogel together with the stem cells further showed potential in the promotion of vascular repair, by inhibiting fibrosis and muscular atrophy, promoting blood flow and stimulating angiogenesis. (Wang et al., 2019).

Interestingly, synergistic behavior in the reduction of the thermo-gelling temperature of methylcellulose was also depicted with the incorporation of a biologically active

molecule, gallic acid, together with a salt. Both gallic acid and sodium chloride were found to lower the gelation temperature of methylcellulose, however, when each was added to the methylcellulose in isolation, the gelation temperature was still above that of the physiological body temperature. When both gallic acid and sodium chloride were then combined simultaneously with methylcellulose in a ratio of 0.3:4:1, a gelation temperature of 36.7°C was achieved. Sustained release of drug together with the gallic acid was seen in the in vitro release studies (Sangfai et al., 2017).

The effects of the incorporation of different salts into methylcellulose-based thermogels on gel stability, transition temperature and the preparation process of thermogels was investigated in detail by Altomare et al. (2016). Gels containing higher concentrations of methylcellulose had a greater viscosity and a corresponding higher G' value, and therefore a slower flow rate. An increase in the respective salt concentration within the thermogel for a constant concentration of methylcellulose resulted in an increase in G', and a shift in the gelation temperature to lower temperatures, below 37°C. Different gelation temperatures were achieved depending on the salt added, for the same concentration of methylcellulose. When sodium sulfate and phosphate buffer saline were incorporated as salts respectively, a gelation temperature of 20°C was achieved. In contrast, when calcium chloride was added, a gelation temperature of 37°C was achieved. Furthermore, the critical salt concentration to achieve a gelation temperature of 37°C differed for each salt, due to the differences in the ionic interactions between the respective salts and methylcellulose. Overall, the thermogel containing 0.05M sodium sulfate and 8% w/v methylcellulose displayed the best results, with a transition temperature of 37°C, good stability and the best handling in terms of in vitro cell tests. This study showed potential for application of these gels in terms of cell sheet bio-fabrication and cell seeding (Altomare et al., 2016).

In theory, to decrease the gelation temperature of a methylcellulose-based thermogel, the concentration of methylcellulose can simply be increased, however, this results in an increase in viscosity such that the gel becomes difficult to handle, as well as a change in the kinetics of the formulation, as discussed above. Alternatively, a salt could be added to the gel to lower the gelation temperature, however, studies have shown a consequent decrease in the strength of such gels when in an aqueous solution or in a cell culture environment. To circumvent these issues and formulate a methylcellulose gel capable of thermogelation at physiological body temperature,

Gaihre et al. (2019) recently blended calcium chloride and alginate with methylcellulose, resulting in the formation of a crosslinked, strong gel within 10 min at 37°C. The resulting formulation served as an injectable medium for the delivery of chitosan microparticles. Furthermore, faster gelation was observed upon the addition of the chitosan microparticles due to the absorption of water from the solution by the microparticles, thereby increasing the viscosity of the solution, as was evidenced by the swelling of the microparticles when added into the solution (Gaihre et al., 2019).

Many strategies have been proposed for the reduction of the gelation temperature of methylcellulose, each with their own setbacks, from the addition of salts, to merely increasing the concentration of methylcellulose within the gel. However, most recently, hybridization of methylcellulose to decrease the gelation temperature has shown promising results in terms of creating formulations that are capable of in situ gelation, making it an attractive prospect in biomedical applications. With further research, more successful hybrid combinations could be found.

2.2.5.1. PCL-PEG Based Thermogels

Poly(caprolactone) is a hydrophobic, biodegradable and biocompatible polymer that has been combined with the hydrophilic poly(ethylene glycol) in the formation of di- and triblock copolymers such as PEG-PCL-PEG and PCL-PEG-PCL (Bae et al., 2005). These crystalline polymers can be lyophilized and reconstituted into thermogels within minutes (Shah et al., 2018). The mechanism of PCL-PEG thermo-gelation has been described as follows: (1) at lower temperatures, a fully sol state is present, with the freely flowing micelles in the solution still small in size, (2) as the temperature is increased slightly, so too do the micelles increase in size, and this state is known as the favorably sol state, (3) as the temperature reaches the sol-gel transition temperature and the concentration is above CGC, we enter the favorably gel state, where micelles increase in size rapidly, (4) lastly, a dense gel is formed, as the micelles aggregate and pack closely together (Wei et al., 2009). The structure and mechanism of thermogelation of PEG-PCL based thermogels can be seen in Figure 2.9 in the case below.

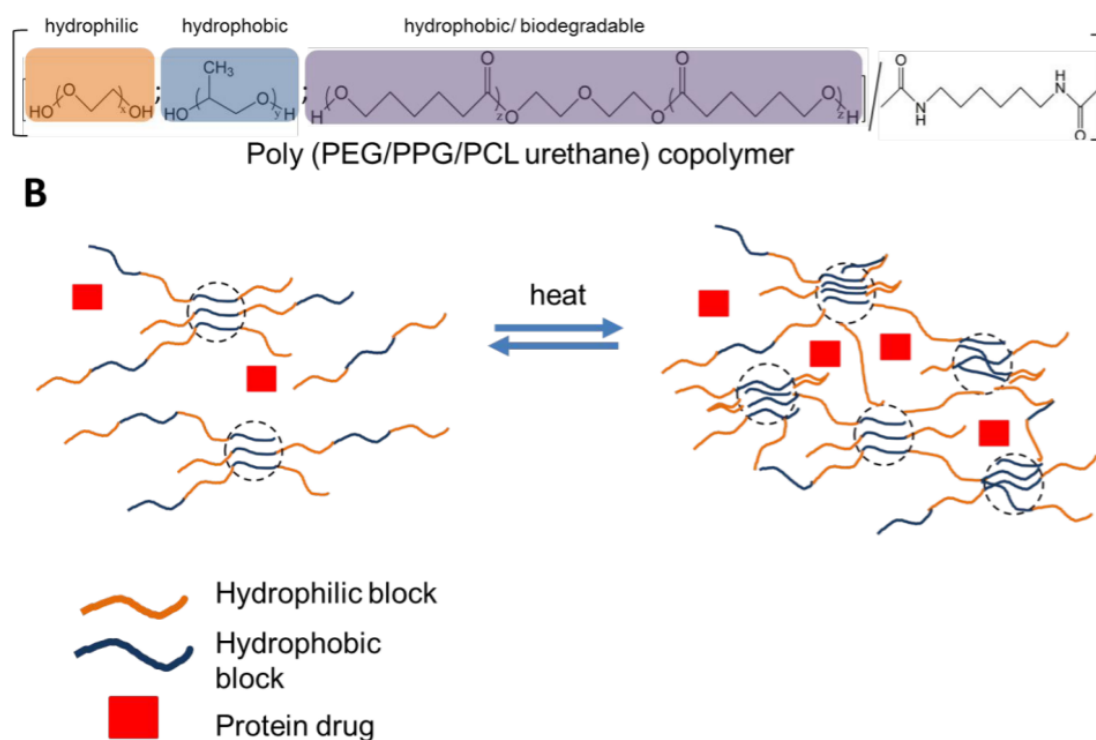


Figure 2.9: Thermogelling polyurethane copolymer for protein delivery. **A.** Schematic of polyurethane copolymer composition based on function. **B.** Protein drug is mixed with polyurethane copolymer. Upon heating, the multiblock copolymer is able to generate crosslinks and form a hydrogel under mild conditions to encapsulate protein drug. Reproduced with permission from Royal Society of Chemistry, Xue, K., Zhao, X., Zhang, Z., Qiu, B., Tan, Q. S. W., Ong, K. H., et al. (2019). Sustained delivery of anti-VEGFs from thermogel depots inhibits angiogenesis without the need for multiple injections. *Biomater. Sci.* 7, 4603–4614. doi:10.1039/c9bm01049a.

Hybridization of PEG-PCL based thermogels has been employed resulting in innovative in situ gel forming systems with improved mechanical strength, solubility, and release profiles, as discussed below.

2.2.5.2. Hybridization of PCL-PEG Based Thermogels

To improve the gel strength optimize the release profile of paclitaxel in the treatment of tumors, Loh et al. (2012) employed hybridization in the development of a poly(PEG-PPG- PCL-urethane) thermo-gelling system (Figure 2.9). The resulting solution formed a gel at physiological body temperature with an elastic modulus of 500 Pa and was able to withstand forces of up to 30 Pa. The CGC of the system was 3% wt. Drug release studies showed almost perfect zero order kinetics with the commonly seen

“initial burst release” suppressed. Thus, this system proved to be ideal for the release of a consistent concentration of drug and it mitigated the danger of side effects that are often seen when chemotherapeutic drugs display an initial burst release, thereby resulting in concentrations of the chemotherapeutic agent rising above the maximum safe concentrations. In addition, when compared to a pluronic F127 gel in the study, the poly(PEG-PPG-PCL-urethane) thermogel showed more favorable results in terms of in vitro stability of the gel (Loh et al., 2012).

This same group of researchers further employed their poly(PEG-PPG-PCL-urethane) thermogel in the delivery of anti-VEGFs to inhibit angiogenesis, mitigating the need for multiple injections. The release rate was now optimized by altering the hydrophile/lipophile balance, and a PEG:PPG ratio of 4:1 showed the best release profile for this application. The anti-VEGFs showed activity for up to 40 days, with anti-angiogenic effects depicted both ex vivo and in vivo. This study is a steppingstone for the development and use of thermogels in the delivery of bioactive protein molecules (Xue et al., 2019).

In another study, gel strength drug release profiles were improved by combining PEG-PCL-PEG, pluronic F127, and poly propylene fumarate (PPF) in a ratio of 4:1:1 in the formulation of an innovative successful pseudo-bone thermogel. The resulting semi-elastic gel showed controlled, sustained release of the incorporated drug at physiological temperature over the entire 14-day period, compared with a shorter duration of drug release up till day 10 when at ambient room temperature. In addition, a viscous modulus of 0.1–0.3 Pa at temperatures between 10 and 20°C was noted, with an increase in gel strength by 45,000 times when physiological body temperature was reached (Kondiah et al., 2017).

The solubility and temperature responsiveness of PEG-PCL based thermogels was improved via the synthesis of a mPEG-b-[PCL-g-(MEO2MA-co-OEGMA)]-b-mPEG block-graft copolymer by Shang et al. (2019). Results showed that due to the hydrogen bonds formed between water molecules and the oligo (ethylene glycol) graft chain that is hydrophilic, the synthesized copolymer showed better solubility in water and formed a clear solution, compared to just PEG-PCL-PEG, which displayed a cloudy solution in water. In addition, the improvement in the thermo-responsiveness of the copolymer

was attributed to the OEGMA and MEO2MA molecules, and the formulation showed a gelation temperature of 30°C (Shang et al., 2019).

Lastly, hybridization of PEG-PCL resulted in the formulation of an ideal penta-block PNIPAAm-PCL-PEG-PCL-PNIPAAm thermogel for application in wound healing. The formulation was able to gel at 37°C with a structure that was three dimensional and consisted of interconnected pores, similar to that of the extracellular matrix. Optimal results in terms of skin tissue engineering were achieved when a 20% formulation was used, with a thermogel pore size ideal for fibroblast attachment, and improved cell adhesion and cell proliferation indicating good biocompatibility. The formulation degraded gradually after 4 weeks and shows potential as an injectable dosage form for the sustained delivery of drugs, as well as in wound healing applications (Oroojalian et al., 2019). A comprehensive summary of the hybrid systems discussed within this article can be found in Table 2.1.

Table 2.1 Summary of the various hybrid thermo-gelling systems discussed in this chapter

Thermo-responsive polymers and their hybrid systems	Responsive Temperature	Advantages of hybridisation and salient features of hybrid system	Current/ Potential application	Reference
Chitosan-β-glycerophosphate based thermogels	37°C	Setbacks: Gelation time, gel strength and cytocompatibility need to be optimized for potential applications.		
Type 1 collagen	37°C	Improvement in gel stiffness, gel compaction, and proliferation of stem cells.	Tissue repair	(Wang and Stegeman n, 2010)
Type 1 collagen	37°C	Reduced toxicity facilitating better cell growth and biocompatibility.	Scaffold for tissue engineering	(Song et al., 2017)

Thermo-responsive polymers and their hybrid systems	Responsive Temperature	Advantages of hybridisation and salient features of hybrid system	Current/Potential application	Reference
NIPAAm	30.1-31.8°C	Reduced toxicity facilitating better cell growth and biocompatibility; Improved solubility.	Treatment of oral mucosal ulcers	(Luo et al., 2020)
4-thio-butylamide and hydroxyapatite	37°C	Good muco-adhesion and sustained release over 20 days.	Tissue engineering and controlled drug delivery	(Liu et al., 2014)
Alginate	37°C	Sustained release of synthetic mRNA at an ideal rate.	Exogenous protein synthesis and tissue engineering	(Steinle et al., 2018)
γ - glycidoxypropyltrimethoxysilane (GPTMS)	36.5-37°C	Decreased gelation time and increased the gel strength.	Injectable biomaterial, bone regeneration/tissue engineering	(Shirosaki et al., 2015)
Gelatin/bioactive glass	37°C	Decreased gelation time and temperature with improved mechanical properties.	Bone tissue engineering	(Moreira et al., 2018)
Poly (N-Isopropylacrylamide) (pNIPAM) based thermogels	LCST = 32°C	Setbacks: Very poor mechanical strength. Shows thermo-responsive behaviour but cannot form a viscous gel on its own.		

Thermo-responsive polymers and their hybrid systems	Responsive Temperature	Advantages of hybridisation and salient features of hybrid system	Current/Potential application	Reference
Cellulose	36°C	Improved mechanical strength of gel.	Blood vessel repair	(Liu et al., 2004)
Poly(NIPAM-co-AM)/PEG/PTA	UCST = 59.6°C	Competitive behaviour resulting in an upper critical solution temperature.	Catalysis and other separation systems	(Guo et al., 2015)
Collagen	37°C	Improved mechanical strength and facilitated the development of different morphologies in stromal cells.	Directing the course of cell fate	(Barnes et al., 2016)
Mesoporous silica (KIT-6)	34°C	Improve mechanical strength and increased adsorption capacity thereby offering a higher loading capacity.	Controlled, sustained release drug delivery, and delivery of other guest molecules	(Kamachi et al., 2016)
Acrylic acid	32°C	The mechanical strength of the gel was greatly improved.	Tissue generation, soft robotics, sensors and manipulators	(Zhang et al., 2016)
ABA triblock copolymer flowerlike flower-like nanogels	32°C	Improved gel elasticity and strength, rapid temperature responsiveness and large swelling ratio.	Controlled drug delivery and tissue engineering	(Yuan et al., 2018)
2-Hydroxyethyl methacrylate (HEMA) and methacrylate	<37°C	Extended and sequential delivery of proteins	Protein delivery, tissue engineering,	(Nelson et al., 2012)

Thermo-responsive polymers and their hybrid systems	Responsive Temperature	Advantages of hybridisation and salient features of hybrid system	Current/Potential application	Reference
polylactide (MAPLA)			intramyocardial injections following ischemic injury	
Sulfonated poly(serinol hexamethylene urea) (SPSHU)	37°C	Decreased initial burst release and sustained release rate. Spatiotemporal control of VEGF.	Delivery of angiogenic factors such as VEGF.	(Lee et al., 2018)
N-[3-(Dimethylamino)propyl]methacrylamide (DMPMA); acrylamide (AM) PVA/PDMAEMA A-PSS PDMAEMA Poly(acrylic acid-co-acrylamide)	-	Successful manipulation of lower critical solution temperature and shape adaptive behaviour. Distinct swelling characteristics in each layer: Dually thermal and pH responsive. Dually thermal and c(H') responsive. Faster, reversible, bidirectional bending in both hot and cold temperature conditions.	Dually thermo-responsive shape adaptive hydrogel actuators.	(Zhang et al., 2019)

Thermo-responsive polymers and their hybrid systems	Responsive Temperature	Advantages of hybridisation and salient features of hybrid system	Current/Potential application	Reference
Pluronic based thermogel	15-30°C	Setbacks: High gelation concentration of $\geq 18\%$. Delicate structure leading to poor in-vitro stability in some cases.		
Methylcellulose	37°C	Decreased gelation concentration with an improvement in cell viability and sustained release over a 28-day period.	Bone remodelling	(Rangabhatla et al., 2016)
Alginate and hyaluronic acid	37°C	Improvement in gel strength, cytocompatibility and cell viability.	Cell injection/Tissue engineering	(Abdi et al., 2012)
Pluronic/alginate	36-40°C	Increased upper and lower transition temperatures, with an increase of approximately a hundred-fold in elastic moduli.	Wound healing, soft tissue engineering, dental applications and injectable drug delivery system	(Quah et al., 2018)
Methacryloisobutyl-polyhedral oligosilsesquioxane (MA-POSS)	33.5°C	Modification of the gelation temperature and degradation temperature.	Biomaterial for drug delivery, food and cosmetic applications.	(Dou, Karim and Loh, 2016)
Carboxymethyl chitosan (CMCS)	32-37°C	Increased gelation time and temperature, as well as increased drug release – tuneable with	Depot and controlled localized and systemic delivery of	(Khan et al., 2018)

Thermo-responsive polymers and their hybrid systems	Responsive Temperature	Advantages of hybridisation and salient features of hybrid system	Current/Potential application	Reference
		concentrations used.	chemotherapeutics.	
Methylcellulose based thermogels	40-50°C	Setbacks: High sol-gel transition temperature between 40-50°C, not compatible with physiological body temperature.		
Mucin	37°C	Decreased gelation temperature and time.	Wound healing	(Nowald et al., 2016)
Kosmotropic ion - KCl		No effect on gelation kinetics.		(Nowald et al., 2016)
Xylitol, sodium phosphate dibasic, and polyethylene glycol	32°C	Decreased gelation temperature and increased gelation speed.	Delivery of placental mesenchymal stem cells for treatment of severe ischemia in limbs.	(Wang et al., 2019)
Gallic acid, and sodium chloride	36.7°C	Decreased gelation temperature and sustained release of drug and gallic acid.	Wound healing	(Sangfai et al., 2017)
Various salts		Increased G', and a shift in the gelation temperature to temperatures below 37°C.	Cell seeding and cell sheet biofabrication	(Altomare et al., 2016)

Thermo-responsive polymers and their hybrid systems	Responsive Temperature	Advantages of hybridisation and salient features of hybrid system	Current/Potential application	Reference
Phosphate buffer Sodium sulphate	20°C 37°C	Good stability and the best handling in terms of in vitro cell tests.		(Altomare et al., 2016)
Calcium chloride	37°C			(Altomare et al., 2016)
Calcium chloride and alginate	37°C	Decreased gelation temperature and time.	Delivery of chitosan microparticles	(Gaihare et al., 2019)
PCL-PEG based thermogels				
Poly (PEG-PPG-PCL-urethane)	37°C	Improvement in gel strength with almost perfect zero order kinetics.	Delivery of chemotherapeutics.	(Loh, Yee and Chia, 2012)
Poly (PEG-PPG-PCL-urethane)		Improvement in release profile of gel.	Delivery of anti-VEGFs to inhibit angiogenesis.	(Xue et al., 2019)
PEG-PCL-PEG, pluronic F127 and poly propylene fumarate (PPF)	37°C	Improvement in gel strength with sustained release of drug over a 14-day period.	Pseudo-bone thermogel	(Kondiah et al., 2017)
mPEG-b-[PCL-g-(MEO2MA-co-OEGMA)]-b-mPEG	30°C	Improved water solubility and reversible thermogelation	Repair of damaged tissues	(Shang et al., 2019)
PNIPAAm-PCL-PEG-PCL-PNIPAAm	37°C	Ideal pore size for fibroblast attachment, improved cell	Skin tissue engineering, wound healing, and sustained	(Oroojalian et al., 2019)

Thermo-responsive polymers and their hybrid systems	Responsive Temperature	Advantages of hybridisation and salient features of hybrid system	Current/Potential application	Reference
		adhesion and cell proliferation.	drug delivery.	

2.3. Concluding remarks

In situ thermo-gelling systems may potentially replace conventional methods of treatment for diseases where sustained release of drugs, genes, proteins, or other molecules are needed. These include chronic conditions which require constant amounts of drug over a long period of time, such as diabetes, cancer treatment and ocular conditions such as glaucoma, to name a few. Thermogels also show great potential for wound healing applications, due to their ability to adequately fill the irregularly shaped wound area, their ease of application and their biodegradability.

There is great potential for the creation of tailor-made drug delivery systems by hybridization of thermo-responsive polymers with other compounds that either may or may not possess thermo-gelling ability but enhance the physico-mechanical properties and in vitro performance of thermo-gelling systems. Generally, natural polymers have the ability to mimic the human extra-cellular-matrix and provide good cell attachment but lack in mechanical strength, whereas synthetic polymers are often sought after due to their mechanical properties, but lack in their biocompatibility compared to natural polymers as well as in the ability to mimic the ECM. Therefore, hybridization of natural polymers with synthetic ones is a common trend that has been exploited by researchers. The creation of new thermo-gelling co-polymers via the hybridization process is an exciting prospect. However, further research in this field is needed to fully understand the thermo-gelling mechanisms and in vivo behavior of these systems to enable us to hybridize these polymers in a manner that will satisfy our dosage form and therapeutic requirements. Therefore, commercialization of thermo-gelling systems may still take a while.

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CHAPTER THREE

DESIGN OF METHYLCELLULOSE BASED THERMORESPONSIVE IN SITU GELLING SYSTEMS

3.1 Introduction

Thermo-responsive polymers have become increasingly popular over the years with a great deal of research being dedicated to the development of novel “smart” materials (Roy, Brooks and Sumerlin, 2013). These smart materials which undergo a drastic change in their physical and viscoelastic properties facilitate in-situ gelation and drug delivery. This makes them ideal for use in irregularly shaped wounds, by enabling the system and the bioactive to fill the entire wound space, resulting in even drug distribution and consistent healing throughout the wound area. There are many natural and synthetic polymers that display thermo-responsive properties. For this research, one polymer from each category (natural, synthetic, and semi-synthetic) was chosen, and then hybridized to create a natural-synthetic system, and a natural-semisynthetic system.

Among the natural polymers, methylcellulose is a popular choice. Cellulose is biocompatible, biodegradable, and abundant in nature, making it ideal and cost effective for biomedical applications (Alavi, 2019; Brinchi et al., 2013). Methylcellulose gels at temperatures between 40 and 50°C, as discussed in chapter two, however, it has a significant elastic modulus at its lower critical solution temperature (Matanović, Kristl and Grabnar, 2014), and will thus lend strength and viscosity to a hybrid system.

Chitosan- β -glycerophosphate is a semi-synthetic polymer that is afforded its thermo-responsive behaviour by the addition of its polyol salt component. Chitosan- β -glycerophosphate based gels are known to mimic extra-cellular matrix and provide a receptive environment for cell growth (Saravanan et al., 2018). While chitosan forms a gel at 37°C in the presence of a polyol salt, it has a low elastic modulus at its lower critical solution temperature and has low mechanical strength. β -glycerophosphate has

been known to be cytotoxic. Therefore, hybridizing chitosan with methylcellulose may improve the mechanical strength and biocompatibility of the system.

Pluronic F127 is a synthetic polymer that forms a gel at concentrations above 18% as discussed in the previous chapter of this dissertation. This high concentration is not ideal in terms of cost effectiveness, high viscosities, and gelation at ambient temperature. However, the low temperatures at which pluronic forms a gel make it ideal for use when combined with methylcellulose, to create a system which gels at an optimal temperature for in-situ gelation.

Rheological analysis of hybrid polymer formulations is often undertaken to analyse the interactions that occur between the polymers, as well as to exact the influence of the incorporation of each polymer (Ngwuluka et al., 2013). This is especially important when dealing with thermo-responsive systems, as the effect of temperature on the strength, viscosity and structural behaviour can be analysed using rheological techniques. These rheological properties will be the key factors in determining the suitability of the formulations for the proposed in-situ gelling system and its application in wound healing. The rheological behaviour of thermo-responsive polymers is often influenced by the concentration of the polymers used, as the thermo-responsive behaviour is often altered by altering the concentration of the polymer. Therefore, the effect of concentration on the thermo-gelling behaviour of the system will be explored within this chapter. The rheological properties (such as the mechanical strength, viscosity, and behaviour of the system as either a liquid or gel) and gelation kinetics (the gelation time and temperature) acquired from the analysis in this chapter aided in determining the final formulation for the proposed system.

3.2 Materials and methods

3.2.1 Materials

Low molecular weight chitosan, β -glycerophosphate disodium salt pentahydrate, glacial acetic acid, Pluronic F127 and Methocel LV A15 were purchased from Sigma-

Aldrich (St. Louis, Missouri, USA). Fibrous nanocellulose was purchased from DLD Scientific (Durban North, SA).

3.2.2 Synthesis of methylcellulose based thermo-responsive sol-gel systems

3.2.2.1 Hybridization of methylcellulose with pluronic and nanocellulose

To prepare the cellulose-pluronic hybrid polymer solutions, a 36% w/v solution of pluronic was prepared by dissolving 36_g of pluronic F127 in 100_mL of cold deionized water and allowing it to stir until dissolved in an ice bath. This concentration was chosen since pluronic F127 has a critical gelation concentration of 18%_wt (Radivojša, Grabnar and Grabnar, 2013), thus 36% w/v was used as the initial concentration to facilitate the critical gelation concentration during hybridization. A 7% w/v methocel:1% w/v nanocellulose solution was then prepared by dispersing 1 g of nanocellulose particles in an amount of deionised water, which was thereafter ultrasonicated (Ultrasonic Cleaner, SE-366, Scientech, India) for 30 min to ensure proper distribution of the nanoparticles. A 7% w/v concentration of cellulose was chosen as it exhibited a gelation temperature of 32°C (Gupta et al., 2006), which was suitable for the current application, and a 1% w/v nanocellulose was settled upon by a trial-and-error method. Methocel was then dissolved in the remainder of the water and the nanocellulose and methocel were then combined and allowed to stir overnight. Thereafter, the pluronic F127 solution and cellulose solution were combined dropwise using the cold method in various ratios, which will be detailed in the table below.

Table 3.1: Various Pluronic-Cellulose hybrid formulations that were investigated

Pluronic-Cellulose Hybrids
Pluronic F127 10:90 Methocel-nanocellulose (P10NC)
Pluronic F127 20:80 Methocel-nanocellulose (P20NC)
Pluronic F127 30:70 Methocel-nanocellulose (P30NC)
Pluronic F127 40:60 Methocel-nanocellulose (P40NC)

3.2.2.2 Hybridization of methylcellulose with chitosan- β -glycerophosphate

To prepare the methylcellulose-chitosan hybrid polymer solutions, a 2% w/v solution of Chitosan was prepared by dissolving 2_g in 100_mL of 0.5% v/v glacial acetic acid. A 2% w/v solution of chitosan was chosen as a 1% w/v solution proved to be not viscous enough, while concentrations over 2% w/v proved to be too viscous to work with for the proposed system. In addition, 2%_wt was reported as the gelation concentration of chitosan when combined with glycerophosphate (Chenite et al., 2001). ~~3% w/v, 5% w/v and 7% w/v~~ Solutions of methylcellulose were prepared by dissolving 3_g, 5_g and 7_g of methocel in 100_mL of deionized water respectively to achieve concentrations of 3% w/v, 5% w/v and 7% w/v, and allowing it to stir overnight. An 87.5% w/v solution of β -glycerophosphate disodium salt pentahydrate was prepared by dissolving 7_g of the glycerophosphate in 5_mL of deionized water to produce a final volume of 8_mL. Thereafter, chitosan and methocel were combined in a dropwise manner and allowed to stir over night. The cold method was used and an amount β -glycerophosphate was added drop wise into the combined cellulose-chitosan mixture and allowed to stir. The final concentration of glycerophosphate was kept constant at ~9% throughout the combinations (Kojarunchitt et al., 2015). The solutions were then refrigerated overnight and allowed to rest before analysis. ~~VA table of the various hybrid combinations- were used for the current study (Table 3.2) will be listed below.~~

Table 3.2: Various Chitosan-Cellulose hybrid formulations that were investigated

Chitosan-Cellulose Hybrids			
Chitosan: 3%	Methylcellulose: β -	Chitosan: 3%	Methylcellulose: β -
Glycerophosphate (3MC-CHT)		Glycerophosphate (3MC-2CHT)	
1:1:0.25		2:1:0.375	
Chitosan: 5%	Methylcellulose: β -	Chitosan: 5%	Methylcellulose: β -
Glycerophosphate (5MC-CHT)		Glycerophosphate (5MC-2CHT)	
1:1:0.25		2:1:0.375	
Chitosan: 7%	Methylcellulose: β -	Chitosan: 7%	Methylcellulose: β -
Glycerophosphate (7MC-CHT)		Glycerophosphate (7MC-2CHT)	

1:1:0.25	2:1:0.375
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3.2.3 Sol-gel phase transition

A tilt bottle test will also be conducted to determine the gel strength and the estimated gelation time. A sample of the gel will be placed into a bottle and then into an incubator at 37°C for 30 minutes. The bottle will be periodically removed from the incubator and tilted, to determine whether the formulation has gelled. The time point at which the sample within the bottle no longer flows when the bottle is tilted will be noted as the gelation time.

3.2.4 Rheological analysis of thermo-responsive behaviour

3.2.4.1 Viscosity and temperature ramp

The viscosity of the polymer solutions at varying concentrations will be measured using a Rheometer (Haake Modular Advanced Rheometer System, ThermoFischer Scientific, Germany). The samples will be run at a temperature range of 0-40°C at a rate of 1°C per minute and at a shear rate of 100 s⁻¹, with the use of a C35/1° Titanium rotor in the polymer solution. Temperature ramp measurements will be conducted at a heating rate of 1°C/min, over a temperature range of 0°C to 40°C, using parallel plate geometry, at a constant strain of 1% and a constant frequency of 6 rad/s. The elastic (storage) modulus (G') and loss modulus (G'') of the gel will be determined, together with their crossover points to indicate the gel transition temperature (Tang et al., 2010).

3.2.4.2 Oscillation stress sweep

An oscillation stress sweep of the formulations will be conducted to determine the mechanical strength of the formulations at 37°C and at 25°C, characterized by their yield stress value and the linear viscoelastic region. This will also determine whether the formulations will be able to withstand the stress applied during the application as

well as any stress that may be experienced in situ. The strength of the formulation may also influence the release kinetics of the formulations.

3.2.4.3 Oscillation frequency sweep

An oscillation frequency sweep of the formulations will be conducted to determine the structural behaviour of the formulations at 37°C and at 25°C, to determine whether the formulations will be able to withstand any forces applied during application, as well as any physiological forces that may be experienced in situ. The structural behaviour of the formulations at 25°C and at 37°C will also inform us of whether the formulations are in a sol or gel state at these temperatures.

3.3 Results and Discussion

3.3.1 Sol-gel phase transition

Table 3.3: Time taken for the various formulations to form a gel

Formulation	Gelation Time
3MC-CHT	20 min
5MC-CHT	10 min
7MC-CHT	7 min
3MC-2CHT	30 min
5MC-2CHT	12 min
7MC-2CHT	10 min
P10NC	Did not form a stiff gel in 30 min
P20NC	10 min
P30NC	7 min
P40NC	5 min

Note. 3MC-CHT is the formulation using an initial 3% concentration of cellulose, and combined in a 1:1 ratio with chitosan; 5MC-CHT is formulated using an initial 5%

concentration of cellulose and combined in a 1:1 ratio with chitosan; and 7MC-CHT employs a 7% concentration of cellulose and is combined in a 1:1 ratio with chitosan. The same is applicable for the formulations labelled “-MC-2CHT”, however these formulations are combined in a 1:2 ratio with chitosan. P10NC refers to the formulation that is combined in a 10:90 ratio of pluronic:methylcellulose-nanocellulose blend; P20NC is combined in a 20:80 ratio as described above, P30NC refers to a 30:70 ratio, and P40NC refers to a blend of pluronic:methylcellulose-nanocellulose blend in a 40:60 ratio.

When a tilt bottle test was conducted, it was found that when methylcellulose was hybridized with chitosan- β -glycerophosphate, increasing concentrations of methylcellulose within the formulations resulted in quicker gelation times, whereas doubling the ratio of chitosan (for all concentrations of cellulose) resulted in an increase in the gelation time. This is due to the fact that increasing the concentration of methylcellulose within the formulation reduces the temperature at which the formulation starts to form a gel, thereby resulting in quicker gel formation at 37°C. Whereas doubling the ratio of chitosan within the formulation resulted in an increase in the temperature at which the formulation starts to gel, thus a slightly longer time was taken for these formulations to form a gel at 37°C.

The opposite was true when pluronic was hybridized with methylcellulose and nanocellulose, as quicker gelation times are noted in the formulations with a greater content of pluronic, as opposed to cellulose. This is because the greater the content of pluronic f127 in the formulation, the lower the temperature at which the elastic modulus starts to increase (Figure 3.123) and the lower the crossover point between the elastic and loss moduli (Table 3.43.2), thus allowing quicker gel formation at 37°C.

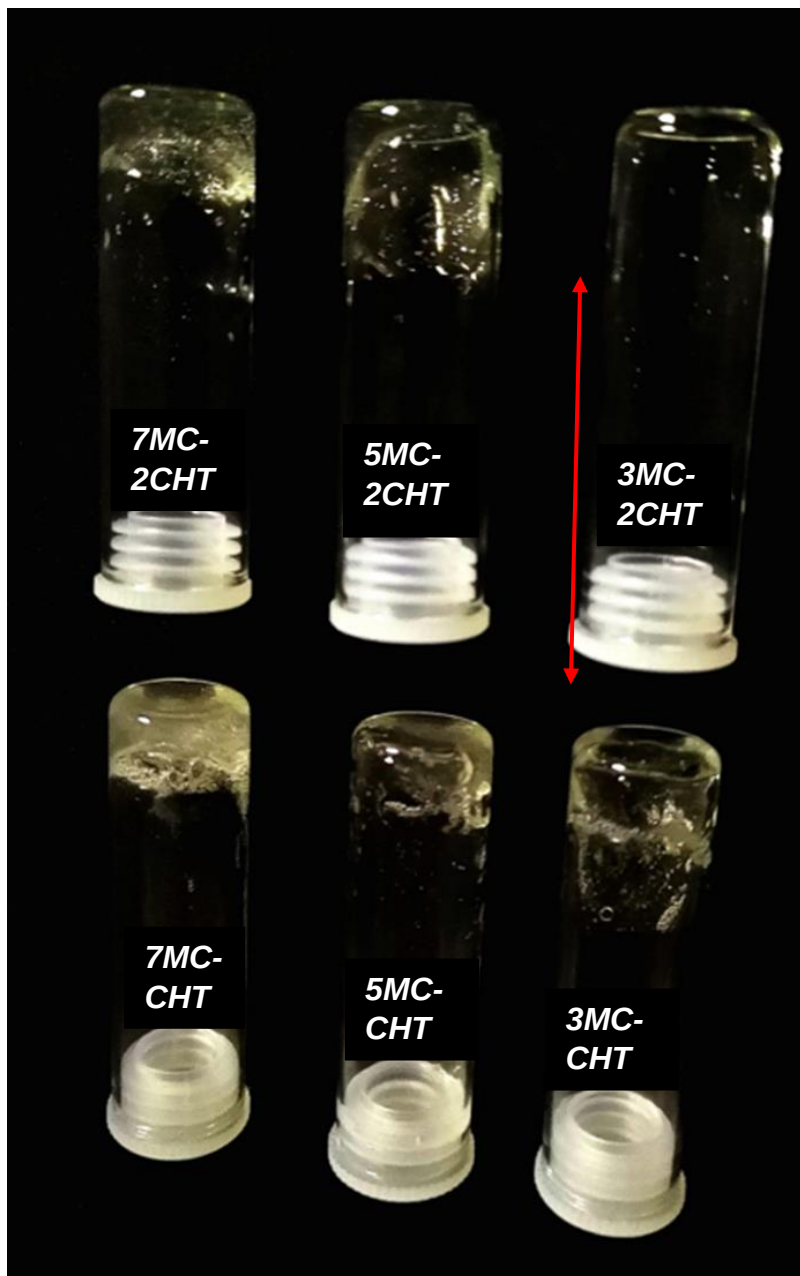


Figure 3.1: The chitosan- β -Glycerphosphate-cellulose formulations after being left at 25°C for 12 minutes. Where 3MC-CHT is the formulation using an initial 3% concentration of cellulose, and combined in a 1:1 ratio with chitosan; 5MC-CHT is formulated using an initial 5% concentration of cellulose and combined in a 1:1 ratio with chitosan; and 7MC-CHT employs a 7% concentration of cellulose and is combined in a 1:1 ratio with chitosan. The same is applicable for the formulations labelled “-MC-2CHT”, however these formulations are combined in a 1:2 ratio with chitosan.

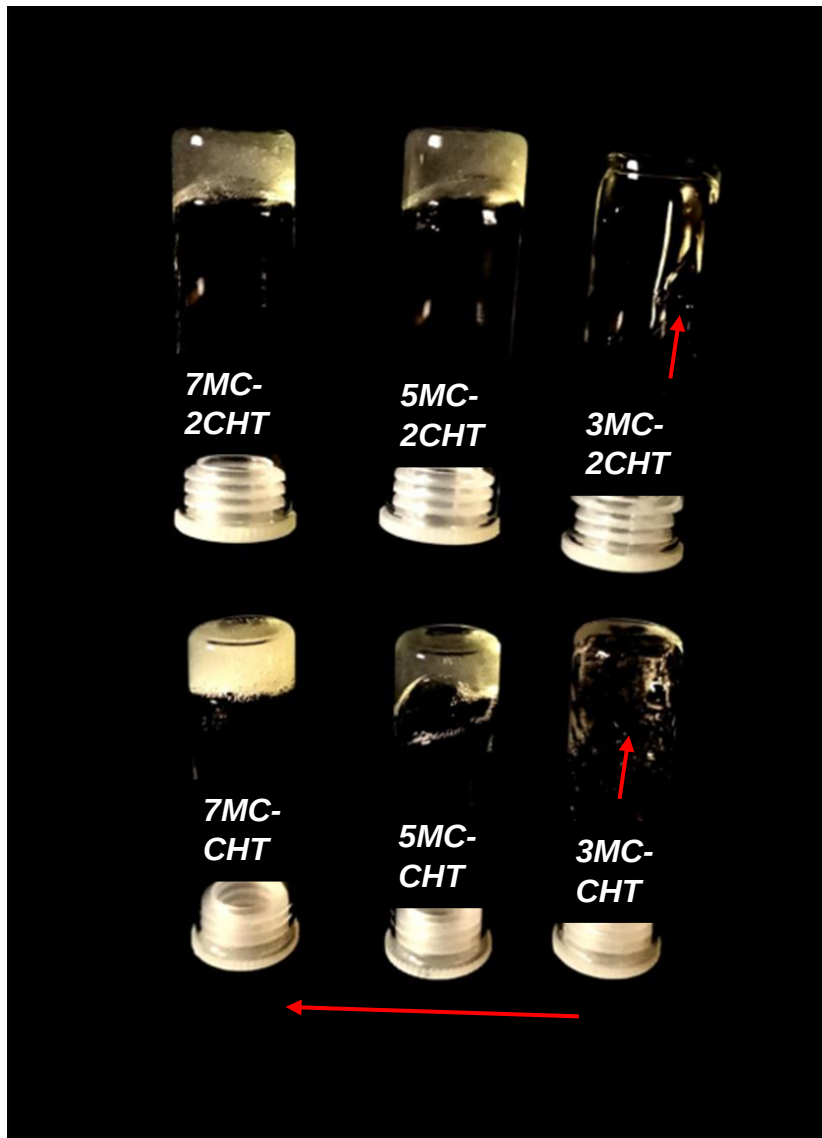


Figure 3.2: The chitosan- β -Glycerphosphate-cellulose formulations after being left at 37°C for 12 minutes. Where 3MC-CHT is the formulation using an initial 3% concentration of cellulose, and combined in a 1:1 ratio with chitosan; 5MC-CHT is formulated using an initial 5% concentration of cellulose and combined in a 1:1 ratio with chitosan; and 7MC-CHT employs a 7% concentration of cellulose and is combined in a 1:1 ratio with chitosan. The same is applicable for the formulations labelled “-MC-2CHT”, however these formulations are combined in a 1:2 ratio with chitosan.

The gels were photographed at the 12 minute mark and it can be seen that formulations containing 3% cellulose (3MC-CHT and 3MC-2CHT) had not yet formed a gel (Figure 3.2). The effect of methylcellulose on the strength of the gel in the chitosan-cellulose based formulations can be seen in formulations labelled ‘3MC-CHT’ to ‘7MC-CHT’, with the opaqueness and stiffness of the gels increasing with increasing cellulose

concentrations (Figure 3.2). In addition, when the formulations were left at room temperature for the same amount of time, formulations with equal ratios of cellulose to chitosan had already started to thicken and began to form a gel (5MC-CHT and 7MC-CHT), whereas doubling the ratio of chitosan to cellulose mitigated this problem, and formulations remained in a more liquid state at room temperature (5MC-2CHT and 7MC-2CHT) (Figure 3.1).

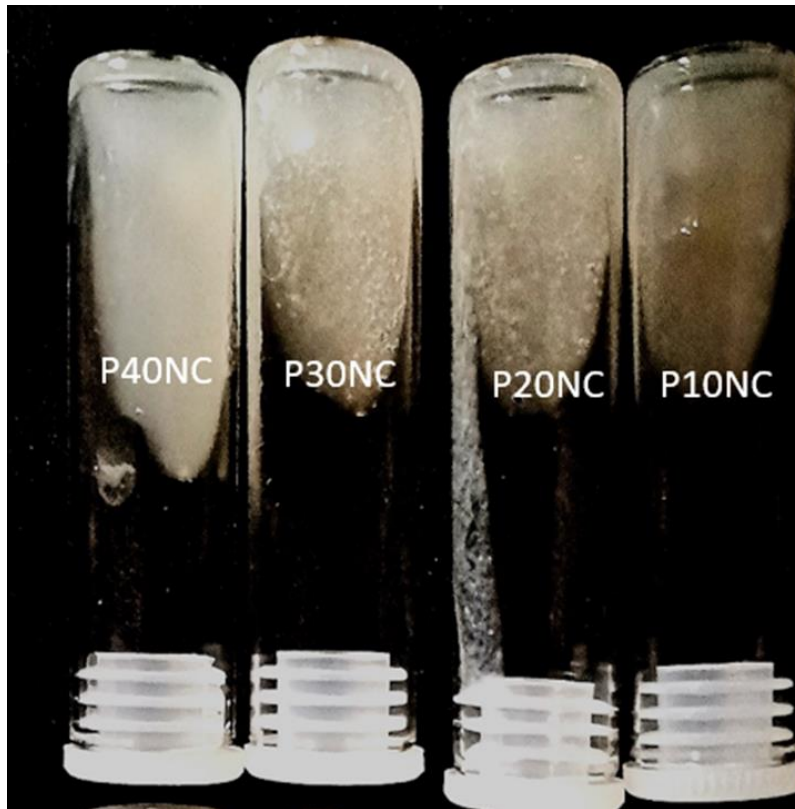


Figure 3.3: The pluronic-cellulose formulations after being left at 25°C for 12 minutes. Where P10NC refers to the formulation that is combined in a 10:90 ratio of pluronic:methylcellulose-nanocellulose blend; P20NC is combined in a 20:80 ratio as described above, P30NC refers to a 30:70 ratio, and P40NC refers to a blend of pluronic:methylcellulose-nanocellulose blend in a 40:60 ratio.

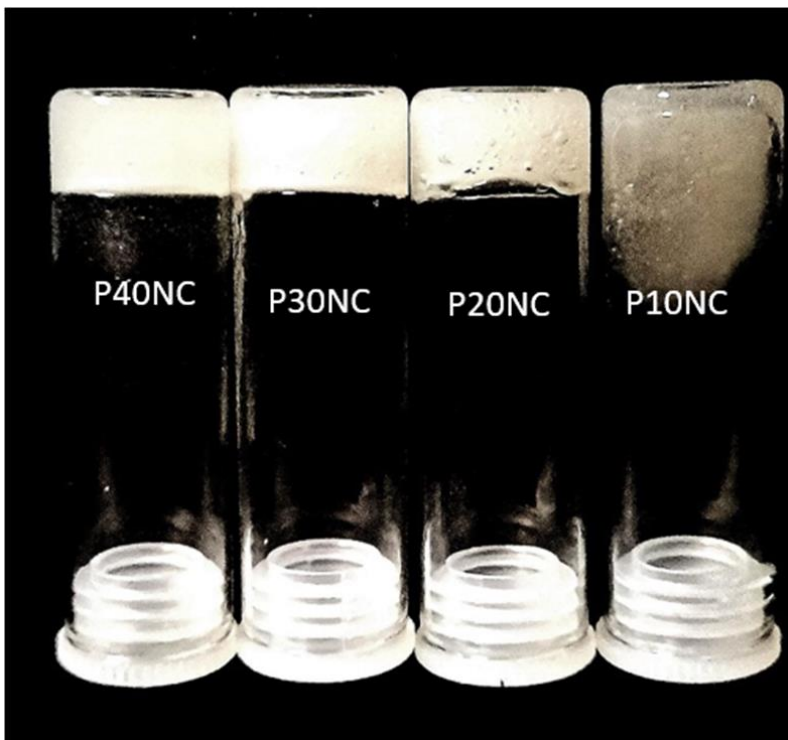


Figure 3.4: The pluronic-cellulose formulations after being left at 37°C for 12 minutes.

Where P10NC refers to the formulation that is combined in a 10:90 ratio of pluronic:methylcellulose-nanocellulose blend; P20NC is combined in a 20:80 ratio as described above, P30NC refers to a 30:70 ratio, and P40NC refers to a blend of pluronic:methylcellulose-nanocellulose blend in a 40:60 ratio.

In the pluronic-cellulose based formulations, it is seen that all formulations wherein the final concentration of pluronic was above 7.2% (P20NC) had formed a gel within 12 minutes (Figure 3.4), however when left at room temperature for the same amount of time, all of the pluronic-cellulose based formulations remained in liquid form (Figure 3.3). P40NC became more opaque when left out at room temperature (Figure 3.3), indicating the beginning of gel formation, but was still able to flow. While the crossover temperature of P40NC was 23.6°C as seen in table three, perhaps the gelation time was much longer than the 12 minutes, the time at which the image in figure 3.3 was taken, and therefore a stiff gel had not yet been formed.

3.3.2 Rheological analysis of thermo-responsive behaviour

3.3.2.1 Chitosan-cellulose based formulations

3.3.2.1.1 Viscosity and temperature ramp

From table 3.4.1 below it is noted that in the chitosan-cellulose based formulations, as the concentration of cellulose increases in the formulations labelled 3MC- to 7MC-, the elastic (storage) modulus (G') and viscosity increase at both room temperature and physiological temperature. The temperature at which the elastic modulus begins to increase also decreases, indicating that the formulations start to form a gel at lower temperatures with higher concentrations of cellulose. This is in keeping with the findings by Arvidson and co-workers (2013), that the gelation temperature decreases with increasing concentrations of cellulose, and this has been attributed to the greater degree of crosslinking per polymer chain (Arvidson et al., 2013). This can further be explained by the disappearance of the exothermic effect at higher concentrations of cellulose, as described by Niemczyk-Soczynska and team (elaborated in section 2.2.4 of this dissertation) due to the densely packed polymeric network at high concentrations (Niemczyk-Soczynska et al., 2019). In the chitosan-based formulations where 'no crossover *' is noted, this is because the elastic modulus is already greater than the loss modulus from the onset. The viscosity in these formulations was also significantly higher than the others, even at room temperature. These high viscosities rendered these formulations (5MC-CHT, 7MC-CHT and 7MC-2CHT) unsuitable for use in the intended system, as a freely flowing solution at ambient temperatures was needed for this application. We can therefore deduce that a 7% cellulose concentration was not suitable when hybridised with chitosan (formulations 7MC-CHT and 7MC-2CHT), as the elastic moduli and viscosities at 25°C were much too high, whereas a 3% cellulose concentration produced elastic moduli and viscosities that were too low, even at 37°C, rendering these formulations (3MC-CHT and 3MC-2CHT) unsuitable as well. A 5% cellulose solution thus proved to be the best for hybridization with chitosan- β -glycerophosphate, as can be seen in the formulations labelled '5MC-' (5MC-CHT and 5MC-2CHT).

Another noteworthy point in the chitosan-based formulations is that when the ratio of chitosan is doubled that of methylcellulose within each formulation, the elastic modulus and viscosity of the resulting formulations were lowered, while the temperatures at

which these formulations started to form a gel were only slightly altered. This technique therefore proved to be useful to mitigate the issue of high viscosities at ambient temperatures, and to allow for the formulation of a freely flowing system at room temperature, without disrupting thermo-responsive behaviour of the system in a way that would affect in-situ gelation. For this reason, formulation 5MC-2CHT was chosen as the optimal formulation as the elastic modulus and viscosity at 25°C were much lower than those in formulation 5MC-CHT, while at the same time the elastic modulus and viscosity at 37°C, and the gelation temperature, were not altered drastically.

Table 3.4.1 Rheological assessment of the chitosan-cellulose hybrid formulations

Formulation	Crossover Temperature (°C)	Temperature at which G' starts to increase (°C)	G' at 25°C (Pa)	G' at 37°C (Pa)	Viscosity at 25°C (mPas)	Viscosity at 37°C (mPas)
3MC-CHT	24.81	23	4.577	135.2	997.1	21 670
5MC-CHT	No crossover *	25	24.49	456	3 991	72 730
7MC-CHT	No crossover *	17	283.9	3 611	45 420	575 700
3MC-2CHT	26.34	25	2.122	89.14	495.2	14 590
5MC-2CHT	26.95	23	0.8579	413.1	244.7	65 850
7MC-2CHT	No crossover *	21	74.81	1750	12 100	280 400

~~From the table above it is noted that in the chitosan-cellulose based formulations, as the concentration of cellulose increases in the formulations labelled 3MC- to 7MC-, the elastic (storage) modulus (G') and viscosity increase at both room temperature and physiological temperature. The temperature at which the elastic modulus begins to increase also decreases, indicating that the formulations start to form a gel at lower temperatures with higher concentrations of cellulose. This is in keeping with the~~

findings by Arvidson and co-workers (2013), that the gelation temperature decreases with increasing concentrations of cellulose, and this has been attributed to the greater degree of crosslinking per polymer chain. This can further be explained by the disappearance of the exothermic effect at higher concentrations of cellulose, as described by Niemczyk-Soczynska and team (elaborated in section 2.2.4 of this dissertation) due to the densely packed polymeric network at high concentrations (Niemczyk-Soczynska et al., 2019). In the chitosan-based formulations where 'no crossover *' is noted, this is because the elastic modulus is already greater than the loss modulus from the onset. The viscosity in these formulations was also significantly higher than the others, even at room temperature. These high viscosities rendered those formulations (5MC-CHT, 7MC-CHT and 7MC-2CHT) unsuitable for use in the intended system, as a freely flowing solution at ambient temperatures was needed for this application. We can therefore deduce that a 7% cellulose concentration was not suitable when hybridised with chitosan (formulations 7MC-CHT and 7MC-2CHT), as the elastic moduli and viscosities at 25°C were much too high, whereas a 3% cellulose concentration produced elastic moduli and viscosities that were too low, even at 37°C, rendering these formulations (3MC-CHT and 3MC-2CHT) unsuitable as well. A 5% cellulose solution thus proved to be the best for hybridization with chitosan- β -glycerophosphate, as can be seen in the formulations labelled '5MC' (5MC-CHT and 5MC-2CHT).

Another noteworthy point in the chitosan-based formulations is that when the ratio of chitosan is doubled that of methylcellulose within each formulation, the elastic modulus and viscosity of the resulting formulations were lowered, while the temperatures at which these formulations started to form a gel were only slightly altered. This technique therefore proved to be useful to mitigate the issue of high viscosities at ambient temperatures, and to allow for the formulation of a freely flowing system at room temperature, without disrupting thermo-responsive behaviour of the system in a way that would affect in-situ gelation. For this reason, formulation 5MC-2CHT was chosen as the optimal formulation as the elastic modulus and viscosity at 25°C were much lower than those in formulation 5MC-CHT, while at the same time the elastic modulus and viscosity at 37°C, and the gelation temperature, were not altered drastically.

The effect of hybridizing chitosan-glycerophosphate with methylcellulose on the gelation temperature can be explained via the following mechanism (Figure 3.5). At low temperatures, water molecules interact with the methoxyl groups in cellulose, methylcellulose behaves as a polyhydroxy material. When cellulose is then added to chitosan at low temperatures, water molecules interact with cellulose and chitosan via hydrogen bonding, resulting in an enhancement in the dissolution of the chitosan chains and reduction in the molecular mobility, thereby preventing chitosan chain association and precipitation. Upon an increase in temperature, energized water molecules are dissipated due to the reduction in the hydrogen bonding intermolecular forces, and gelation occurs via hydrophobic intermolecular chain associations. The increase in temperature affords the molecules energy allowing the chains to orient themselves favourably for the formation of a structured three-dimensional network. The effect of glycerophosphate on the gelation temperature can be explained by the presence of hydroxyl groups in glycerophosphate, which also played a role in the prevention of association and precipitation of chitosan chains (Tang et al., 2010). Glycerophosphate is known to have ionic interactions and is known to be an ionic physical crosslinker, therefore in the presence of chitosan and methylcellulose, where cellulose has -OH groups, there are ionic interactions that can occur thereby facilitating physical crosslinking.

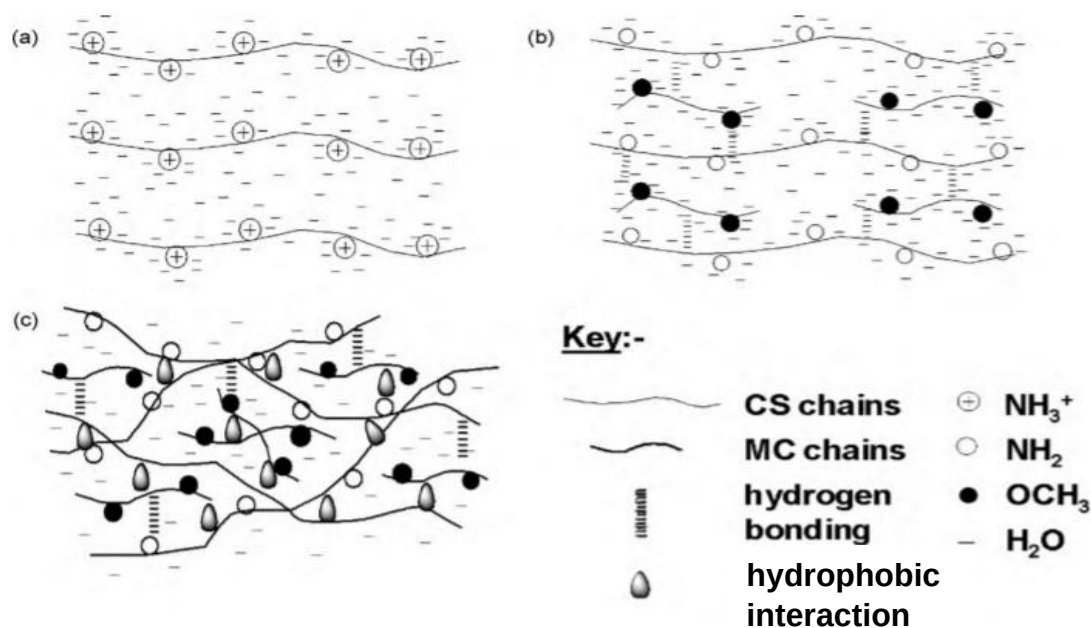


Figure 3.5: Mechanism of gelation between chitosan and methylcellulose. (a) chitosan solution at low temperatures; (b) Chitosan-methylcellulose solution at room temperature, where the presence of hydrogen bonding can be seen; (c) Chitosan-methylcellulose at higher temperatures, where hydrogen bonding exists together with hydrophobic intermolecular forces. Adapted with permission from Elsevier, Tang. et al. 2019. Production and characterisation of novel injectable chitosan/methylcellulose/salt blend hydrogels with potential application as tissue engineering scaffolds, Carbohydrate Polymers, 82, 833-841.

The synergistic result that was observed when chitosan- β -glycerophosphate was hybridised with methylcellulose can once again be seen in the graphs below. As the concentration of methylcellulose was increased, the elastic modulus (Figure 3.6) and viscosity (Figure 3.7) were increased, and the temperature at which the formulation began to form a gel was decreased. In the graph below (Figure 3.6), this can be seen by noting that as the concentration of cellulose increased, the temperature at which G' starts to increase was lowered. It can further be seen that doubling the ratio of chitosan to cellulose (for all concentrations of methylcellulose) resulted in a significant decrease in the elastic modulus (Figure 3.6) and viscosity (Figure 3.7) of the formulations at lower temperatures. This thermo-responsive behaviour dictated that the system be refrigerated during storage, so that it may remain unaltered in a sol state.

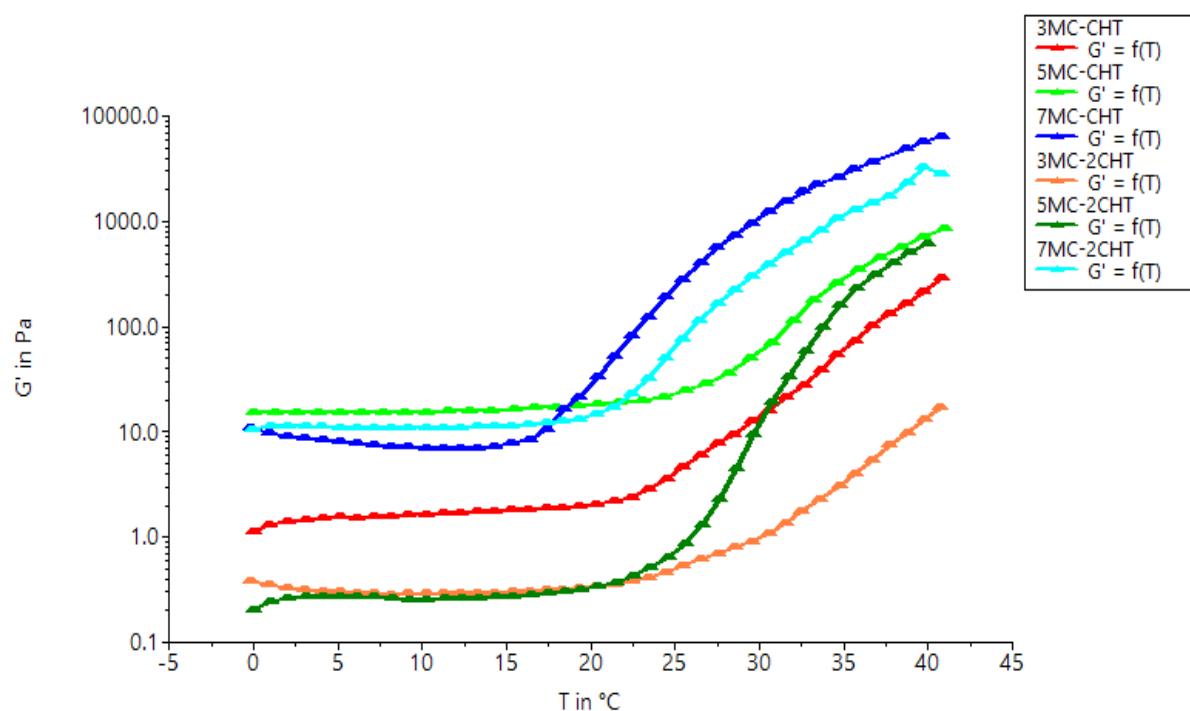


Figure 3.6: Temperature ramp of the various chitosan- β -glycerophosphate-cellulose formulations showing the change in their elastic moduli with temperature.

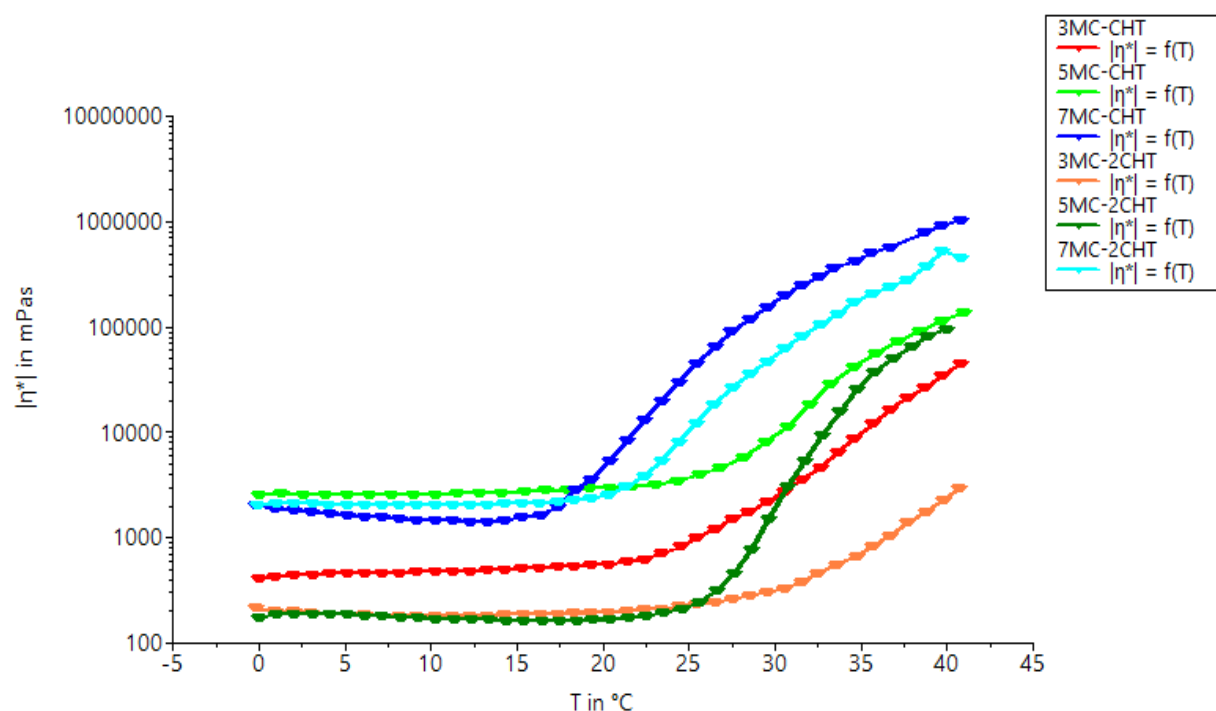


Figure 3.7: Graph depicting the change in viscosity of the various chitosan- β -glycerophosphate-cellulose formulations with temperature.

3.3.2.1.2 Oscillation stress sweep

The viscoelastic region of a formulation is determined by conducting an oscillation stress sweep. This viscoelastic region denotes the mechanical strength of the formulation, and its ability to withstand physiological stressors that might be present once the formulation has been applied. In turn, it also impacts the degradation and release kinetics of the formulation, as the stability and strength of the formulation correlate with the yield value (Ngwuluka et al., 2013). This yield stress value is denoted by the point at which the elastic modulus (G') starts to decline, indicating the occurrence of structural deformation. An increase in the elastic modulus of a formulation correlates to an increase in the degree of intermolecular crosslinking within the formulation (Moura, Figueiredo and Gil, 2007), thereby resulting in an increase in the mechanical strength of the formulation (Anseth, Bowman and Brannon-Peppas, 1996). The degree of crosslinking is also proportionate to the yield stress value, and therefore the extension of the viscoelastic region. Since these formulations are thermo-responsive, and prepared without the addition of any chemical crosslinkers, crosslinking occurs physically, via gelation in response to temperature.

In the rheograms below of the chitosan-cellulose hybrids (Figure 3.8.1), it can be seen that the concentration of methylcellulose was proportionate to the linear viscoelastic region, as well as to the G' value at which this viscoelastic region occurs. This can be attributed to the fact that increasing the concentration of methylcellulose within the formulations resulted in a decrease in the gelation temperature, and thus a greater degree of physical crosslinking was seen at 37°C. As the ratio of chitosan was doubled within the formulation, the linear viscoelastic region was shortened, and the elastic modulus at which the viscoelastic region occurred was also decreased. Once again, this can be attributed to the change in the gelation temperatures, as doubling the ratio of chitosan to cellulose resulted in an increase in the gelation temperatures of the formulations, compared to their counterparts with equal chitosan:cellulose ratios, thus a lesser degree of physical crosslinking was seen at 37°C, and consequently a decrease was seen in the mechanical strength of the formulations. As expected, at 37°C, all formulations exhibited greater elastic moduli (Figure 3.8.1) than loss moduli (Figure 3.8.2), denoting that all formulations displayed gel like behaviour.

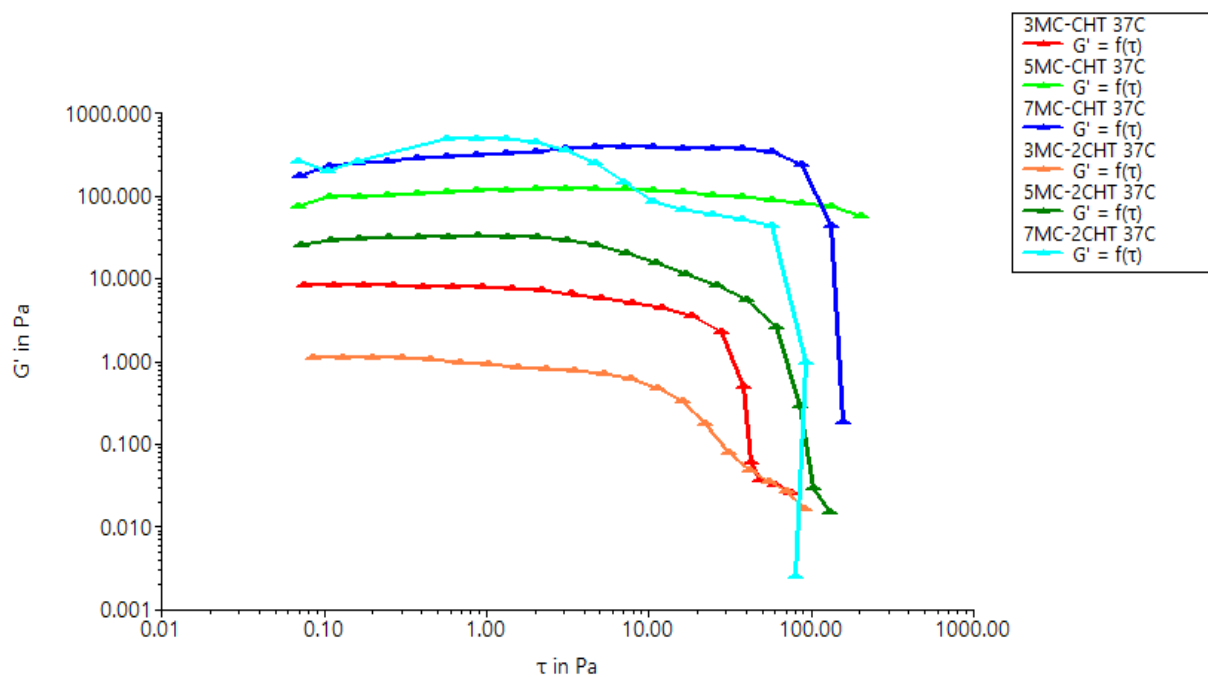


Figure 3.8.1: Graph depicting the change in the elastic moduli of the various chitosan- β -glycerophosphate-cellulose formulations with stress applied at 37°C.

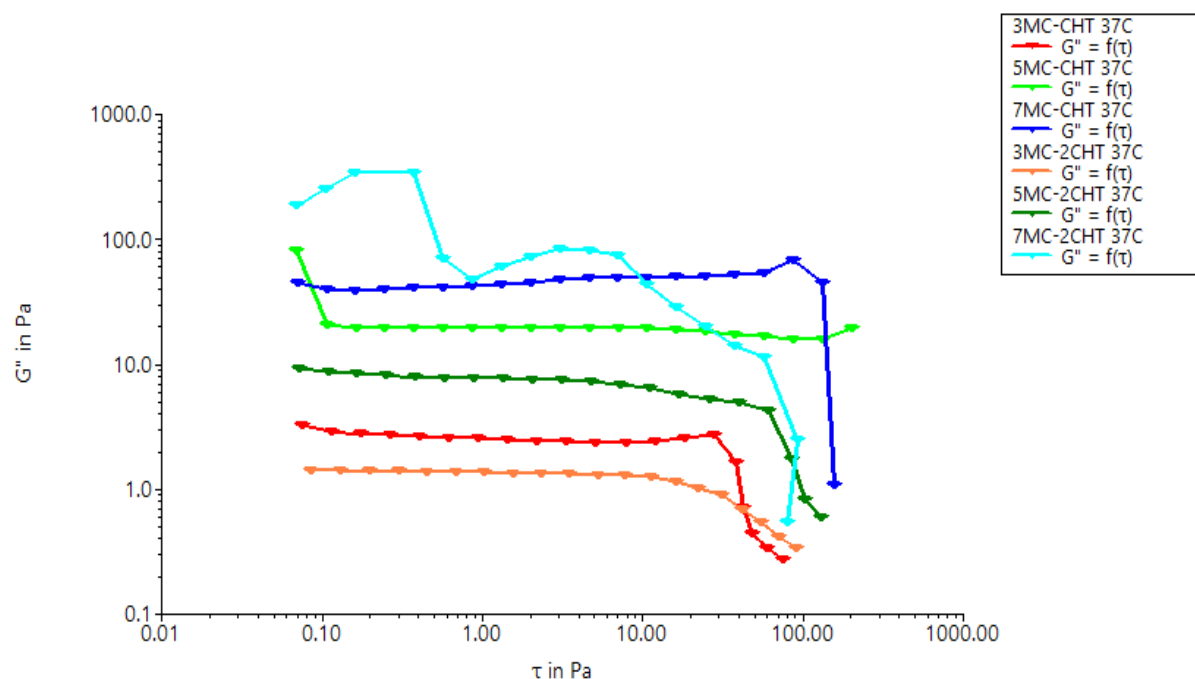


Figure 3.8.2: Graph depicting the change in the loss moduli of the various chitosan- β -glycerophosphate-cellulose formulations with stress applied at 37°C.

For formulations 3MC-CHT, 3MC-2CHT and 5MC-2CHT, the loss modulus is greater than the elastic modulus at 25°C (Figure 3.9.1; Figure 3.9.2), indicating that these formulations were in a liquid state at room temperature. Formulations 5MC-CHT, 7MC-CHT and 7MC-2CHT however exhibit greater elastic moduli than loss moduli at 25°C (Figure 3.9.1; Figure 3.9.2), suggesting that even at room temperature, these formulations display more gel like behaviour, this is in keeping with what was seen in the tilt bottle test in figure 3.1.

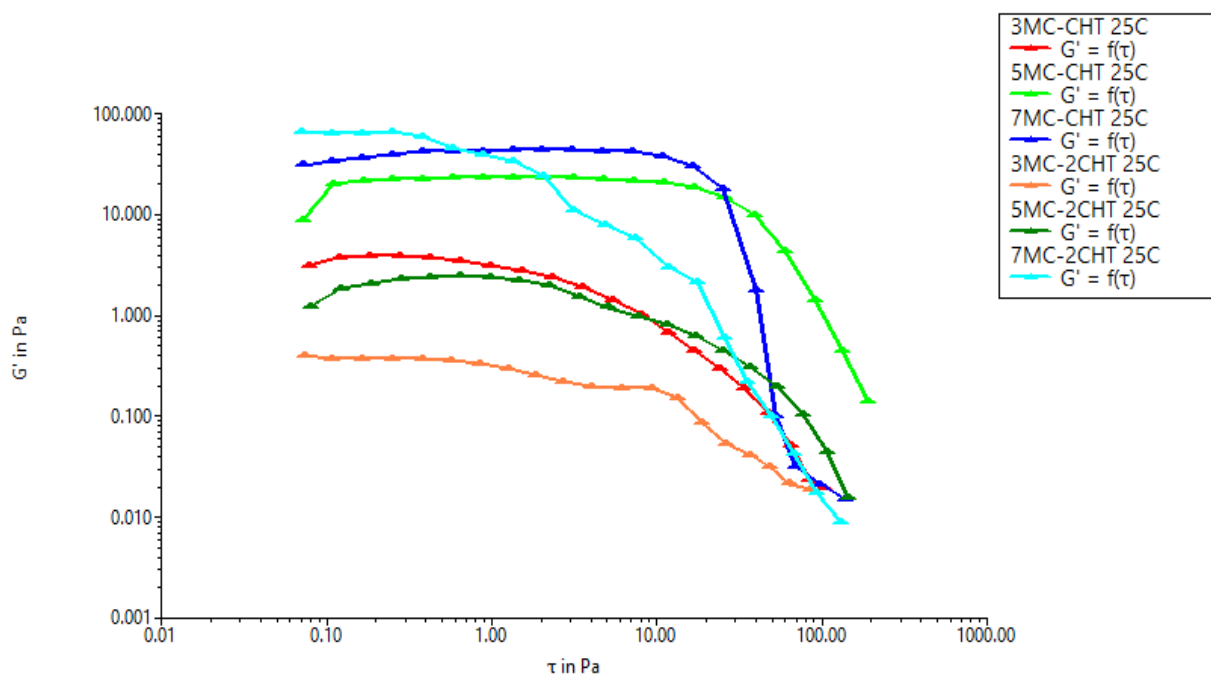


Figure 3.9.1: Graph depicting the change in the elastic moduli of the various chitosan- β -glycerophosphate-cellulose formulations with stress applied at 25°C.

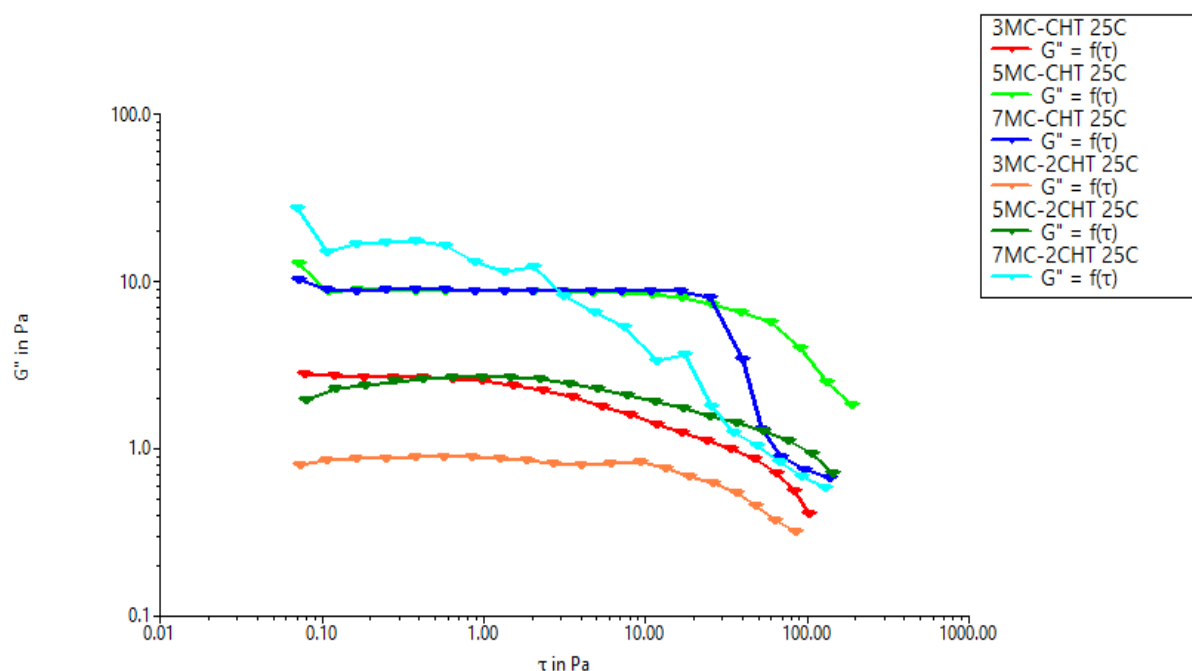


Figure 3.9.2: Graph depicting the change in the loss moduli of the various chitosan-β-glycerophosphate-cellulose formulations with stress applied at 25°C.

3.3.2.1.3 Oscillation frequency sweep

Frequency sweeps are used in rheology to structurally characterize materials to determine whether they behave as a gel (a three dimensional entangled network), a paste (an entangled solution), or a liquid (a particle solution). In the rheograms below (Figure 3.10), the elastic modulus is greater than and almost parallel to the loss modulus for all formulations at 37°C, indicating that these formulations behaved as entangled networks. However, in formulation 3MC-2CHT, the elastic modulus was greater at lower frequencies, but as the frequency increased (angular frequency— ω), a crossover occurred and the loss modulus became slightly higher. This indicates that formulation 3MC-2CHT behaved as a viscous liquid when subjected to higher frequencies at 37°C.

The effect of increasing concentrations of methylcellulose can clearly be seen in the rheograms depicted in figure 3.11, where greater storage moduli are exhibited at higher concentrations of methylcellulose at 25°C, indicating an improvement in the strength of the formulations. Formulation 3MC-CHT behaves as an entangled network at lower frequencies, however as the frequency increased above 10 rad/s, a more

viscous liquid-like behaviour is exhibited. Formulation 5MC-CHT and 7MC-CHT both have elastic moduli that are greater than, and parallel to their loss moduli, indicating that they behave as entangled networks, with the value of the elastic modulus increasing from formulation 5MC-CHT (5% cellulose) to formulation 7MC-CHT (7% cellulose). However, these elastic moduli values are much lower than those displayed in the same formulations at 37°C. The effect of doubling the ratio of chitosan can also be seen if one looks at the rheogram for formulation 5MC-2CHT, and compares it with that of formulation 5MC-CHT. While the elastic and loss moduli increase with increasing frequency, the loss modulus is greater than the elastic modulus at frequencies greater than 1 rad/s, indicating that increasing the ratio of chitosan resulted in the formulation behaving as a liquid at 25°C.

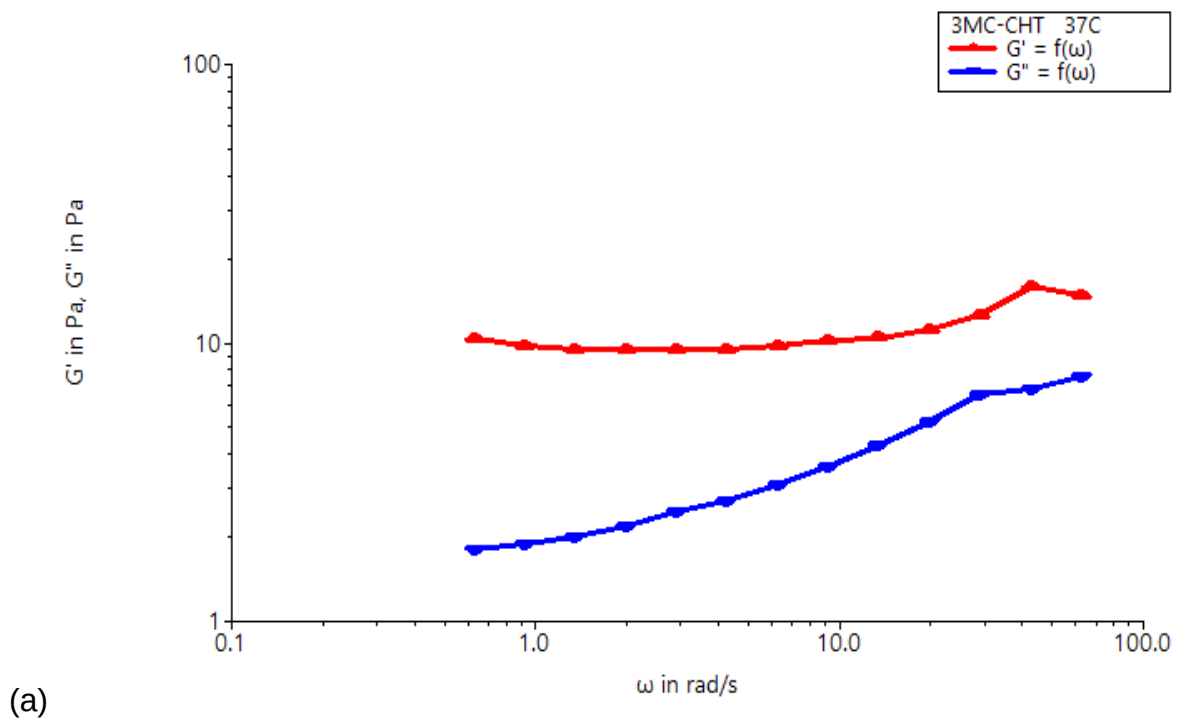
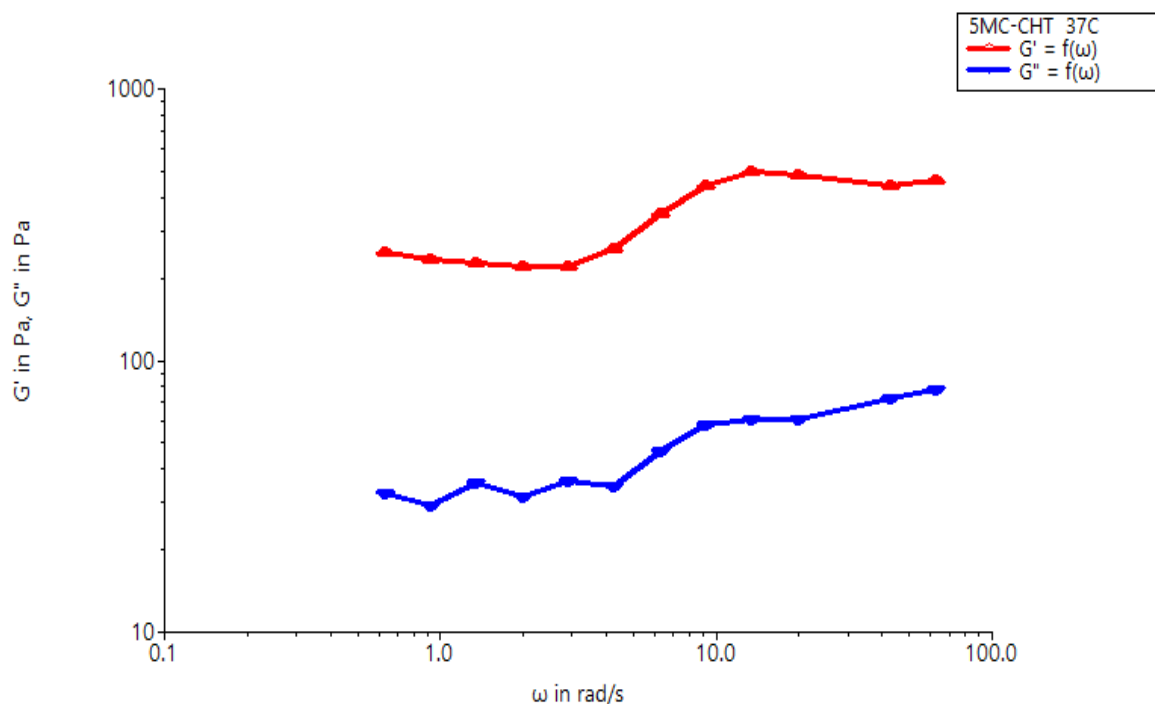


Figure 3.10: Graph depicting the change in the elastic and loss moduli of the various chitosan- β -glycerophosphate-cellulose formulations with frequency at 37°C. (a) Formulation 3MC-CHT; (b) Formulation 5MC-CHT; (c) Formulation 7MC-CHT; (d) Formulation 3MC-2CHT; (e) Formulation 5MC-2CHT; (f) Formulation 7MC-2CHT.

(b)



(c)

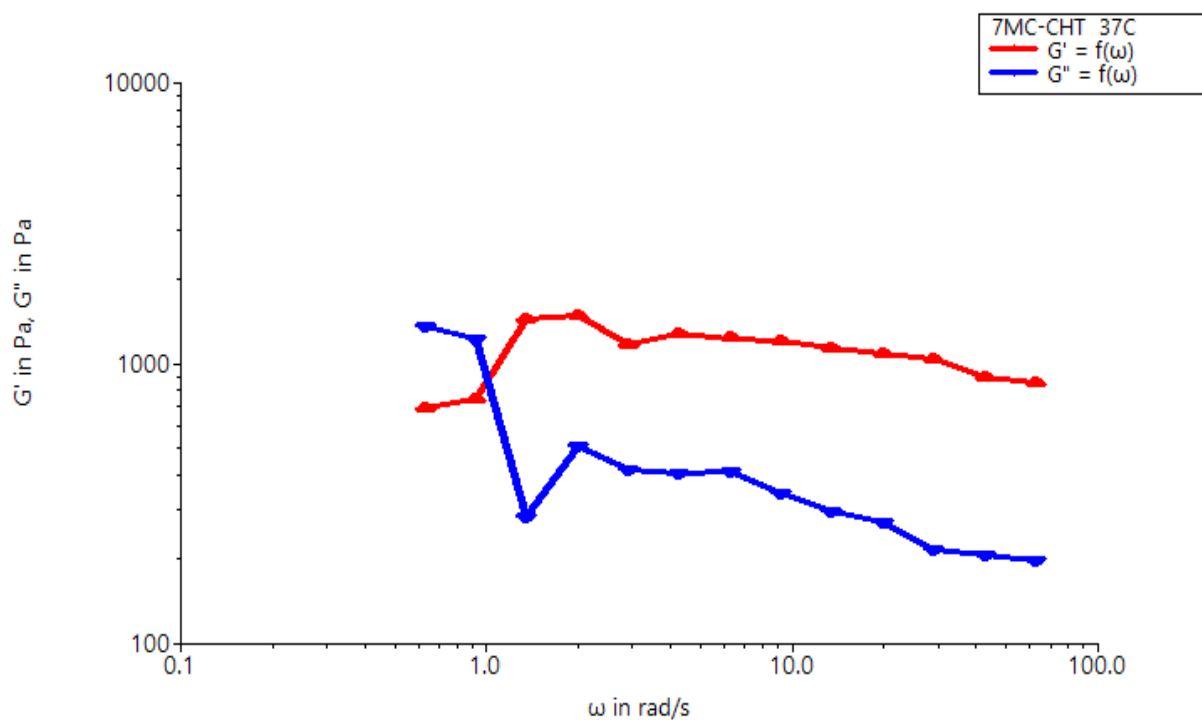
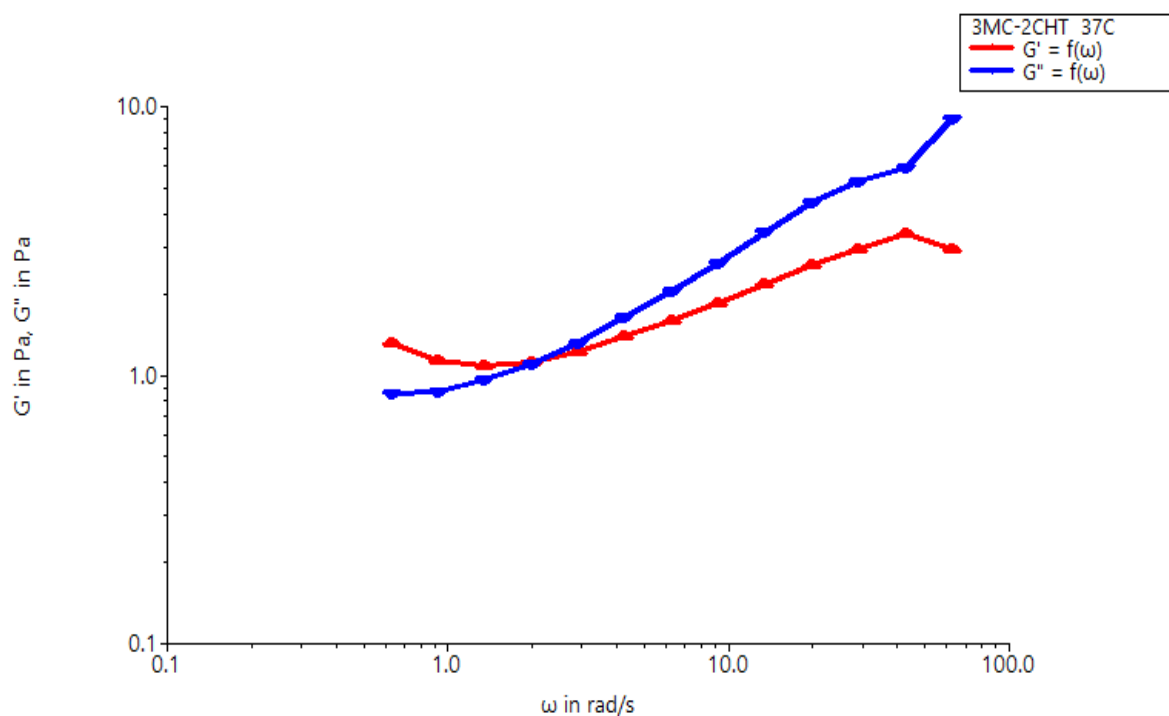


Figure 3.10: Graph depicting the change in the elastic and loss moduli of the various chitosan- β -glycerophosphate-cellulose formulations with frequency at 37°C. (a) Formulation 3MC-CHT; (b) Formulation 5MC-CHT; (c) Formulation 7MC-CHT; (d) Formulation 3MC-2CHT; (e) Formulation 5MC-2CHT; (f) Formulation 7MC-2CHT.

(d)



(e)

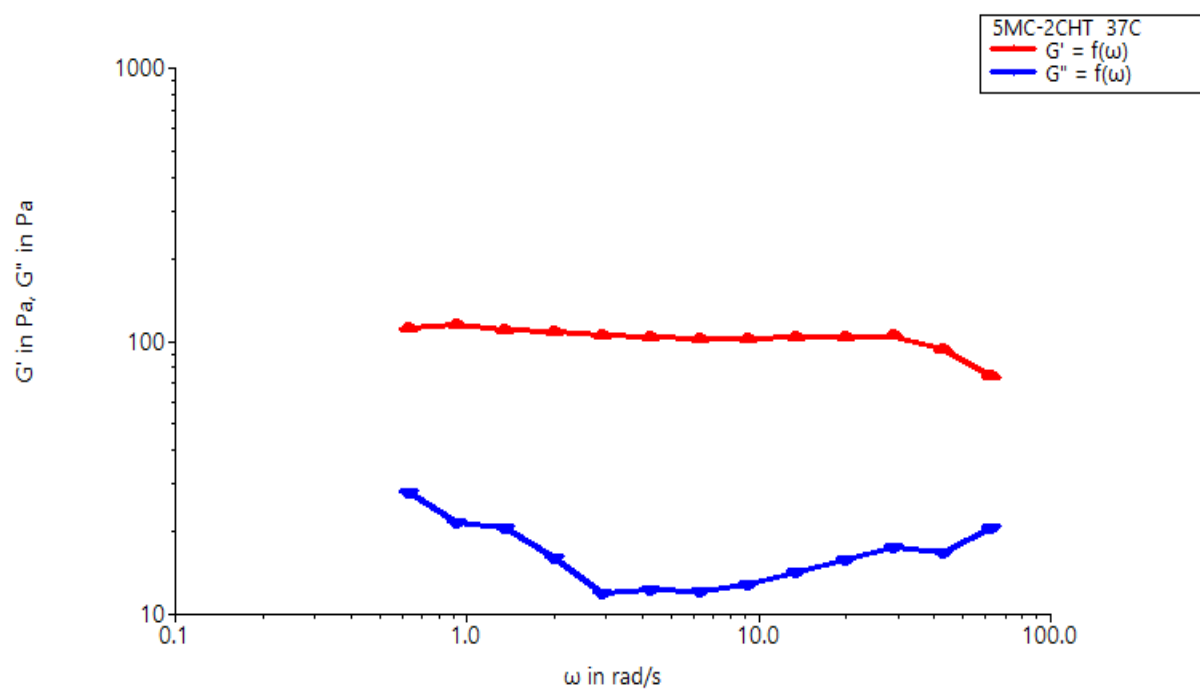


Figure 3.10: Graph depicting the change in the elastic and loss moduli of the various chitosan- β -glycerophosphate-cellulose formulations with frequency at 37°C. (a) Formulation 3MC-CHT; (b) Formulation 5MC-CHT; (c) Formulation 7MC-CHT; (d) Formulation 3MC-2CHT; (e) Formulation 5MC-2CHT; (f) Formulation 7MC-2CHT.

(f)

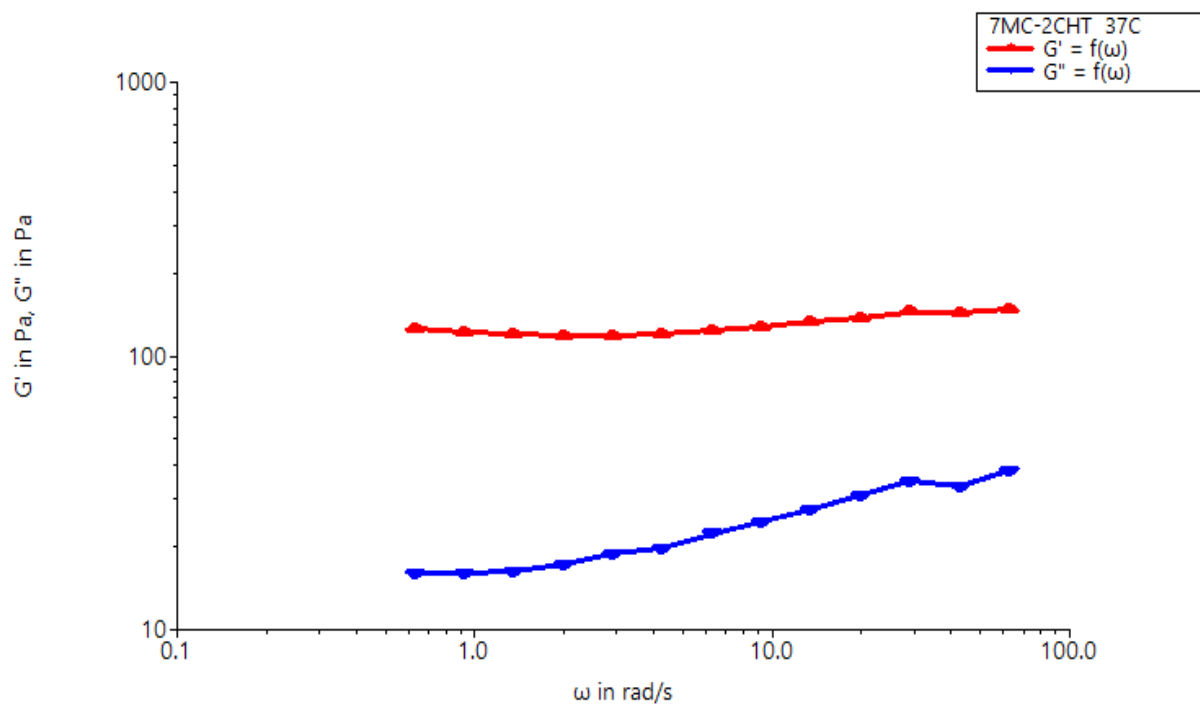
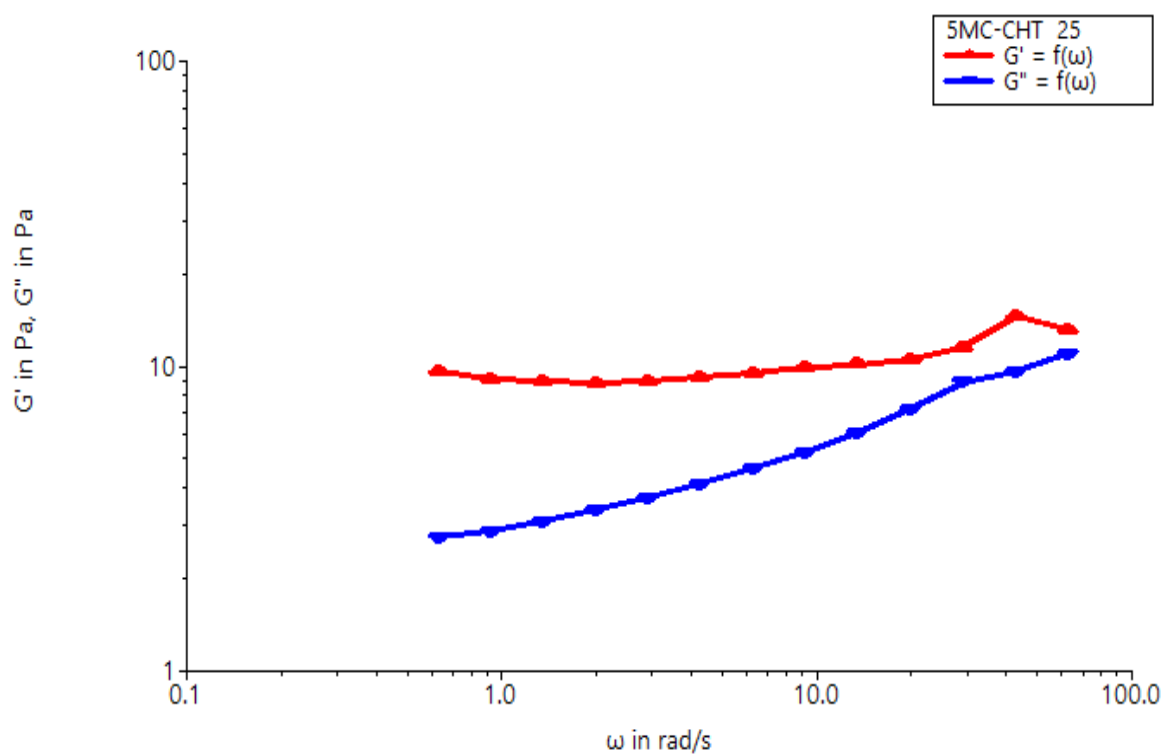
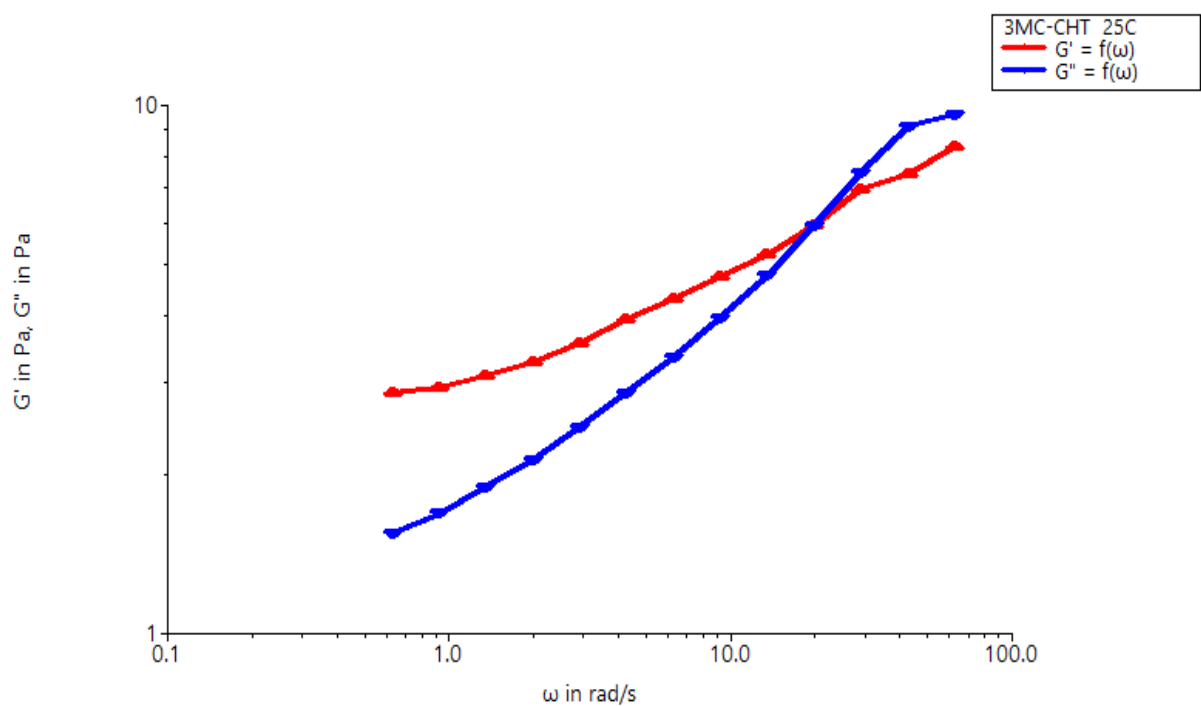


Figure 3.10: Graph depicting the change in the elastic and loss moduli of the various chitosan- β -glycerophosphate-cellulose formulations with frequency at 37°C. (a) Formulation 3MC-CHT; (b) Formulation 5MC-CHT; (c) Formulation 7MC-CHT; (d) Formulation 3MC-2CHT; (e) Formulation 5MC-2CHT; (f) Formulation 7MC-2CHT.

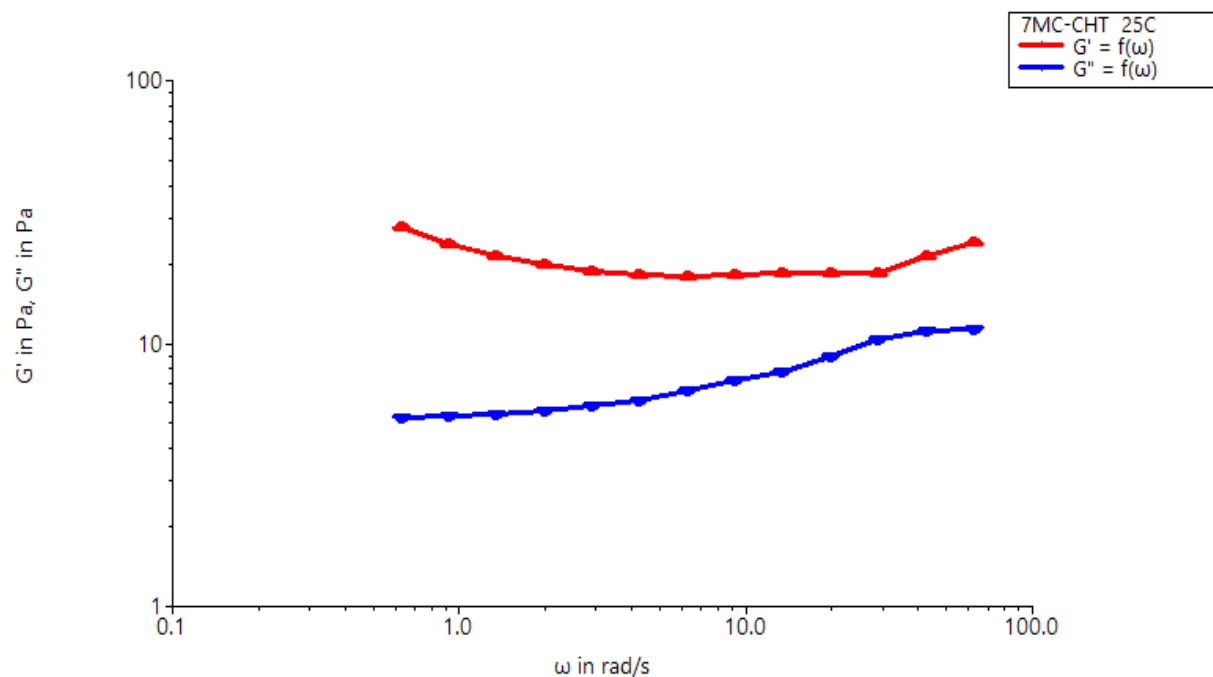
(a)



(b)

Figure 3.11: Graph depicting the change in the elastic and loss moduli of the various chitosan- β -glycerophosphate-cellulose formulations with frequency at 25°C. (a) Formulation 3MC-CHT; (b) Formulation 5MC-CHT; (c) Formulation 7MC-CHT; (d) Formulation 5MC-2CHT.

(c)



(d)

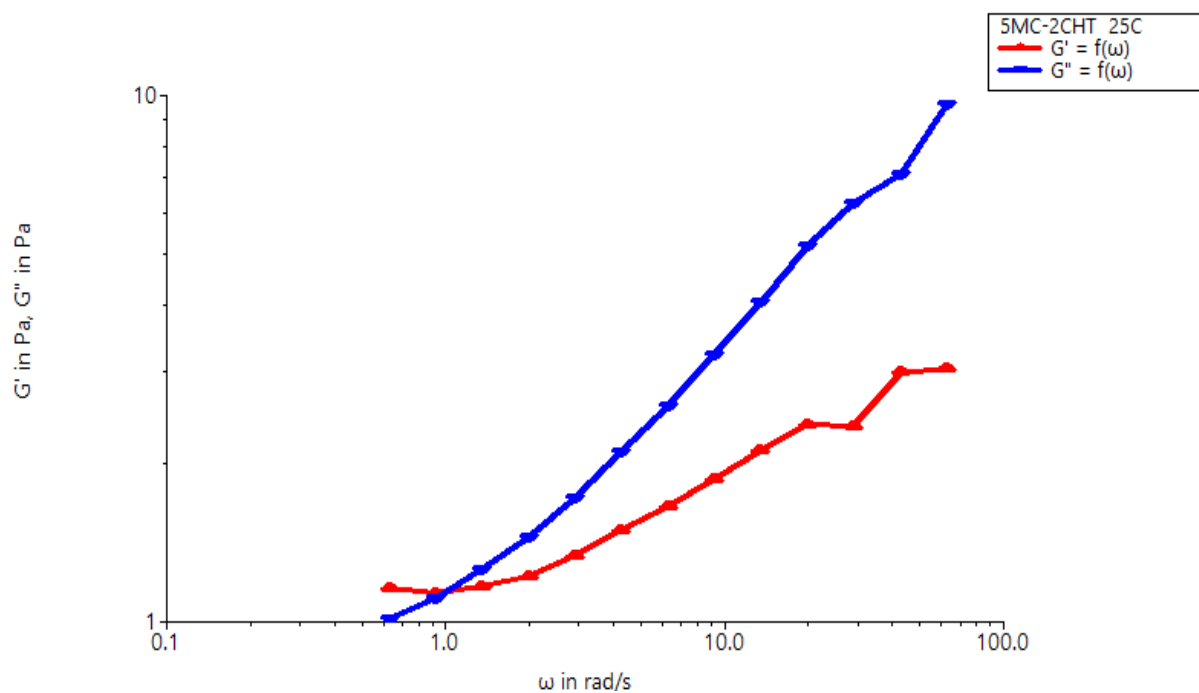


Figure 3.11: Graph depicting the change in the elastic and loss moduli of the various chitosan- β -glycerophosphate-cellulose formulations with frequency at 25°C. (a) Formulation 3MC-CHT; (b) Formulation 5MC-CHT; (c) Formulation 7MC-CHT; (d) Formulation 5MC-2CHT.

3.3.2.2 Pluronic-cellulose based formulations

3.3.2.2.1 Viscosity and temperature ramp

The hybridization of cellulose with pluronic resulted in a decrease in the gelation temperature, as indicated by the decrease in the crossover temperature, as well as the temperature at which the elastic modulus began to increase (Table 3.43.2). This is in keeping with the fact that pluronic f127 gels at much lower temperatures than cellulose, thus increasing the concentration of pluronic within the formulation would decrease the temperature at which the formulation starts to form a gel. The inverse relation between pluronic concentrations and sol-gel temperature is consistent with other studies (Sharma and Bhatia, 2004; Rangabhatla et al., 2016). The strength of the formulations, however, can be attributed to the cellulose component of the formulation, with higher elastic moduli and viscosities seen at 25°C in those formulations with a greater content of cellulose (Figure 3.12; Figure 3.13). While the viscosity and elastic modulus began to increase around 35°C in P10NC, there was no crossover between the elastic and loss moduli, and it did not form a stiff gel within the temperature range studied. P40NC on the other hand formed a gel below 25°C, and therefore depicted a relatively high viscosity and elastic modulus at room temperature.

Table 3.4.2 Rheological assessment of the pluronic-cellulose hybrid formulations

Formulation	Crossover Temperature (°C)	Temperature at which G' starts to increase (°C)	G' at 25°C (Pa)	G' at 37°C (Pa)	Viscosity at 25°C (mPas)	Viscosity at 37°C (mPas)
P10NC	No crossover	35	51.80	2 386	10 030	387 000
P20NC	33.86	31	3.407	260.3	1 572	43 590
P30NC	31.24	29	1.197	1 026	975.6	174 800
P40NC	23.60	22	204.4	5 899	35 230	962 200

~~The hybridization of cellulose with pluronic resulted in a decrease in the gelation temperature, as indicated by the decrease in the crossover temperature, as well as the temperature at which the elastic modulus began to increase (table 3.3.2). This is in keeping with the fact that pluronic f127 gels at much lower temperatures than cellulose, thus increasing the concentration of pluronic within the formulation would decrease the temperature at which the formulation starts to form a gel. The inverse relation between pluronic concentrations and sol-gel temperature is consistent with other studies (Sharma and Bhatia, 2004,; Rangabhatla et al., 2016). The strength of the formulations, however, can be attributed to the cellulose component of the formulation, with higher elastic moduli and viscosities seen at 25°C in those formulations with a greater content of cellulose (figure 3.12; figure 3.13). While the viscosity and elastic modulus began to increase around 35°C in P10NC, there was no crossover between the elastic and loss moduli, and it did not form a stiff gel within the temperature range studied. P40NC on the other hand formed a gel below 25°C, and therefore depicted a relatively high viscosity and elastic modulus at room temperature.~~

In a previous study by Rangabhatla et al. it was found that when pluronic f127 was hybridized with methylcellulose, the lowest concentration of pluronic at which a gel was formed was 12% (Rangabhatla et al., 2016). In a more recent study, the incorporation of cellulose nanocrystals into pluronic reduced the gelation concentration of pluronic to 16.6% (Orasugh et al., 2019). The decrease in the gelation temperature with the incorporation of nanocellulose is a result of the intermolecular hydrogen bonds between the pluronic molecules and the hydroxyl groups in nanocellulose. The interaction between pluronic molecules and methylcellulose molecules also results in a decrease in the intermolecular hydrogen bonding of these molecules with water, thereby reducing the water activity of the system and resulting in a decrease in the gelation temperature of the formulations (Pandit and Kisaka, 1996; Rangabhatla et al., 2016). Upon the incorporation of both methocel and nanocellulose into the pluronic-cellulose formulations, the gelation concentration of pluronic was reduced to 7.2%, almost half of what was previously reported. This is depicted by the formulation labelled P20NC, which formed a gel at 33.86°C. Therefore, P20NC was chosen as the formulation of interest to be studied further from the pluronic-cellulose based formulations.

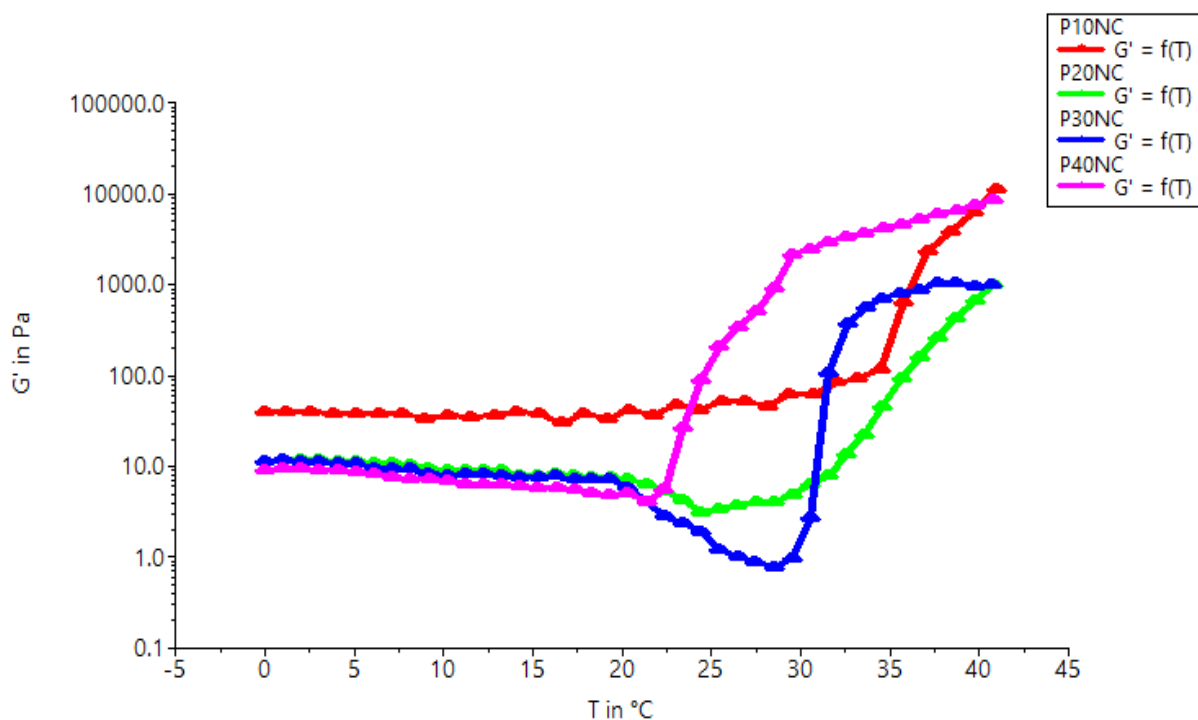


Figure 3.12: Temperature ramp of the various pluronic-cellulose formulations showing the change in their elastic moduli with temperature.

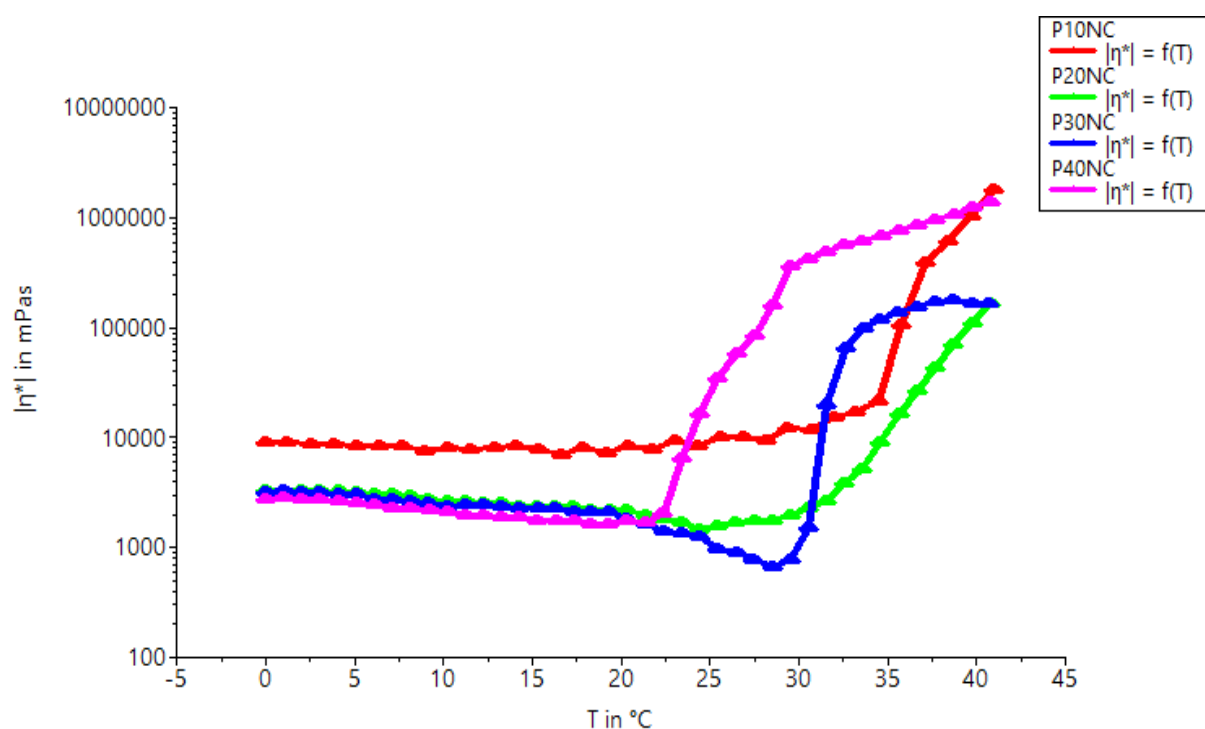


Figure 3.13: Viscosity of the various pluronic-cellulose formulations with temperature.

3.3.2.2.2 Oscillation stress sweep

At 37°C, pluronic f127 had a proportionate effect on the linear viscoelastic region of the formulations, as well as the G' value at which these viscoelastic regions occurred (Figure 3.14). This is due to the fact that pluronic f127 facilitated the gelation of the formulations below 37°C by lowering the gelation temperatures, resulting in a greater degree of physical crosslinking with increasing pluronic f127 concentrations. In contrast, at 25°C (in the absence of physical crosslinking), the strength that was lent to the formulations by their cellulose components resulted in slightly extended viscoelastic regions and higher G' values in those formulations with a greater cellulose content (Figure 3.15). At 25°C, there is no physical crosslinking present due to the formulations being in their sol state, therefore a decline in the elastic moduli of the formulations is seen as stress is applied, and the viscoelastic regions are not as clearly defined as they are at 37°C.

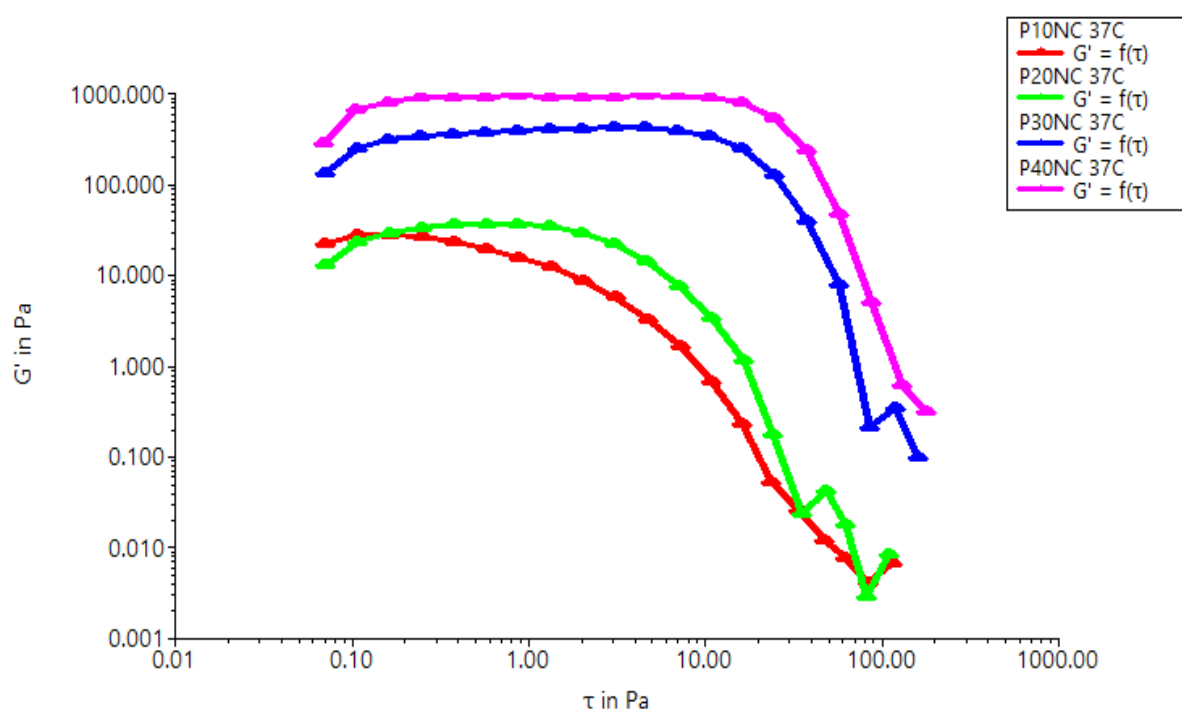


Figure 3.14: Graph depicting the change in the elastic moduli of the various pluronic-cellulose formulations with stress applied at 37°C.

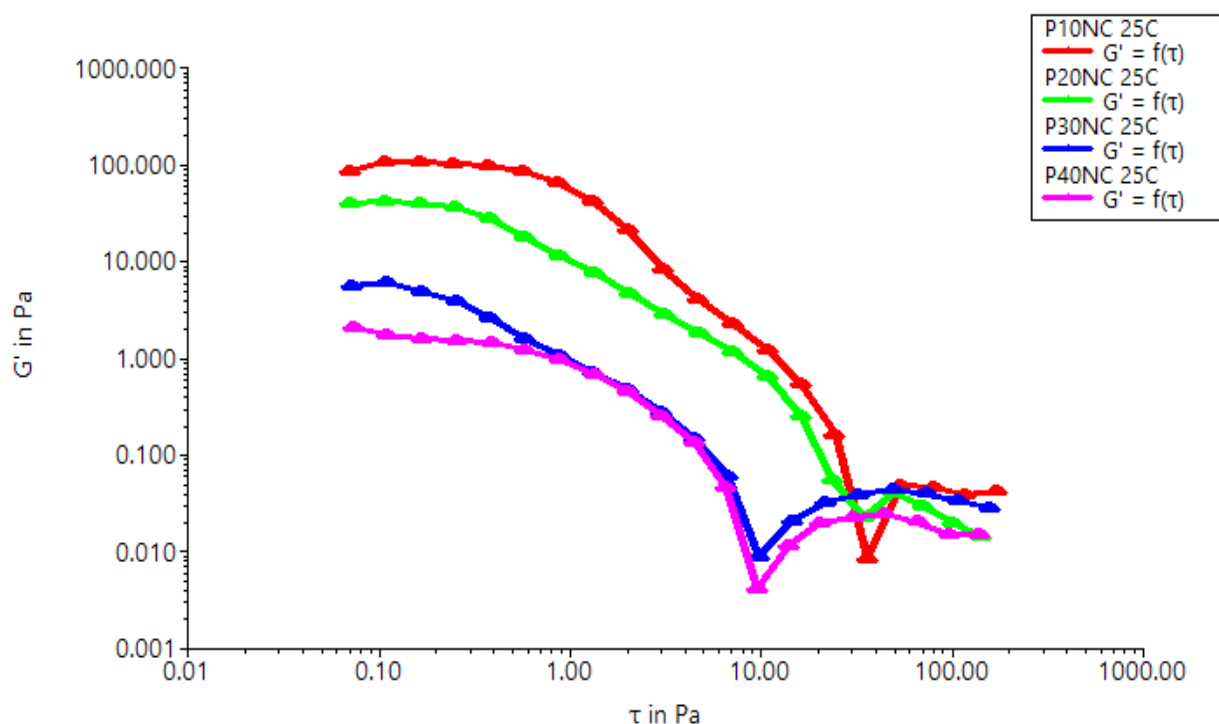


Figure 3.15: Graph depicting the change in the elastic moduli of the various pluronic-cellulose formulations with stress applied at 25°C.

3.3.2.2.3 Oscillation frequency sweep

The effect of increasing concentrations of pluronic on the structure of the formulations can be seen in the rheograms in figure 3.16, with higher elastic moduli values as the concentration of pluronic increases. All pluronic-cellulose based formulations depicted greater elastic moduli than loss moduli at all frequencies at 37°C, with the elastic moduli parallel to the loss moduli. This indicates that all formulations behaved as entangled networks at 37°C. P10NC was the only formulation where the elastic modulus was less than 100Pa at all frequencies, indicating that even though it behaved as an entangled network at 37°C, this was very weak.

Since all pluronic-cellulose formulations displayed liquid like behaviour at 25°C with the loss modulus greater than and parallel to the elastic modulus, only the rheogram for P20NC (the pluronic-cellulose formulation of interest) was shown in figure 3.17.

(a)

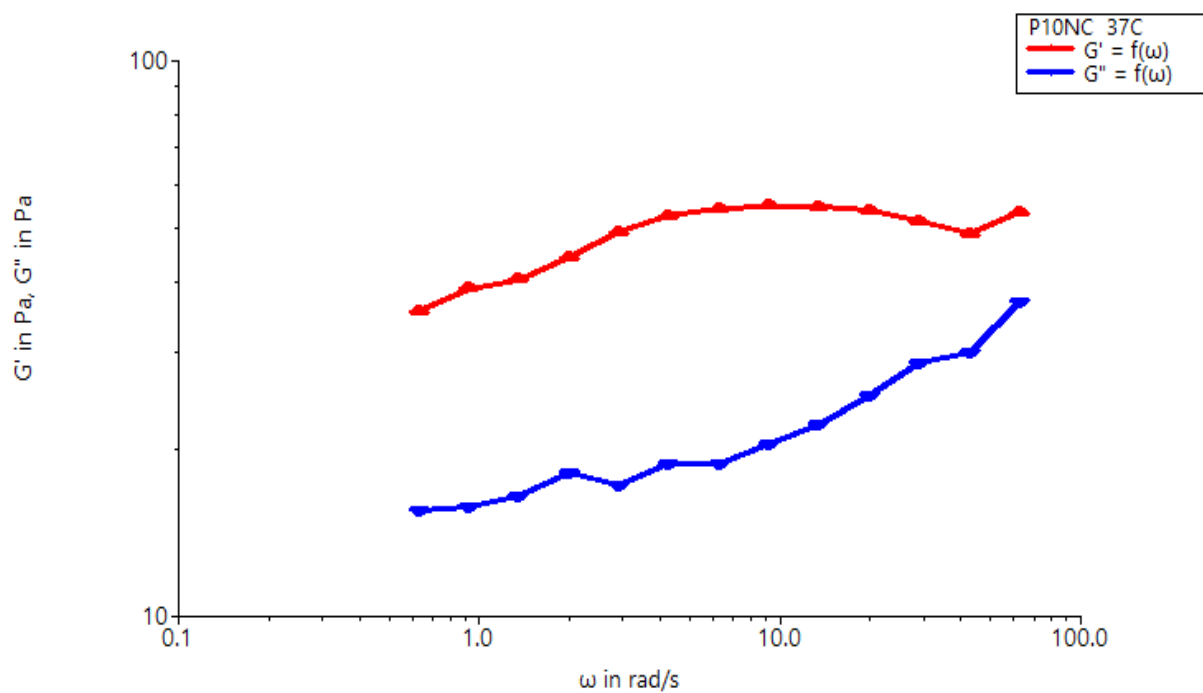
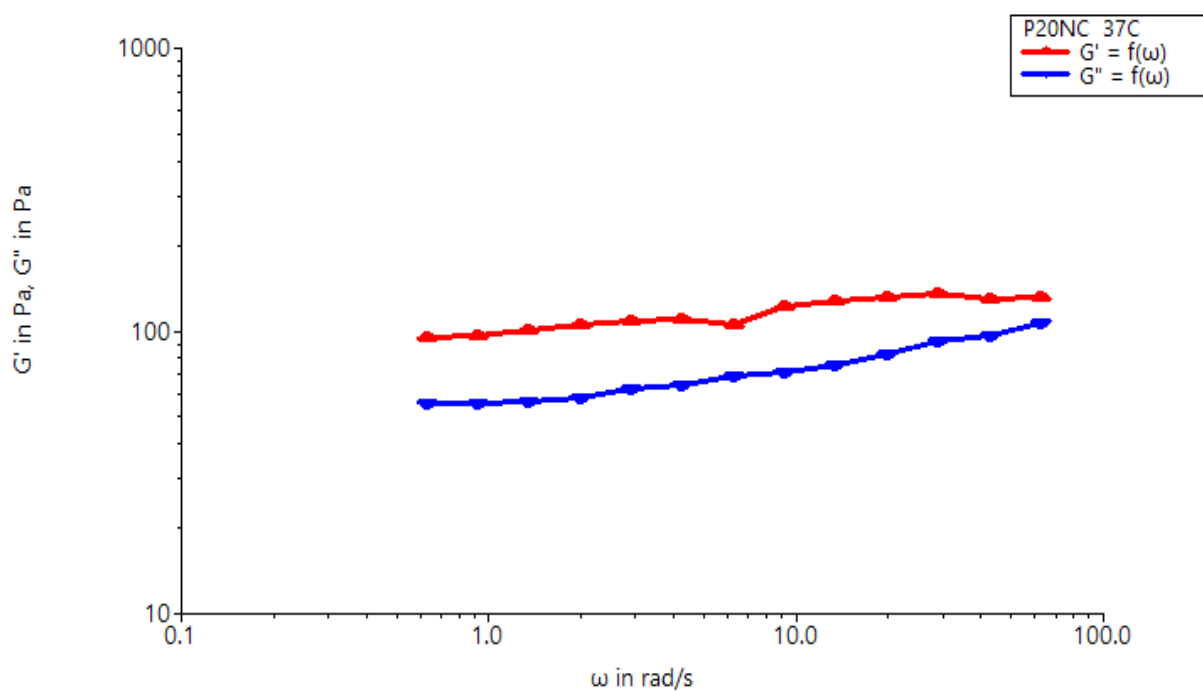


Figure 3.16: Graph depicting the change in the elastic and loss moduli of the various pluronic-cellulose formulations with frequency, at 37°C. (a) P10NC; (b) P20NC; (c) P30NC; (d) P40NC.

(b)



(c)

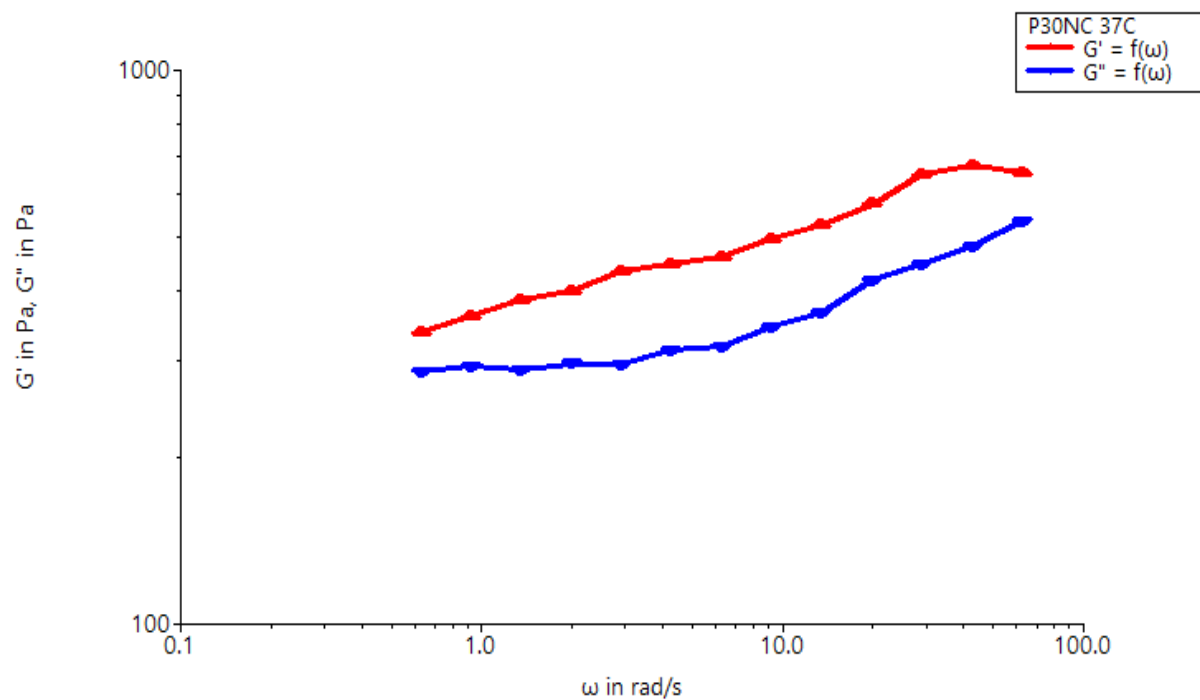


Figure 3.16: Graph depicting the change in the elastic and loss moduli of the various pluronic-cellulose formulations with frequency, at 37°C. (a) P10NC; (b) P20NC; (c) P30NC; (d) P40NC.

(d)

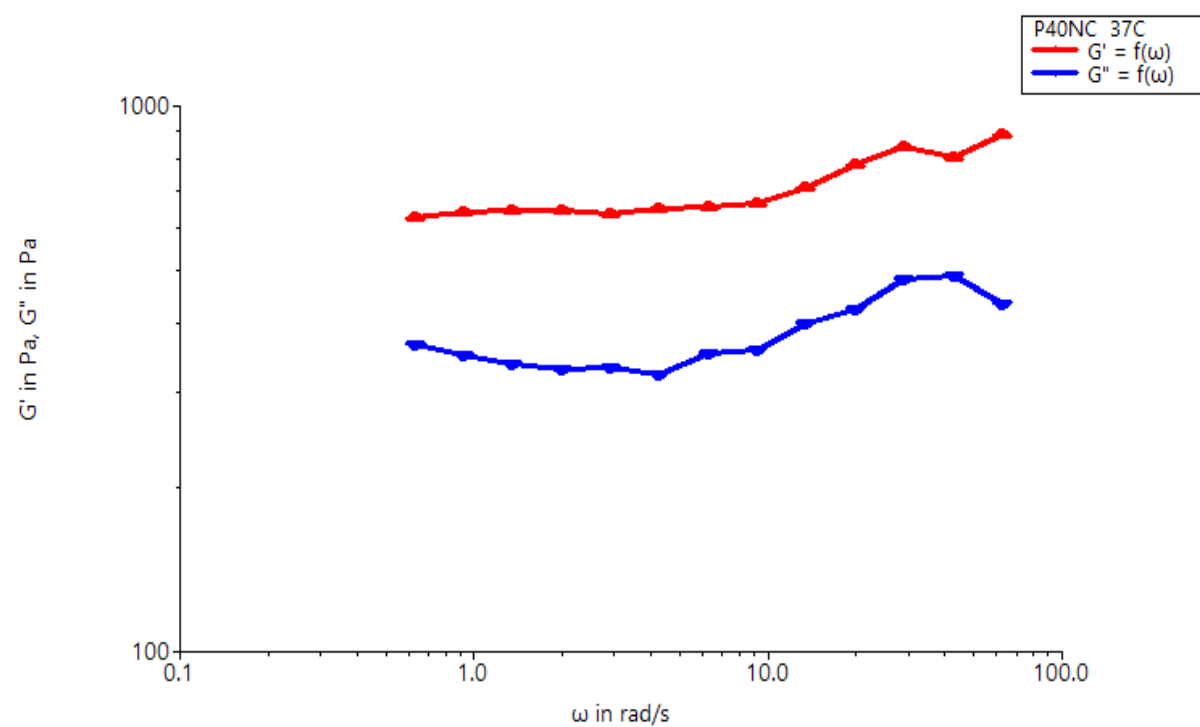


Figure 3.16: Graph depicting the change in the elastic and loss moduli of the various pluronic-cellulose formulations with frequency, at 37°C. (a) P10NC; (b) P20NC; (c) P30NC; (d) P40NC.

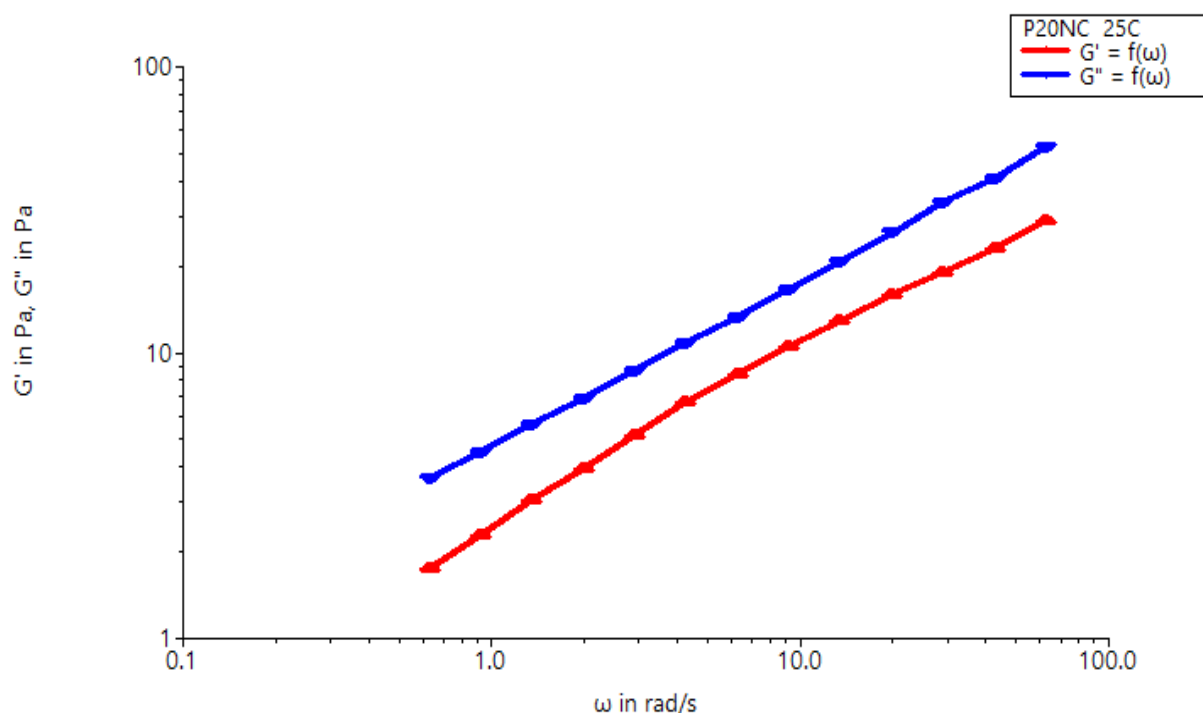


Figure 3.17: Graph depicting the change in the elastic and loss moduli of P20NC with frequency, at 25°C.

3.4 Concluding remarks

In conclusion, the effect of polymer concentration on the rheological properties of thermo-responsive polymer systems is significant and guides their behaviour. Hybridizing polymers with different gelation temperatures and mechanical strengths enables us to manipulate these features, and in turn the rheological properties of the system, to create a system that exhibits these characteristics in a manner that is suited to our needs. The lower the gelation temperature of a formulation, the quicker the formation of gels at physiological temperature, and the greater the mechanical strength of the formulation at 37°C, due to a greater degree of physical crosslinking.

While both the chitosan-cellulose based formulation (formulation 5MC-2CHT) and the pluronic-cellulose based formulation (P20NC) are capable of forming a gel at physiological temperature while remaining in sol state at ambient temperature, formulation 5MC-2CHT showed a greater mechanical strength at 37°C and an

extended viscoelastic region, compared to formulation P20NC, in its gelled state. The chitosan-based formulation (5MC-2CHT) also displayed a lower viscosity at 25°C than the pluronic-based formulation (P20NC), making it more suitable for use in the proposed system. While the pluronic-based formulation (P20NC) had a slightly faster gelation time (10 min) than the chitosan-based formulation (12 min), the rheological properties in terms of strength and viscosity still rendered the chitosan-based formulation (5MC-2CHT) superior in the current application. The low elastic modulus and viscosity of formulation 5MC-2CHT at ambient temperature enables easy application of the formulation to the wound site via syringe.

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CHAPTER FOUR

PHYSICO-CHEMICAL CHARACTERIZATION OF HYBRID CELLULOSE BASED THERMORESPONSIVE IN SITU GELLING SYSTEMS

4.1 Introduction

Rheological properties of thermogels can often further be explained by physicochemical characterization of the formulations and by assessing the molecular, thermal and phase transitions of the system. Analytical methods such as Fourier Transform infrared Spectroscopy allows researchers to detect changes within the chemical makeup of formulations, both in their sol and gel state, and helps us further understand their mechanisms of crosslinking and gelation. Thermal stability of formulations can be assessed by methods such as differential scanning calorimetry and thermogravimetric analysis (Zohuriaan and Shokrolahi, 2004). Rheological properties of gels have also been known to elicit an effect on the swelling, erosion, and release kinetics of a formulation (Andrews et al., 2009). This in turn can be supported by determining the morphology of the system, which provides researchers with insight regarding the porosity of a formulation, as well as the effects of hybridization and manipulation of polymers on the degree of crosslinking, organization, and structural behaviour of the formulation.

The incorporation of a drug or bioactive molecule into a formulation is by virtue of the properties it possesses in promoting and enhancing the achievement of biomedical outcomes. Trans ferulic acid (TFA) was incorporated as a bioactive in this system due to its proposed antibacterial, antioxidant and anti-inflammatory properties (Ou and Kwok, 2004; Bairagi et al., 2018). These properties are particularly useful during the initial phases of wound healing. Therefore, it is imperative to determine the release kinetics of the formulation to ascertain whether an adequate amount of drug is released within an appropriate timeframe for maximal effect to be had within the initial stages of wound healing.

This chapter details the physiochemical analysis of the formulations to determine the changes in their chemical make-up, thermal stability, swelling, erosion and degradation behaviour, as well as the release kinetics of the formulation.

4.2 Materials and methods

4.2.1 Materials

Trans ferulic acid 99% was purchased from Sigma Aldrich (St. Louis, Missouri, USA). Low molecular weight chitosan, β -glycerophosphate disodium salt pentahydrate, glacial acetic acid, Pluronic F127 and Methocel LV A15 were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). Fibrous nanocellulose was purchased from DLD Scientific (Durban North, SA).

4.2.2 Methods

4.2.2.1 Preparation of lyophilized samples for analysis

The formulations were prepared as discussed in chapter three, section 3.2.2. of this dissertation. Thereafter, 2 mL of each formulation was placed in a vial and in an incubator at 37°C and allowed to form a gel, while another vial containing the formulation was kept in an ice bath. The samples were snap-frozen using liquid nitrogen so that they retained their sol or gel structures at 4°C and 37°C respectively. The samples were then lyophilized for approximately 18 hours until dry, before being analysed as detailed below.

4.2.2.2 Assessment of molecular, thermal and phase transitions by physiochemical analysis of the biodegradable thermo-responsive hybrid system

4.2.2.2.1 Fourier Transform Infrared Spectroscopy (FTIR)

Fourier Transform Infrared Spectroscopy (FTIR) analysis produces a “fingerprint” of a sample in terms of the chemical bonds within the sample. These studies will be carried out on the final product, as well as on each of the individual components making up the final product, and changes in the chemical structure will be analysed. This will be

done to assess the compatibility of the polymers with each other and determine the structural arrangement of the hydrogel at 4°C and at 37°C. Wavenumbers between 650 – 4000cm⁻¹ will be used when running the samples, with a 10cm⁻¹ resolution. A Perkin Elmer Spectrum 2000 FTIR spectrometer with a MIRTGS detector will be used (PerkinElmer Spectrum 100, Llantrisant, Wales, UK).

4.2.2.2.2 Differential Scanning Calorimetry (DSC)

Differential Scanning Calorimetry evaluates the heat capacity (Cp) of a material in response to temperature. Melting points and temperatures at which the system exhibits a phase change will be determined via this analysis. A known mass of the final product will be used, and the heating rate will be set at 10°C per minute, with a temperature range of 10 - 300°C, in the presence of Nitrogen (N₂) gas. A METTLER Toledo DSC1 STARe system (Switzerland) will be used to assess the abovementioned thermodynamic properties of the gel system.

4.2.2.2.3 Thermogravimetric Analysis (TGA)

Thermogravimetric analysis will be used to determine the degradation and ionic interactions within the polymer system. The physical and chemical changes within the system will be assessed by measuring the change in mass with time, as the temperature increases. The temperature range will be set at 30 - 900°C, and the analysis will be conducted using a PekinElmer Top Loading TGA 4000 (PerkinElmer, Beaconsfield, UK).

4.2.2.3 Morphological analysis of the hydrogel

To determine the microscopic structure and morphology of the hydrogel, a Nova NanoSEM 650 will be used. Prior to analysis, the sample will be heated to 37°C and thereafter undergo snap-freezing using liquid Nitrogen. The samples will then be lyophilized, and sputter coated with carbon and a palladium/gold film. The morphology of the gel will be determined from the images generated from the scanning electron microscope (SEM).

4.2.2.4 In vitro swelling and erosion of gels

The analysis of in vitro swelling and erosion of the MC/PF/NC and MC/CHT/BGP gels was carried out by the gravimetric analysis method as reported by Li and co-workers (Li et al., 2014). The initial weight of a 5 mL polytop vial was recorded (W_v). One mL of the respective polymer solutions was then allowed to form a gel at 37°C and the polytop vial containing the gel was weighed (W_i). Thereafter, 2 mL of phosphate buffered saline was added to each polytop vial and incubated at 37°C 25rpm (LM-530-2, MRC Laboratory Instruments Ltd. Hahistadrut, Holon, Israel). The buffer solutions were removed at regular intervals and the gels were weighed at each interval (W_t). To determine the swelling or erosion of the gel, the percentage weight of the gel remaining at each time point was expressed via the following equation:

$$\frac{W_t - W_v}{W_i - W_v} \times 100$$

Equation 4.1

4.2.2.5 Incorporation of bio-active trans-ferulic acid

The pluronic-cellulose (P20NC) and chitosan-cellulose (5MC-2CHT) formulations were prepared as discussed in chapter three of this thesis, section 3.2.2, with the addition of trans ferulic acid to the cellulose components of each formulation during preparation (before hybridizing with the pluronic or chitosan components respectively), at a final concentration of 10 µg/mL. Trans ferulic acid displays poor solubility in water at high concentrations, however, due to the low concentration used in this study, solubilisation was not an issue (National Center for Biotechnology Information. PubChem Compound Summary for CID 445858, Ferulic acid. <https://pubchem.ncbi.nlm.nih.gov/compound/Ferulic-acid>. Accessed Jan. 12, 2022.).

4.2.2.6 In vitro drug release

The release of the bio-active trans-ferulic acid from the MC/PF/NC and MC/CHT/BGP gels was determined using a method previously described by Yang and team (Yang et al., 2009). The concentration of trans-ferulic acid in all samples was 10 µg/mL. Two

millilitres of each solution was placed in a 10mL polytop vial and allowed to form a gel at 37°C. Thereafter, 8 mL of phosphate buffered saline (pH 7.4) was gently added to the surface of each gel and the vials were placed in an orbital shaker at 37°C 25 rpm (LM-530-2, MRC Laboratory Instruments Ltd. Hahistadrut, Holon, Israel). Two mL aliquots were removed and filtered at predetermined timepoints (0.5 h, 1 h, 2 h, 4 h, 6 h, 8 h, 10 h, 12 h, 24 h) and replaced with the equivalent fresh saline. The release profiles were subsequently analysed using a NanoPhotometer NP80 (Implen, CA, USA) at a wavelength of 310 nm. The cumulative percentage release of trans ferulic acid at each timepoint was calculated as follows:

Cumulative Percentage Release=

$$\frac{\text{Total amount of bioactive released per timepoint}}{\text{Total amount of bioactive incorporated into the sample}} \times 100$$

Equation 4.2

4.3 Results and Discussion

4.3.1 Fourier Transform Infrared Spectroscopy (FTIR)

FTIR spectra of pristine chitosan, cellulose and β -glycerophosphate (Figure 4.1) are presented alongside the hybrid formulations (Figure 4.2; Figure 4.3). Pristine chitosan exhibited absorption peaks in the ~ 3200 - 3500cm^{-1} range, indicative of strong hydroxyl bonds (O-H stretching and HO-CH₂OH intra- and inter- molecular hydrogen bonds) and amide bonds, $\sim 2875\text{cm}^{-1}$ (corresponding to the stretching of C-H in CH₂), ~ 1556 - 1645cm^{-1} (corresponding to C-O stretching of the secondary amide), $\sim 1376\text{cm}^{-1}$ (indicative of the bending of C-H in CH₂), $\sim 1317\text{cm}^{-1}$ (the stretching of C-N in the secondary amide), $\sim 1150\text{cm}^{-1}$ (C-O stretching of ring ether), and $\sim 1060\text{cm}^{-1}$ (symmetric C-O stretching of the primary alcohol) (van de Velde and Kiekens, 2004; Skwarczynska et al., 2018).

Pristine cellulose depicted peaks at $\sim 3400\text{cm}^{-1}$ corresponding to O-H stretching, $\sim 2900\text{cm}^{-1}$ indicative of the C-H stretching in CH₂ and CH₃, $\sim 1450\text{cm}^{-1}$ characteristic of C-H bending in CH₂, $\sim 1250\text{cm}^{-1}$ corresponding to C-O stretching of the ether

linkages, and $\sim 1050\text{cm}^{-1}$ characteristic of the C-O symmetric stretching of the primary alcohol (Rangabhatla et al., 2016; Tang et al., 2010).

Characteristic peaks in the spectrum of glycerophosphate appear at $\sim 976\text{cm}^{-1}$ corresponding to the aliphatic P-O-C stretching, $\sim 1050\text{cm}^{-1}$ characteristic of the $-\text{HPO}_4$ group, and $\sim 920\text{cm}^{-1}$ representing the PO_4^{2-} group (Skwarczynska et al., 2018).

In addition, pristine pluronic (Figure 4.4) exhibited peaks at $\sim 2879\text{cm}^{-1}$ corresponding to C-H stretching, a broad peak in the $\sim 1100\text{-}1060\text{cm}^{-1}$ region indicative of C-O stretching vibrations within the molecule as previously described (Su, Wang, and Liu, 2002; Dmitrenko et al., 2019).

In the chitosan-cellulose based formulations, as the concentration of methylcellulose is increased at 37°C , there is an increase in the intensity of the peaks (Figure 4.2), and this intensity decreases when the ratio of chitosan to methylcellulose is doubled (in comparison to formulations with a 1:1 chitosan: cellulose), as expected (Figure 4.3; Appendix E). In formulations 5MC-CHT, 7MC-CHT, 5MC-2CHT and 7MC-2CHT there is a marked increase in the intensity of all peaks at 37°C , compared to the peaks at 4°C , and the spectra are parallel to each other. This indicates that these formulations have formed a gel at 37°C . However, no new peaks are seen, indicating that no additional bonds are formed, and gelation does indeed occur via physical crosslinking in response to an increase in temperature. In formulation 3MC-CHT, there is a crossing over of the spectra at 4°C and 37°C , with the peaks in the $\sim 3000\text{-}3500\text{ cm}^{-1}$ region displaying a sharper intensity at 4°C than at 37°C , indicating that the formulation did not form a gel. In formulation 3MC-2CHT, although the peaks at 37°C are slightly more intense than those at 4°C , the spectra seem to cross-over each other at certain points, as opposed to being entirely parallel to each other, as seen in the formulations with greater cellulose concentrations. This suggests that while there may have been some degree of crosslinking beginning to occur in formulations containing 3% cellulose, these forces were not as strong as those in the formulations containing 5% and 7% cellulose, and a stiff gel was not formed.

In the pluronic-cellulose based formulations, as the concentration of pluronic increased, there is an increase in the intensity of the peaks at $\sim 2879\text{cm}^{-1}$ and in the region of $\sim 1100\text{-}1060\text{cm}^{-1}$ as expected (Appendix F), as these peaks are characteristic of pluronic f127. Similarly, an increase in the intensity of the peaks associated with cellulose is seen as the concentration of cellulose increases within the formulation, such as the peaks at $\sim 3400\text{cm}^{-1}$, $\sim 1450\text{-}1300\text{cm}^{-1}$, and $\sim 1250\text{cm}^{-1}$. Once again, the hybrid pluronic-cellulose formulations did not display the formation of any new bonds, but merely a merging of the spectra of the pristine polymers, indicating that crosslinking was of a physical nature, as opposed to chemical. In formulation P10NC, it is seen that the spectra at 4°C (Figure 4.5) and at 37°C (Figure 4.6) are almost identical and overlap each other perfectly, indicating that this formulation does not form a gel at physiological temperature. In formulation P20NC however, the spectrum at 37°C is entirely parallel to the spectrum at 4°C , with all peaks depicting a greater intensity at 37°C than at 4°C , indicating the occurrence of physical crosslinking and thus gelation when the formulation was heated to 37°C . In formulations P30NC and P40NC, there is an increase in the intensity of the peaks associated with pluronic ($\sim 2880\text{cm}^{-1}$ and $\sim 1100\text{-}1060\text{cm}^{-1}$) at 37°C as compared to at 4°C , however the complete spectra are not entirely parallel to each other and overlap at certain points, with the peaks associated with cellulose ($\sim 3400\text{cm}^{-1}$, 1650cm^{-1} , 1350cm^{-1} , and 1250cm^{-1}) being more intense at 4°C than at 37°C . This is in keeping with the fact that the strength of the formulation is attributed to the cellulose component of the formulation at 4°C when the formulation is in its sol state, and that the addition of increasing concentrations of pluronic is responsible for altering the gelation characteristics of the formulation at 37°C .

The differences in intensity of the peaks can be easily observed in the stacked FTIR spectra, which can be found in appendix E and appendix F of this dissertation.

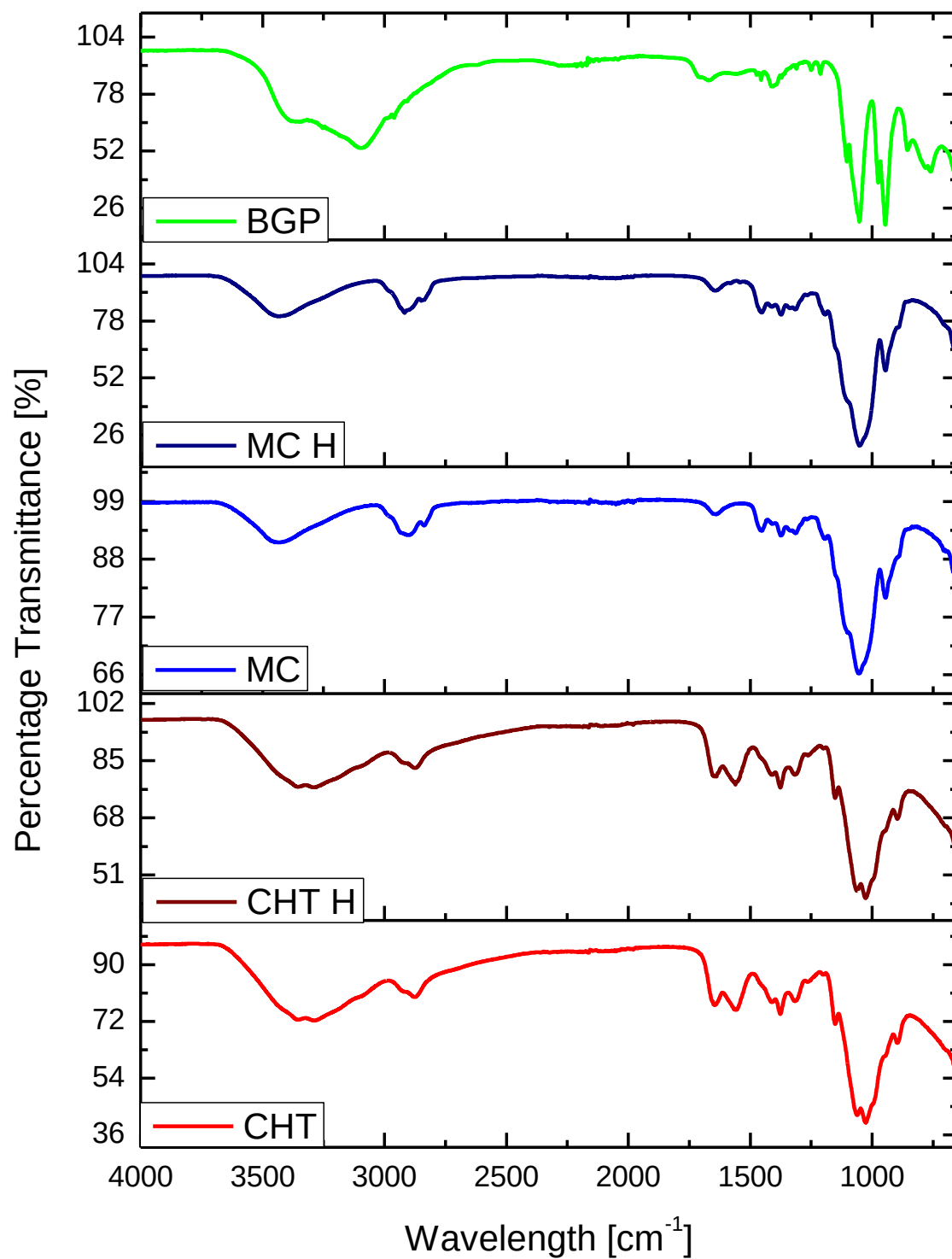


Figure 4.1: Graph depicting changes in the peaks in pristine polymers chitosan, methylcellulose, and β -glycerophosphate-pentahydrate in their cooled (4°C) and heated (37°C) states, using Fourier transform infrared spectroscopy.

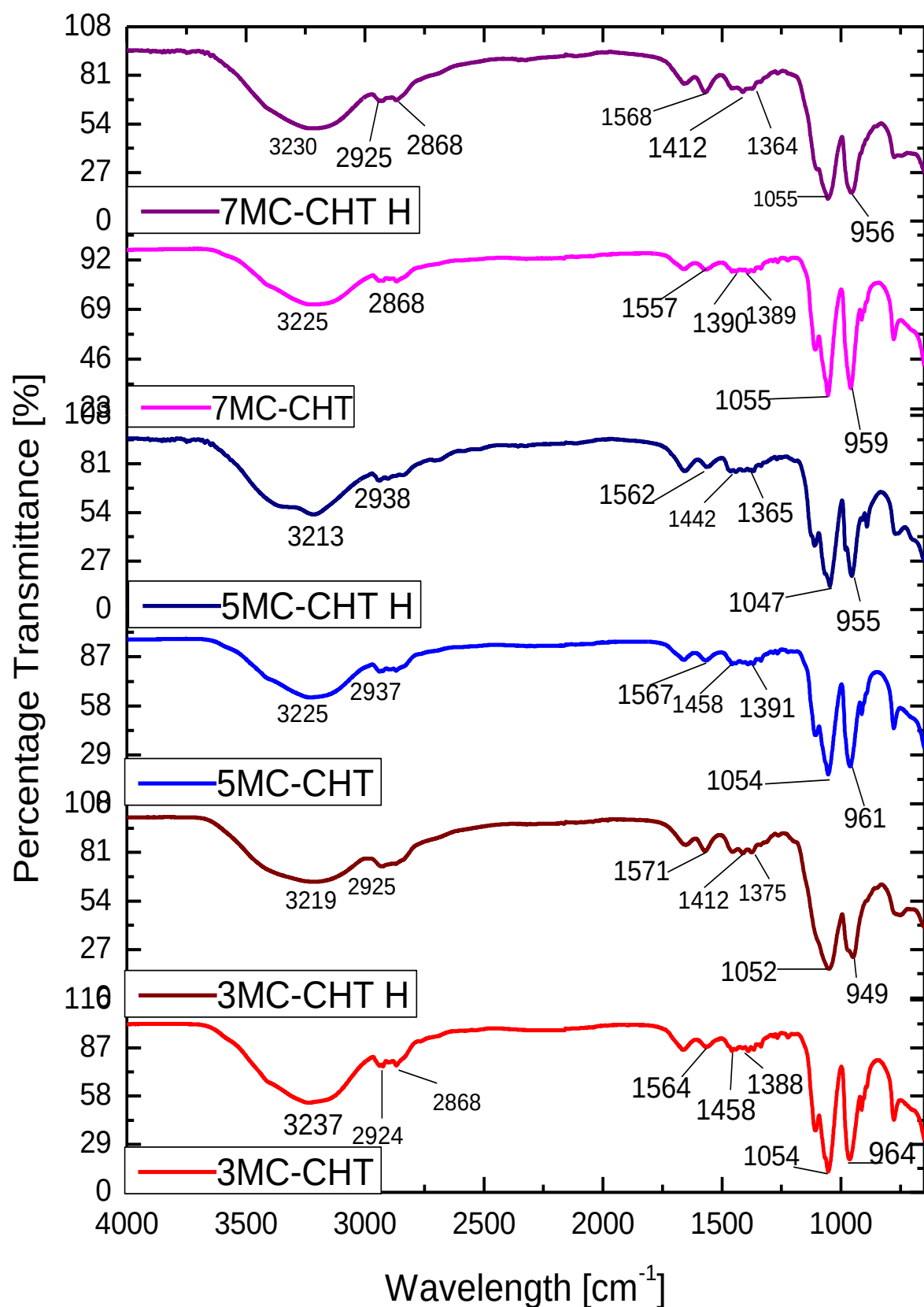


Figure 4.2: Graph depicting changes in the peaks in the hybrid polymer combinations of chitosan, methylcellulose, and β -glycerophosphate-pentahydrate in their cooled (4°C) and heated (37°C) states, using Fourier transform infrared spectroscopy.

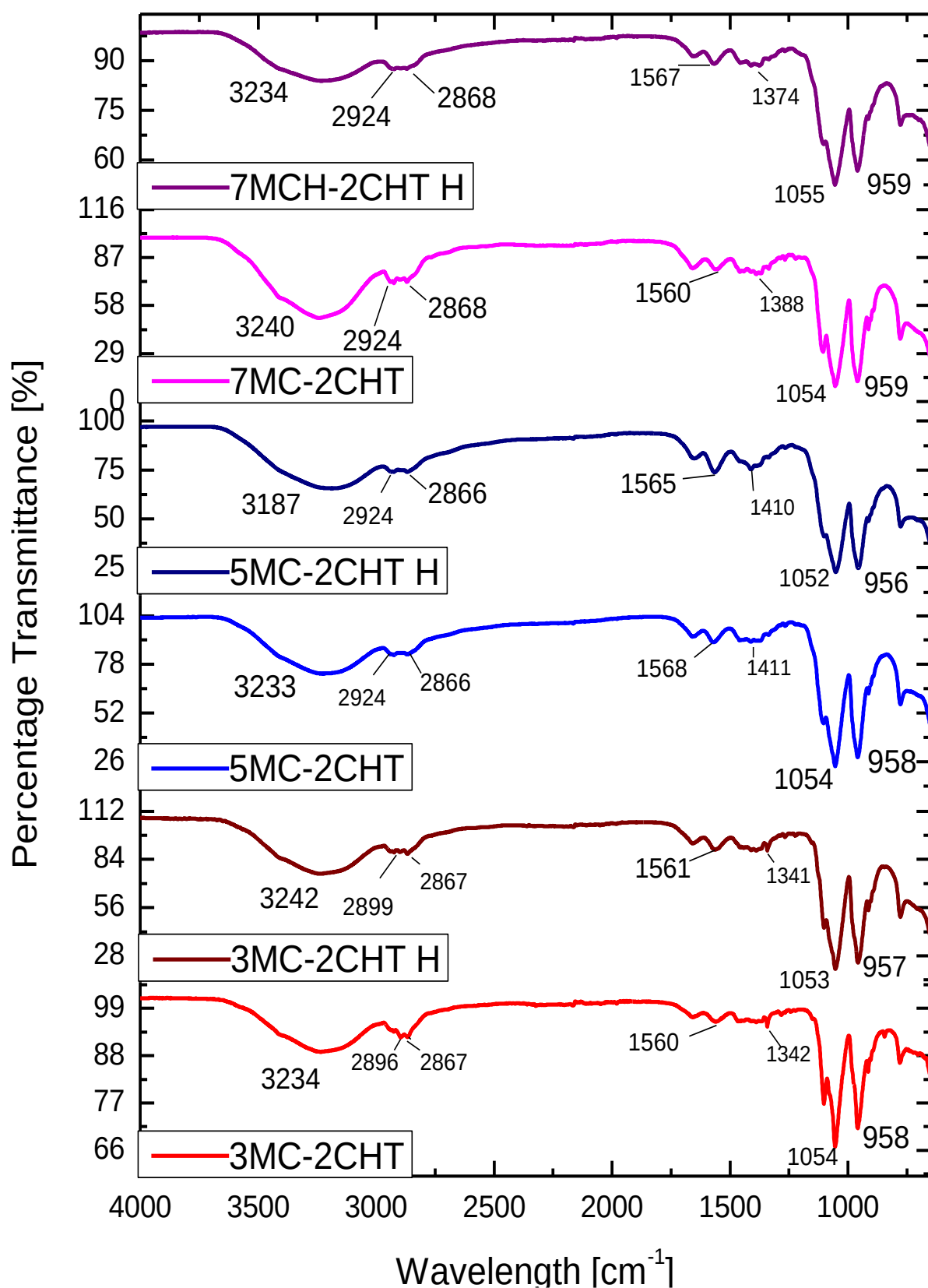


Figure 4.3: Graph depicting changes in the peaks of the hybrid polymer combinations of chitosan, methylcellulose, and β -glycerophosphate-pentahydrate in their cooled (4°C) and heated (37°C) states, using Fourier transform infrared spectroscopy.

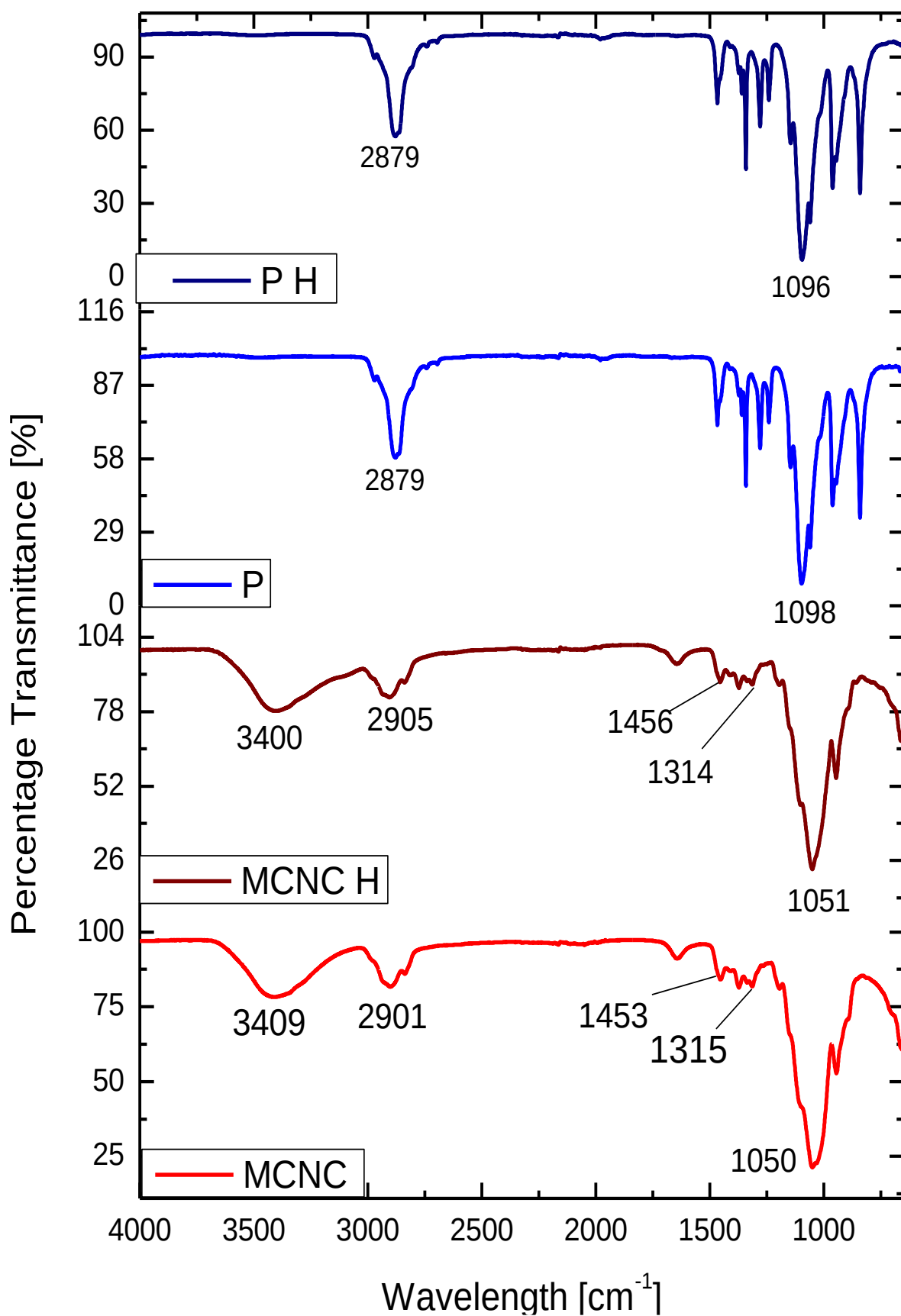


Figure 4.4: Graph depicting changes in the peaks of the pristine polymers of pluronic F127 and the methocel-nanocellulose blend in their cooled (4°C) and heated (37°C) states, using Fourier transform infrared spectroscopy.

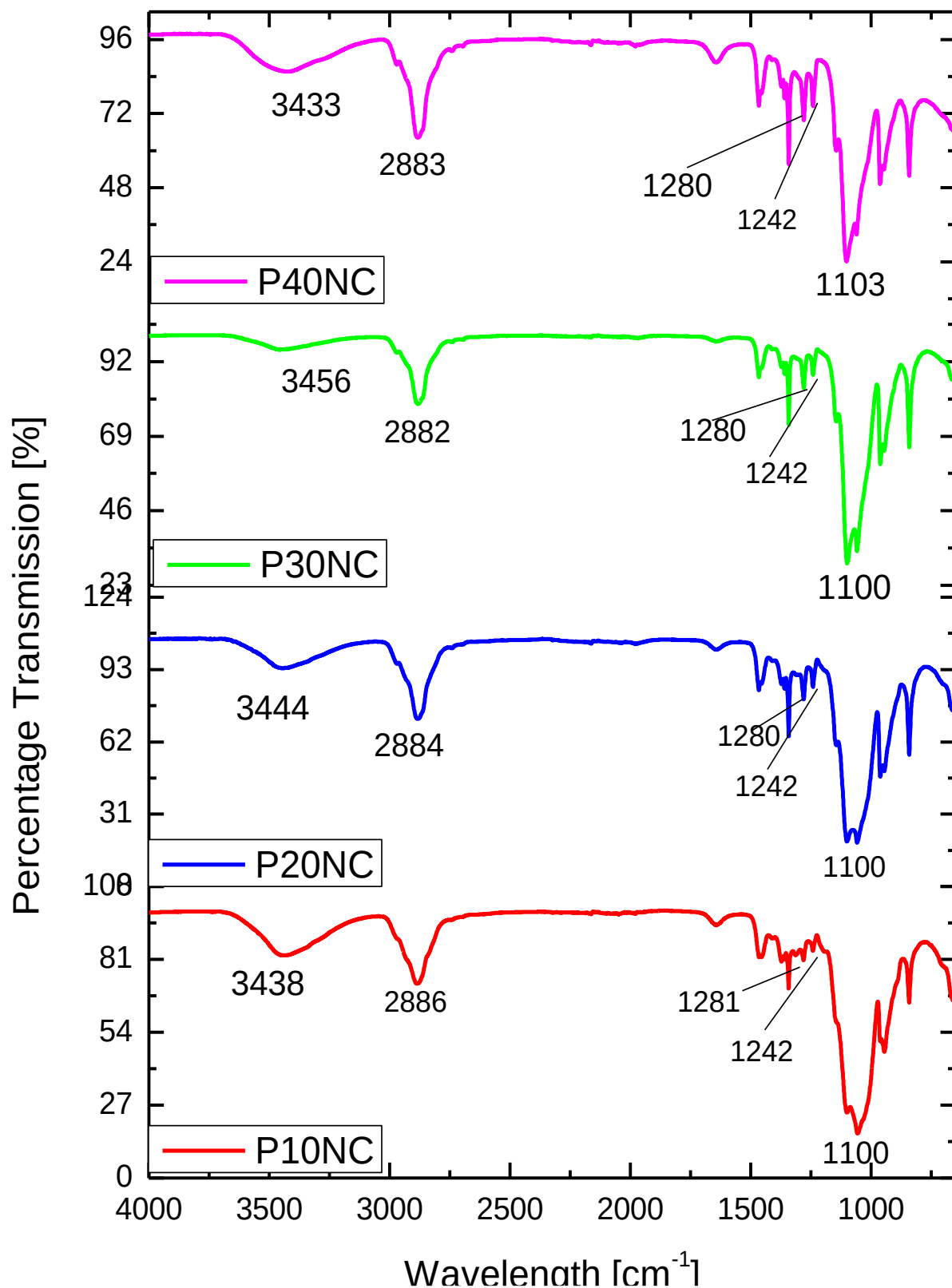


Figure 4.5: Graph depicting changes in the peaks of the hybrid polymer combinations of pluronic F127, methocel and nanocellulose at 25°C using Fourier transform infrared spectroscopy.

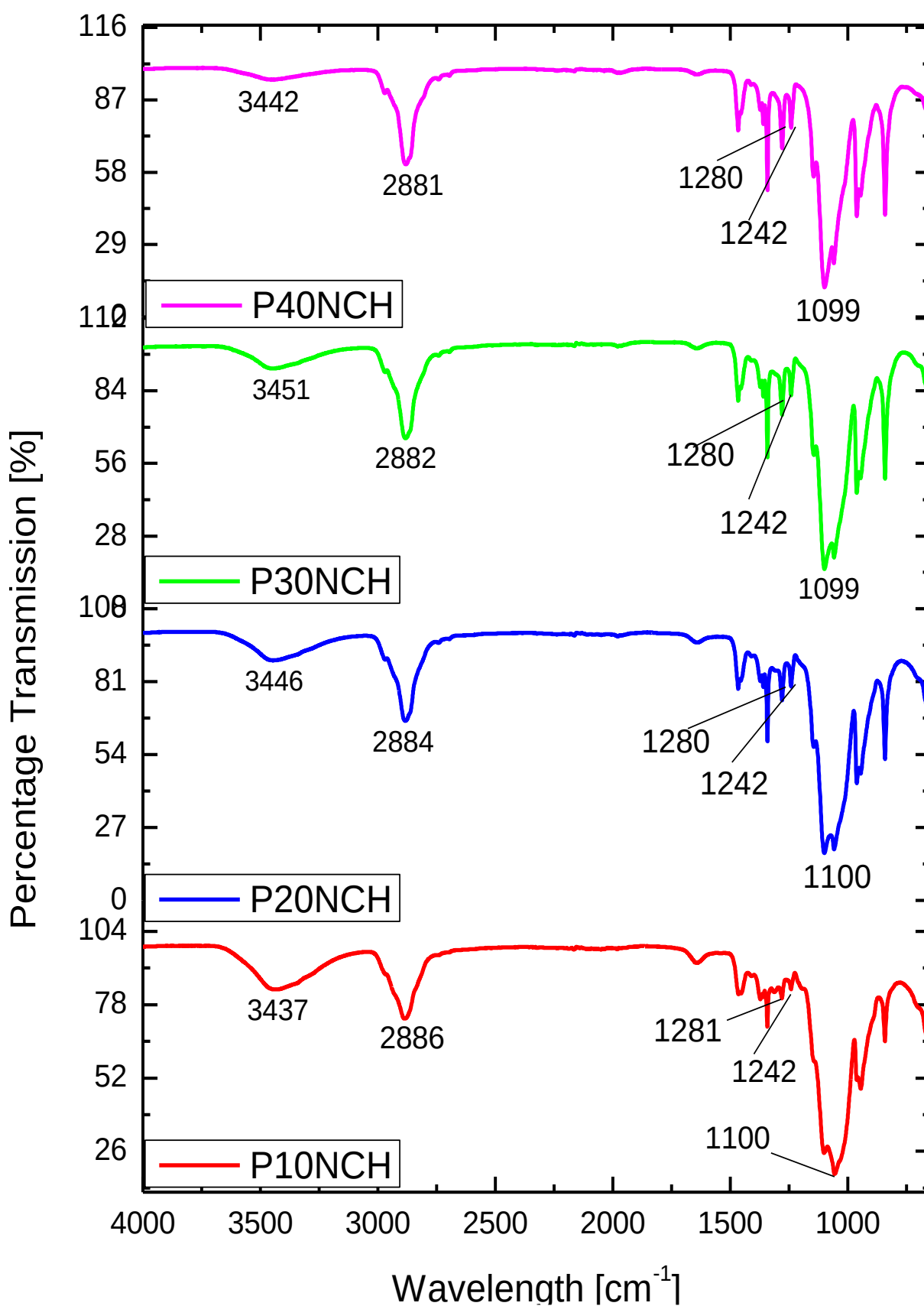


Figure 4.6: Graph depicting changes in the peaks of the hybrid polymer combinations of pluronic F127, methocel and nanocellulose at 37°C, using Fourier transform infrared spectroscopy.

4.3.2 Differential Scanning Calorimetry (DSC)

In the chitosan-cellulose hybrids, the increase in cellulose concentrations resulted in an increase in the intensity of the peaks, as well as a shift in peaks towards the right, such as the endothermic peaks in the $\sim 91^{\circ}\text{C}$ region (Figure 4.8) (characteristic with the endothermic peak seen in this region in pristine glycerophosphate) (Figure 4.7), and at $\sim 102\text{-}110^{\circ}\text{C}$ (characteristic with the endothermic peak seen in pristine chitosan) (Figure 4.7). The endothermic peaks characteristic of glycerophosphate in the region of $\sim 250^{\circ}\text{C}$ are generally lower in the hybridized formulations than in pristine glycerophosphate, with the peaks shifting towards higher temperatures from $\sim 233^{\circ}\text{C}$ - 238°C (Figure 4.8) as the concentration of cellulose increases. These shifts towards higher temperatures in endothermic peaks may indicate greater thermal stability of the formulations in that the formulation displays melting and degradation behaviour at higher temperatures and a greater amount of energy is needed to break the inter and intra-molecular forces within the formulation and elicit a phase change. This demonstration correlates with the rheological data that depicted an improvement in the gel strength and a greater degree of physical crosslinking with increasing concentrations of cellulose within the formulations (Figure 3.8.1), as well as with the increase in the intensity of the peaks in the FTIR spectra as the concentration of cellulose was increased (Figure 4.2; Figure 4.3; Appendix E). The chitosan-cellulose formulations do not display any sharp peaks indicative of crystalline regions and can thus be said to be amorphous.

Additionally, looking at formulation 5MC-2CHT in comparison to formulation 5MC-CHT (Figure 4.9) at 4°C , there is a leftwards shift in peaks as the ratio of chitosan is increased, which may indicate a weaker formulation with weaker bonds at storage temperature. Whereas at the physiological temperature of 37°C , formulation 5MC-2CHT exhibits a rightwards shift in the endothermic peaks towards higher temperatures as expected in comparison to formulation 5MC-CHT (Figure 4.8), with a shift from $\sim 94\text{-}105^{\circ}\text{C}$ (characteristic of pristine glycerophosphate) (Figure 4.7) and $\sim 110\text{-}126^{\circ}\text{C}$ (characteristic of pristine chitosan) (Figure 4.7), together with an increase in the intensity of the peaks (Figure 4.8). This is in keeping with the FTIR spectra which showed a decrease in the intensity of the peaks at 4°C when the ratio of chitosan was doubled (Figure 4.3; Appendix E), as well as with rheological data that showed that increasing the ratio of chitosan in the formulation resulted in a decrease in the

mechanical strength and viscosity of the formulation at storage temperature (Table 3.3.1; Figure 3.7, Figure 3.8.2), thereby mitigating the issue of gelation at lower temperatures without disrupting thermo-responsive behaviour of the system in a way that would negatively affect the gelation of the system at 37°C.

In the pluronic-cellulose hybrids, an increase in the concentration of pluronic resulted in a rightwards shift in the endothermic peaks (characteristic of pluronic) (Figure 4.10) around the range of ~55°C to slightly higher temperatures (Figure 4.11; Figure 4.12), as well as an increase in the intensity of these peaks, which may signify that increasing the concentration of pluronic resulted in increased thermal stability in terms of melting points of the formulation, and a greater amount of energy was needed to break the inter and intra-molecular forces within the formulation. This is in keeping with the FTIR data which showed an increased intensity of the peaks associated with pluronic as the concentration of pluronic increased (Figure 4.5; Figure 4.6; Appendix F), as well as with the rheological data which depicted that increasing the concentration of pluronic resulted in a decrease in the temperature at which gelation starts to occur (Table 3.3.2; Figure 3.12), thereby resulting in a greater degree of physical crosslinking at 37°C, and greater gel strength at 37°C (Figure 3.14). This sharp peak at ~55°C indicates that the crystalline regions within pluronic are melting, however the rest of the spectrum is indicative of amorphous cellulose regions due to the absence of further sharp peaks. Simultaneously, exothermic peaks characteristic of cellulose in the range of ~180°C decreased in intensity with increasing concentrations of pluronic (and thus decreasing concentrations of cellulose) as expected, until they almost completely levelled out in P40NC.

Once again, the DSC spectra confirm that no chemical crosslinking or changes occurred, as the overall shape of the spectra remained constant with only minor changes (such as broadening bands or increased intensity of peaks) seen, due to varying concentrations. These spectra also confirm the successful blending of chitosan- β -glycerophosphate with methylcellulose, as well as the incorporation of pluronic f127 into the methylcellulose-nanocellulose blend. The overall thermal stability of these formulations at these accelerated temperatures gives us an indication of their probable stability at ambient, physiological and storage temperatures.

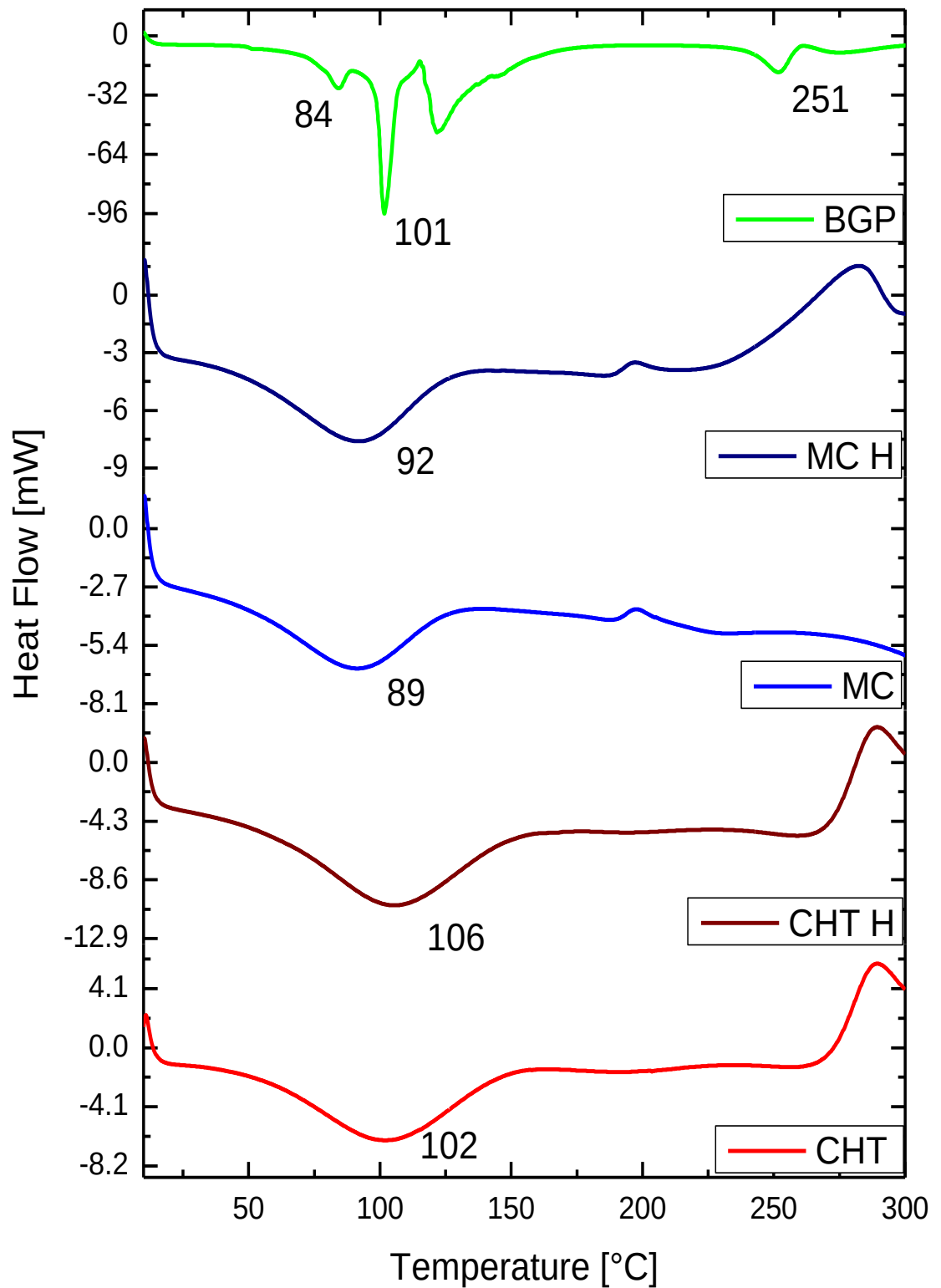


Figure 4.7: Graph depicting thermal changes in the pristine polymer combinations of chitosan, methylcellulose, and β -glycerophosphate in their sol (4°C) and gel states (37°C).

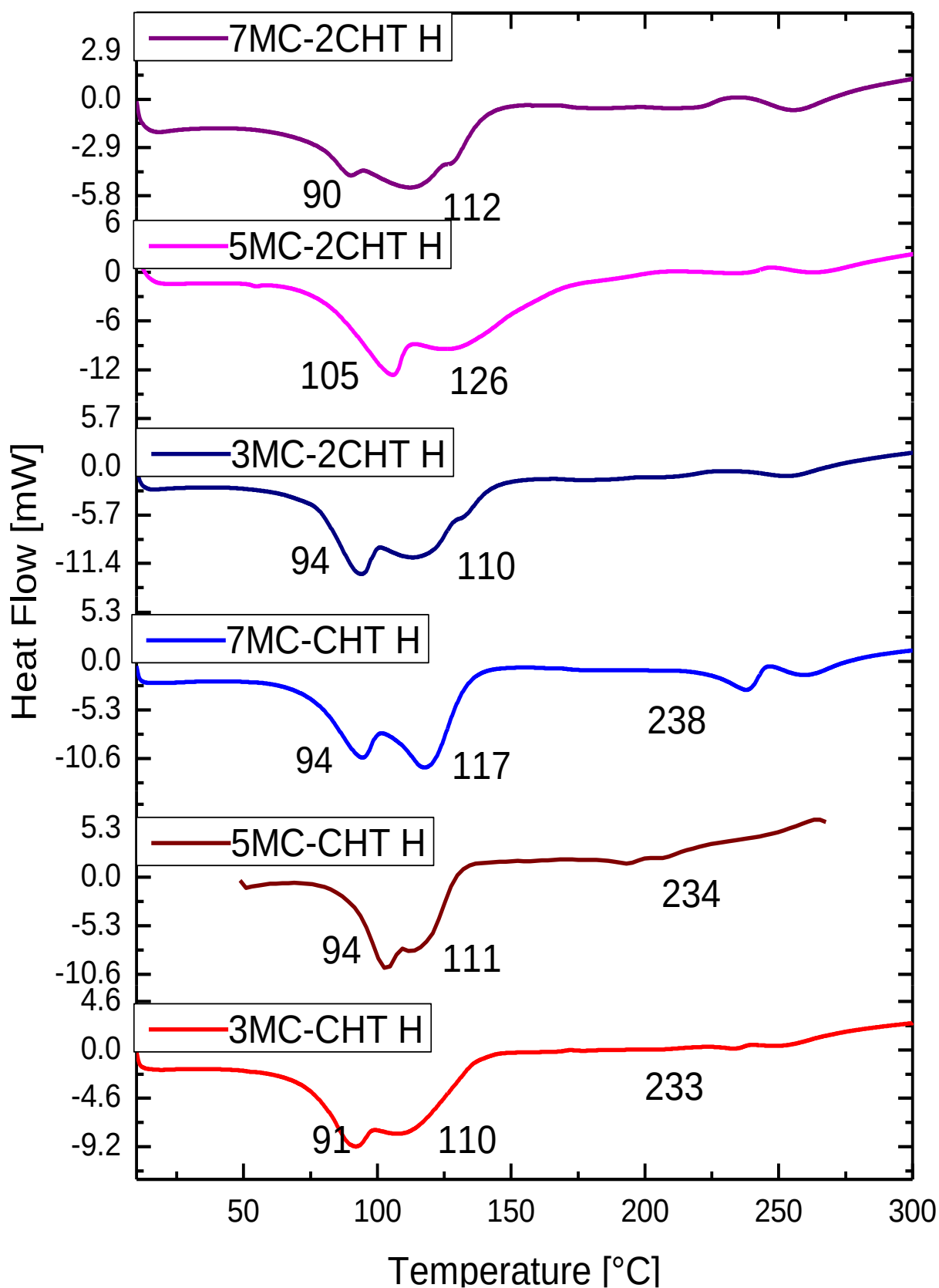


Figure 4.8: Graph depicting thermal changes in hybrid polymer combinations of chitosan, methylcellulose, and β -glycerophosphate in their gel state (37°C).

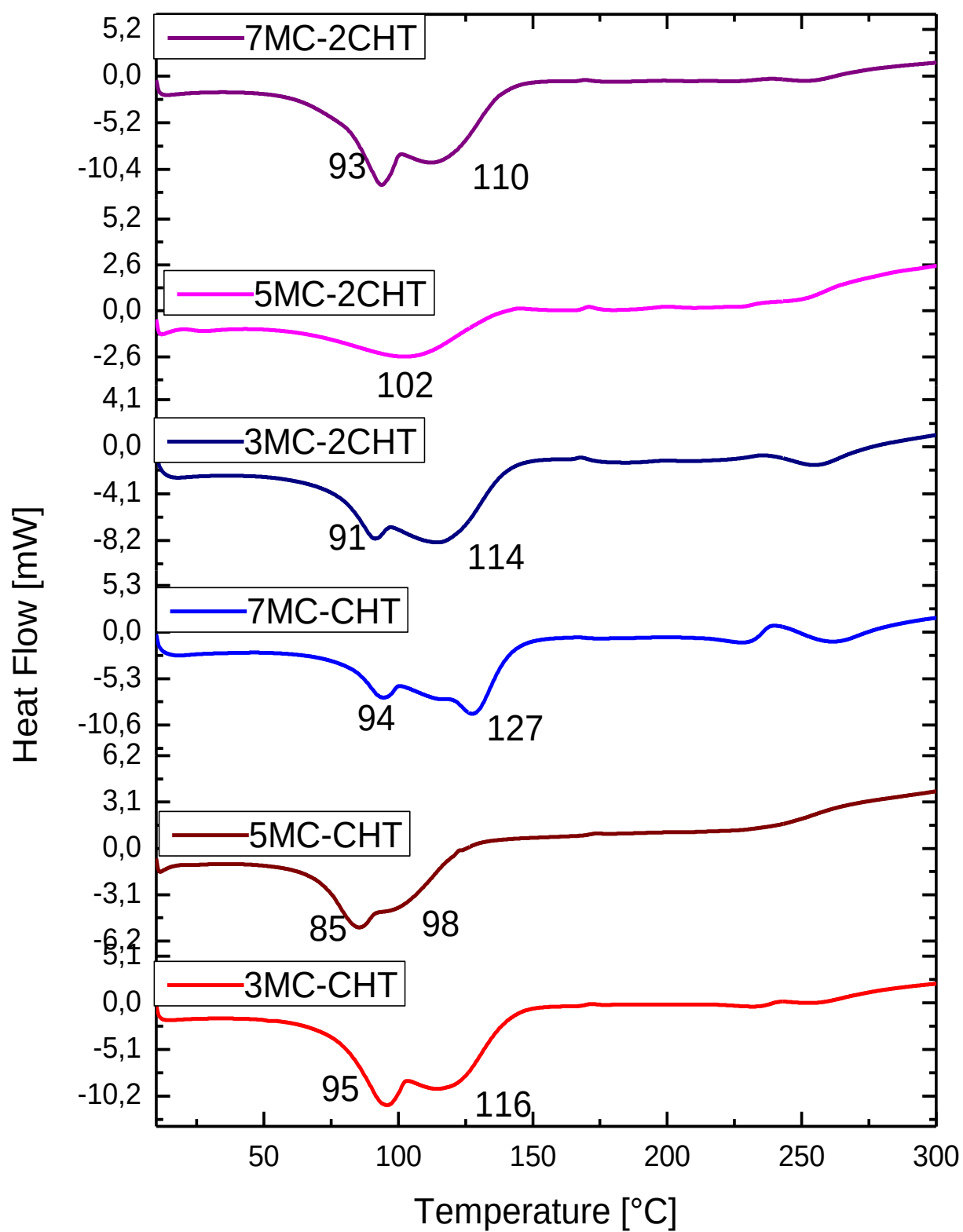


Figure 4.9: Graph depicting thermal changes in hybrid polymer combinations of chitosan, methylcellulose, and β -glycerophosphate in their sol state (4°C).

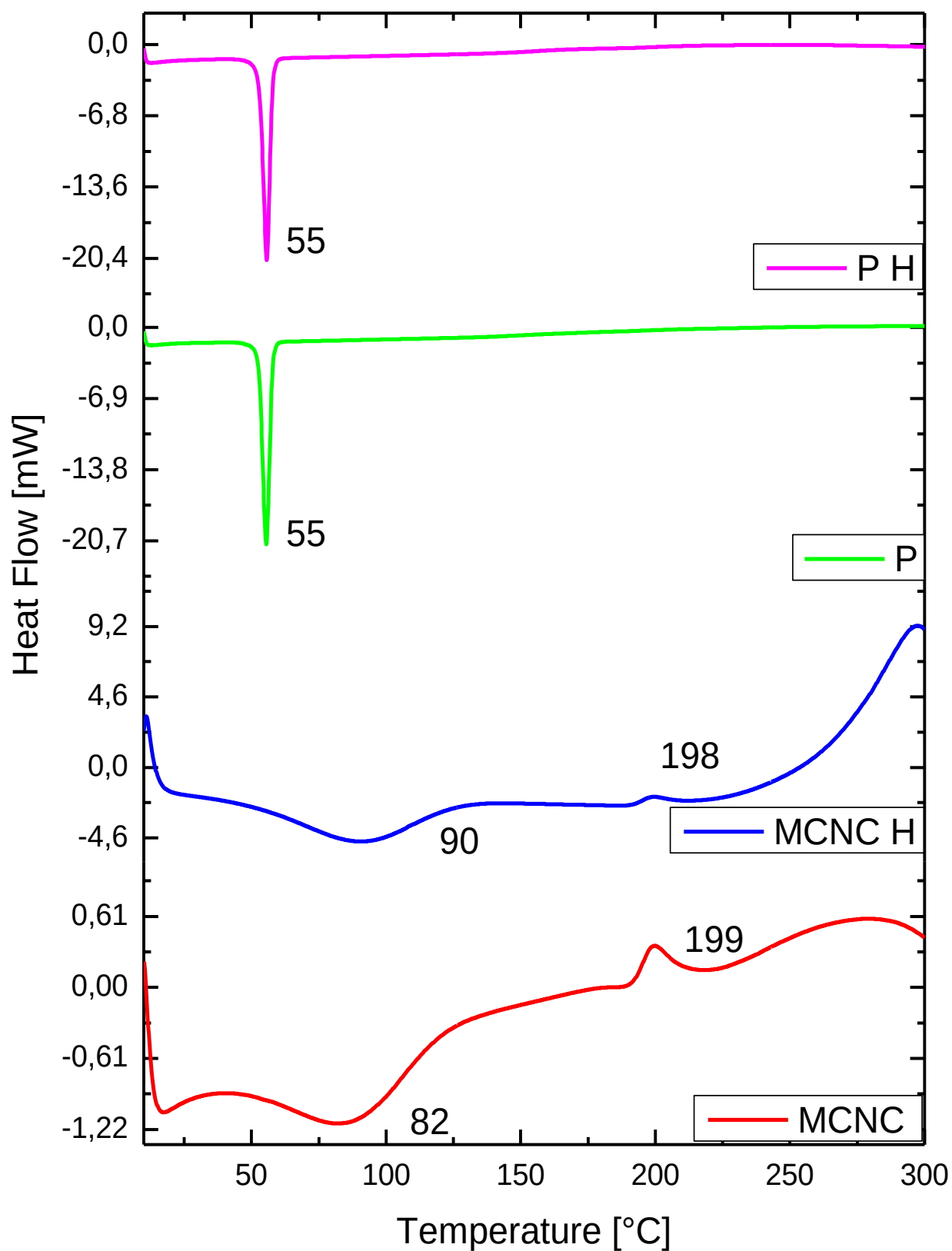


Figure 4.10: Graph depicting thermal changes in the pristine polymer combinations of pluronic and methocel and nanocellulose in their sol (4°C) and gel states (37°C).

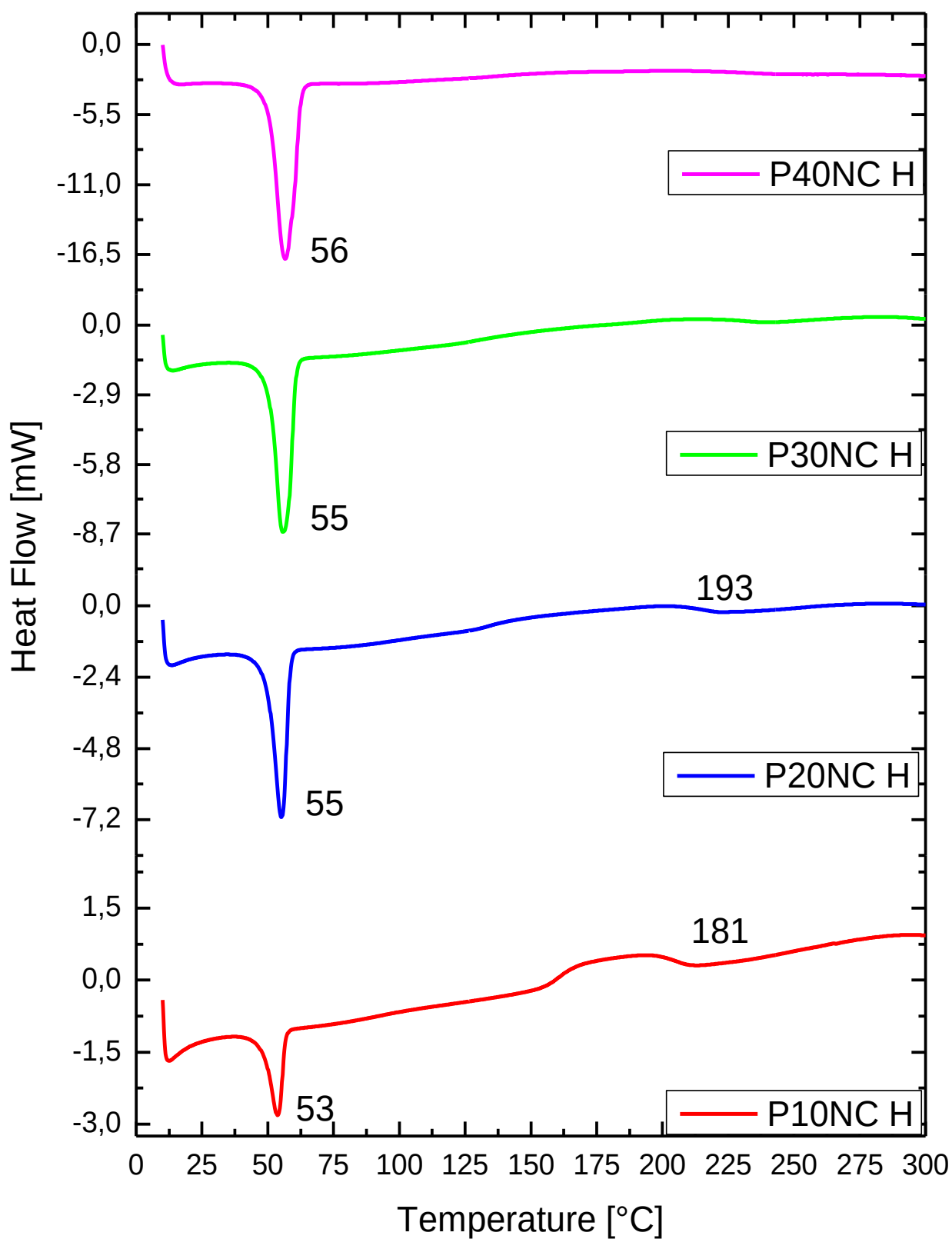


Figure 4.11: Graph depicting thermal changes in hybrid polymer combinations of pluronic and methylcellulose in their gel state (37°C).

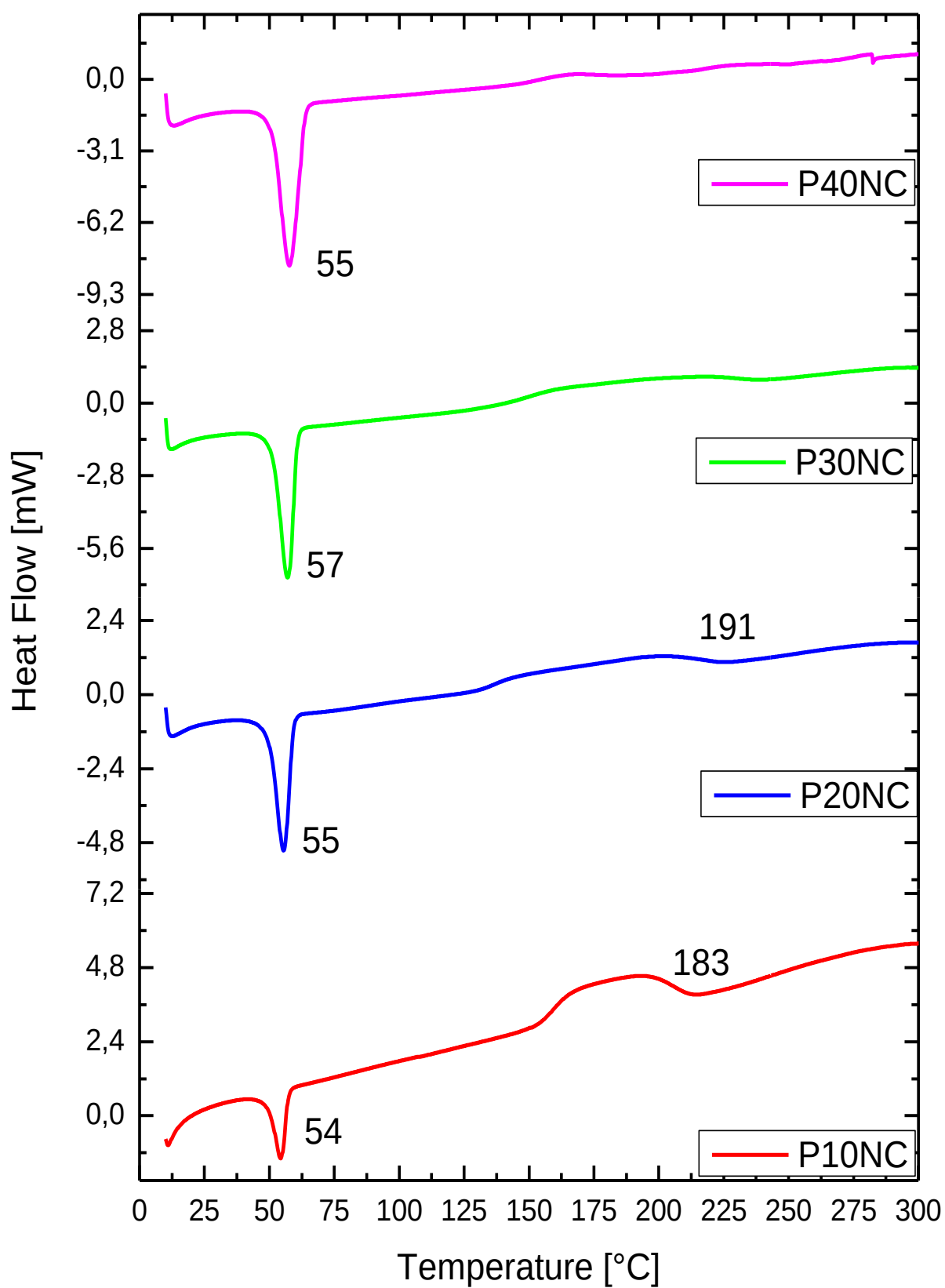


Figure 4.12: Graph depicting thermal changes in hybrid polymer combinations of pluronic and methylcellulose in their sol state (4°C).

4.3.3 Thermogravimetric Analysis (TGA)

A stepwise degradation is seen in the chitosan-cellulose based formulations, with an initial degradation seen from $\sim 60^{\circ}\text{C}$ to 100°C , attributed to the presence of chitosan- β -glycerophosphate, and thereafter thermal stability till $\sim 255^{\circ}\text{C}$, as is seen in the degradation profile of β -glycerophosphate, and the final step being attributed to the degradation of cellulose, at $\sim 320^{\circ}\text{C}$. Increasing concentrations of methylcellulose did not have an effect on degradation of the formulation with temperature, and doubling the ratio of chitosan has not altered the degradation of the formulation either.

In the pluronic-cellulose hybrids, with increasing concentrations of pluronic, there is an increase in the thermal stability of the formulations, and the degradation temperature increases from around 330°C to 365°C . This is expected as the degradation of pluronic occurs at higher temperatures than cellulose. However, in P40NC, degradation occurs from 50°C to 140°C , as is seen in methylcellulose, and thereafter thermal stability is seen from 140°C to 365°C , in keeping with the degradation pattern of pluronic.

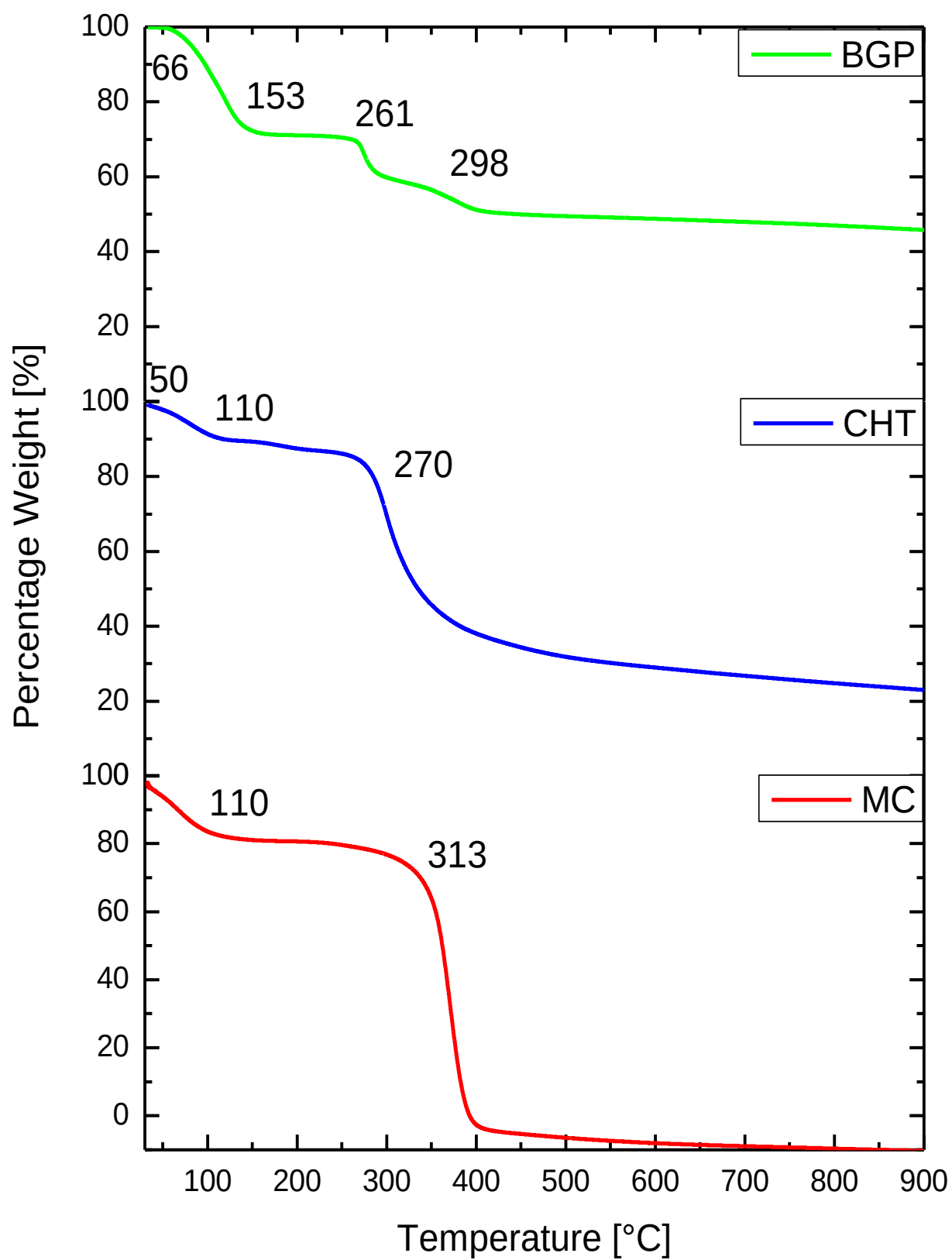


Figure 4.13: Graph depicting degradation of pristine chitosan, methylcellulose and β -glycerophosphate with temperature.

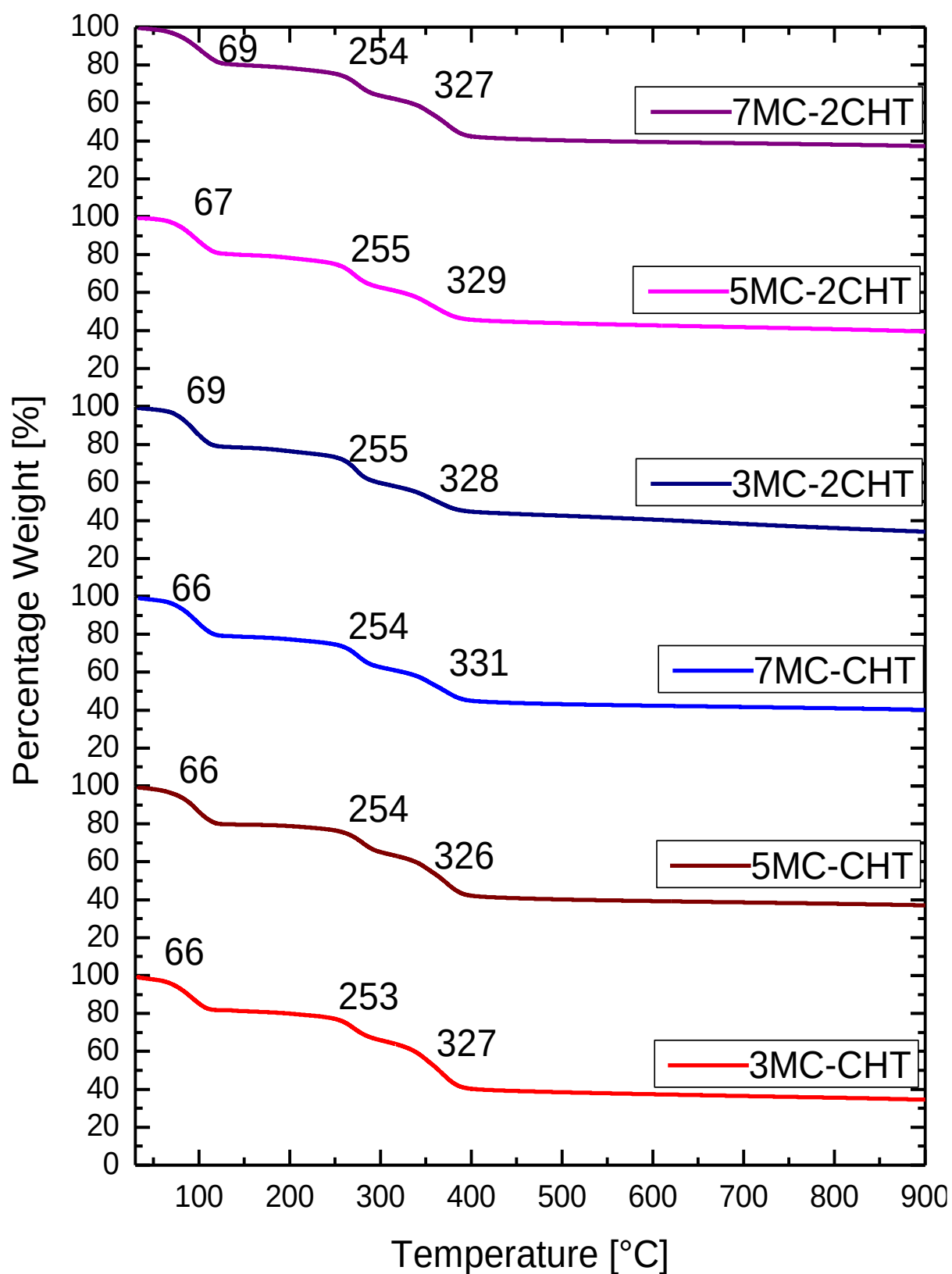


Figure 4.14: Graph depicting degradation of the hybrid polymer systems formulated using chitosan, methylcellulose and β -glycerophosphate with temperature.

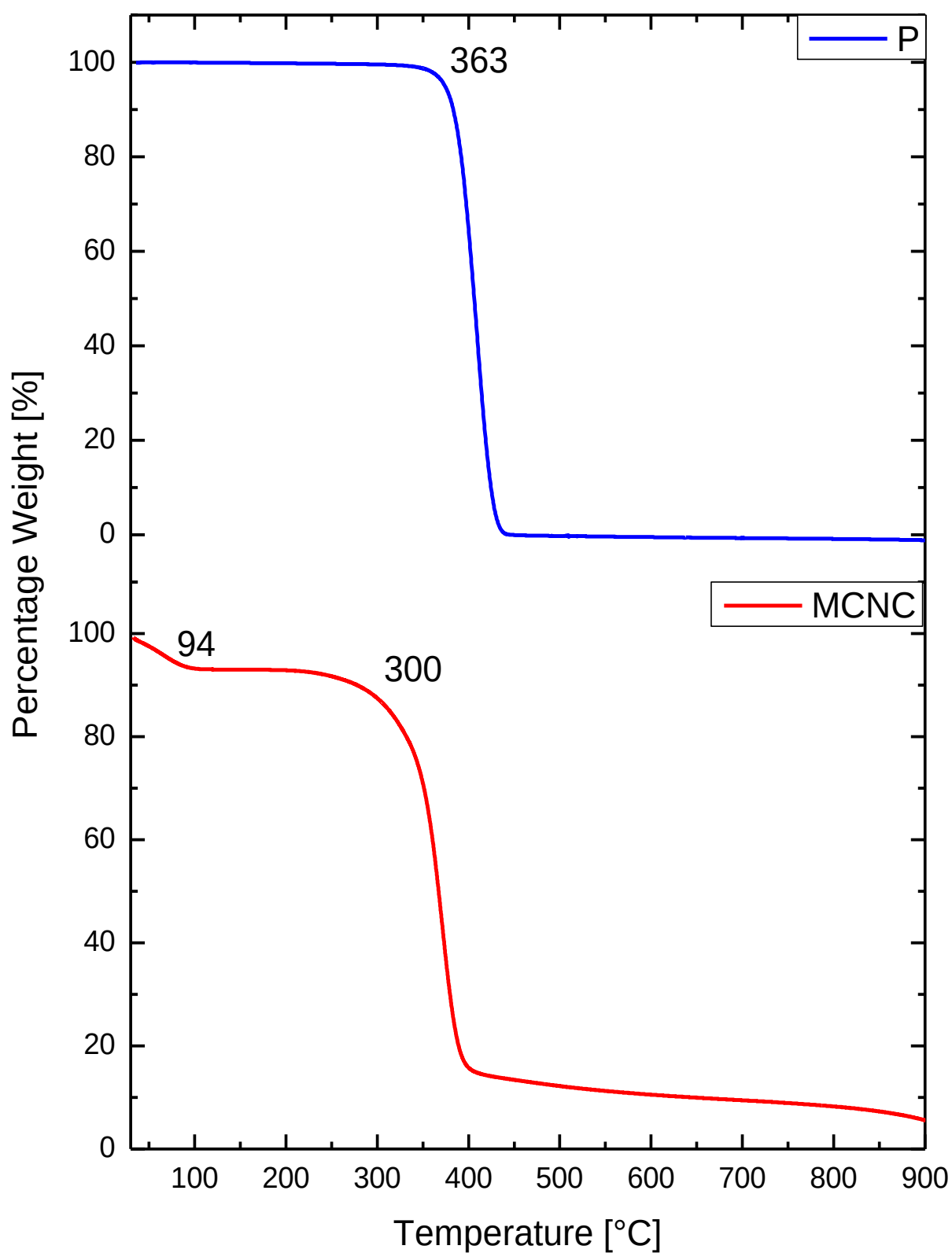


Figure 4.15: Graph depicting degradation of pristine pluronic and methylcellulose with temperature.

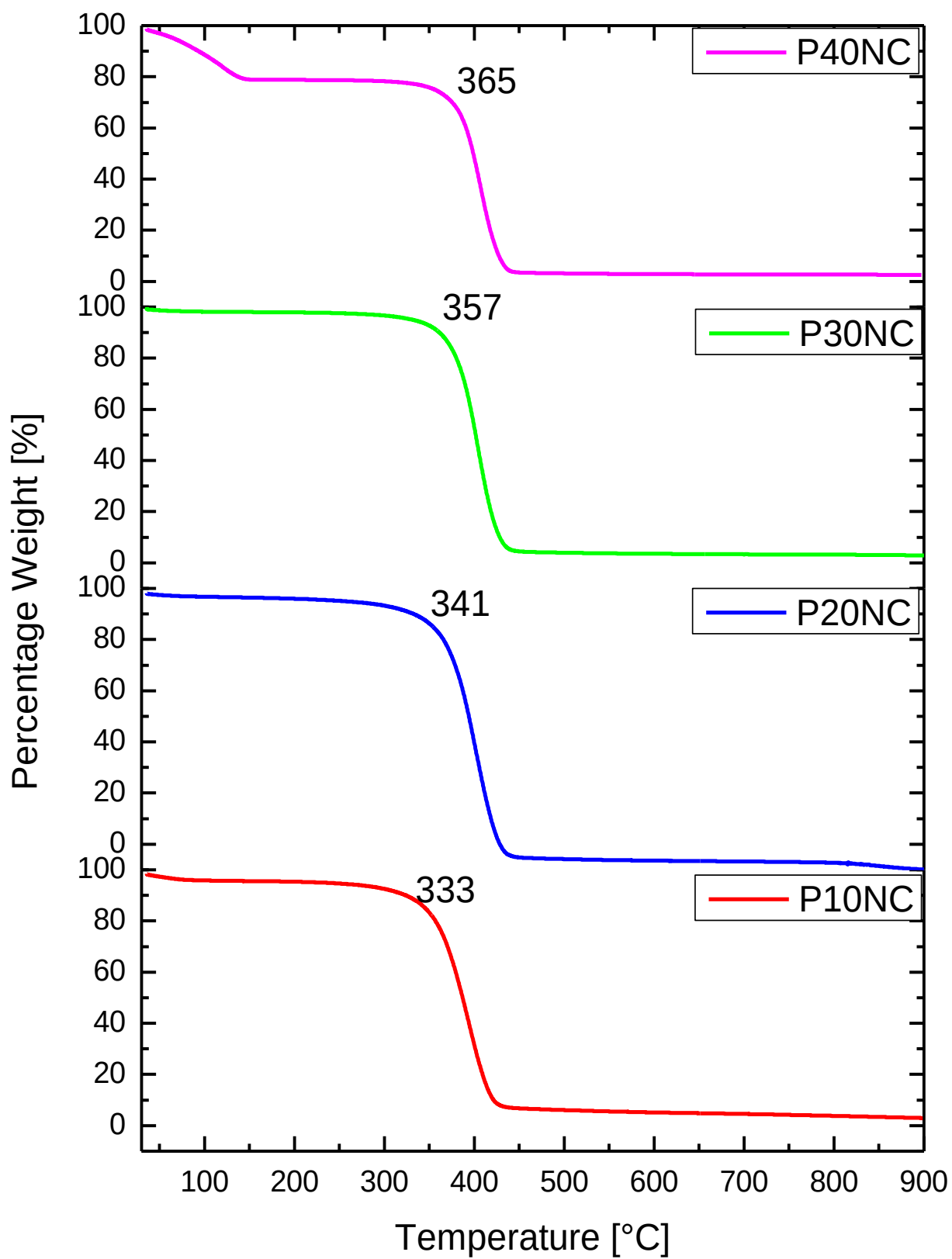


Figure 4.16: Graph depicting degradation of the hybrid polymer systems formulated using pluronic and methylcellulose with temperature.

4.3.4.1 Morphological analysis of the hydrogel

The SEM micrographs of formulation 5MC-2CHT depict a more particulate, loose and 'broken up' surface in figure 4.17, indicating that the formulation is in sol state at 4°C, as compared to a figure 4.18 at 37°C which is more intact and well structured, indicating that physical crosslinking has occurred. This is in keeping with the rheology discussed in chapter three, which shows that physical crosslinking occurs at 37°C, resulting in gel formation.

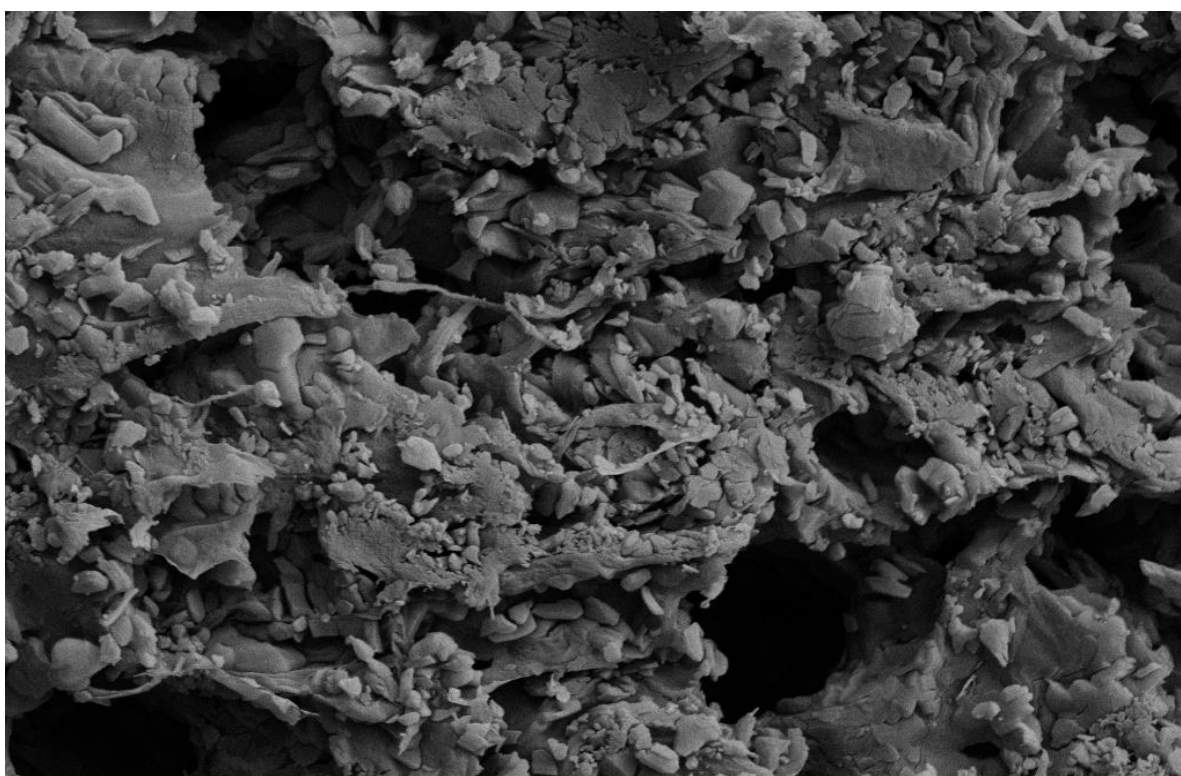


Figure 4.17: SEM image of the surface morphology of formulation 5MC-2CHT (Chitosan- β -glycerophosphate-cellulose) at 4°C.

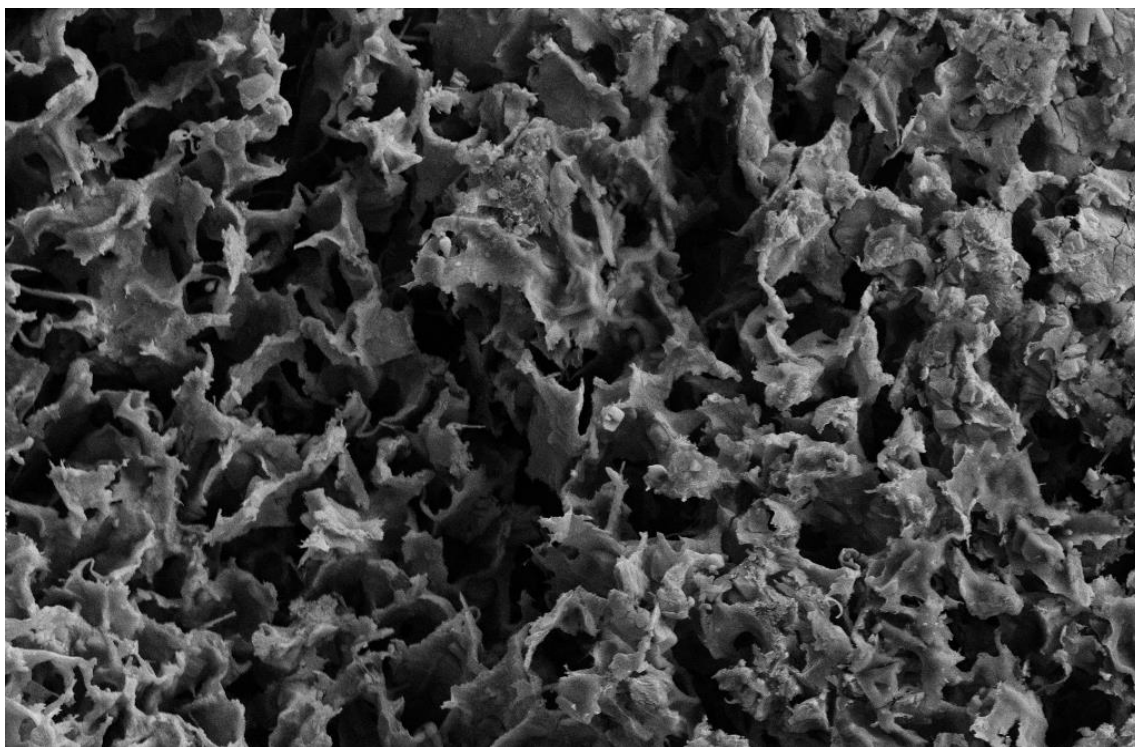


Figure 4.18: SEM image of the surface morphology of formulation 5MC-2CHT (Chitosan- β -glycerophosphate-cellulose) at 37°C

4.3.4.2 Effect of increasing methylcellulose concentrations in the chitosan-cellulose formulations

The effect of increasing concentrations of methylcellulose can also be seen in the micrographs in figures 4.19-4.21. Once again, a loose and particulate-like structure is seen in the formulation containing 3% methylcellulose (formulation 3MC-CHT) (Figure 4.19), indicating that the formulation is in a sol state. A more intact structure with solid-looking walls is seen in the formulation containing 5% methylcellulose (formulation 5MC-CHT) (Figure 4.20), indicating that this formulation has formed a gel at 37°C, and an even more tightly-knit structure is seen in the formulation containing 7% methylcellulose (formulation 7MC-CHT) (Figure 4.21). This clearly demonstrates the proportionality between the degree of physical crosslinking and the concentration of methylcellulose within the formulations, and can also be seen physically in formulations 3MC-CHT to 7MC-CHT in figure 3.2, by observing the opacity of the formulations.

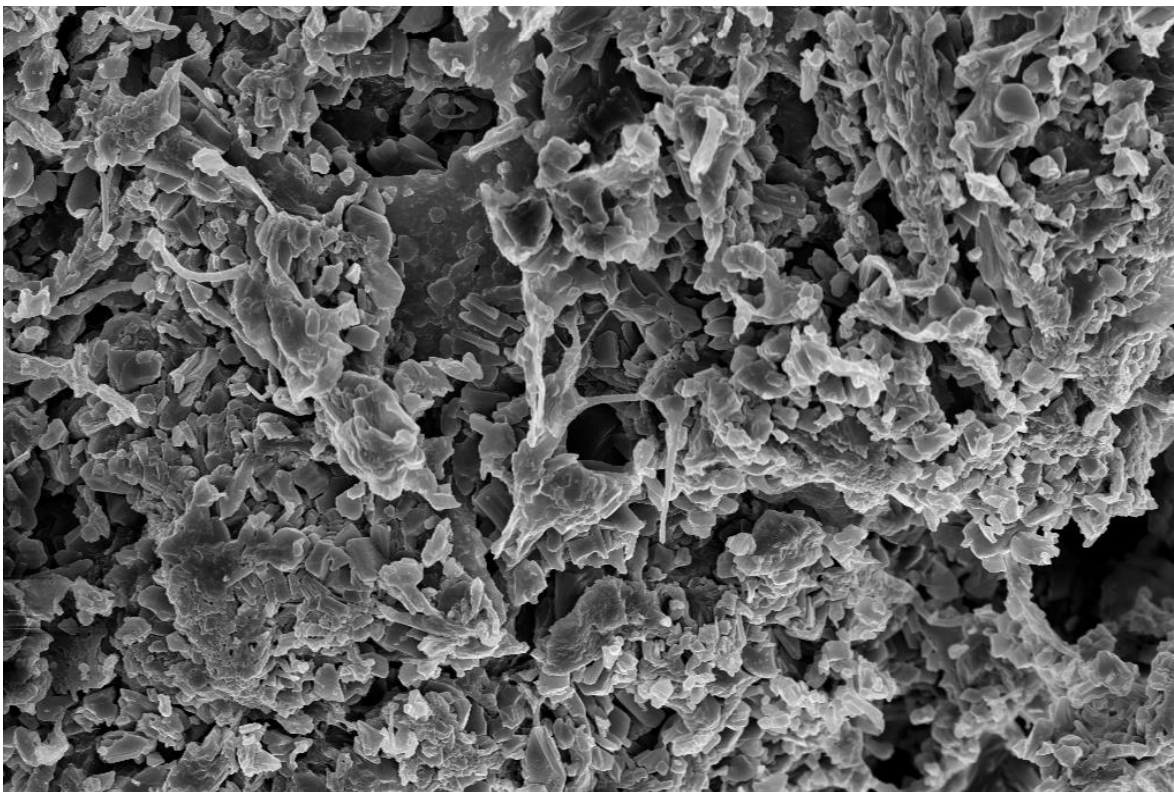


Figure 4.19: SEM image of the surface morphology of a hybrid chitosan- β -glycerophosphate-cellulose formulation at 37°C, formulated using a 3% methylcellulose (formulation 3MC-CHT).

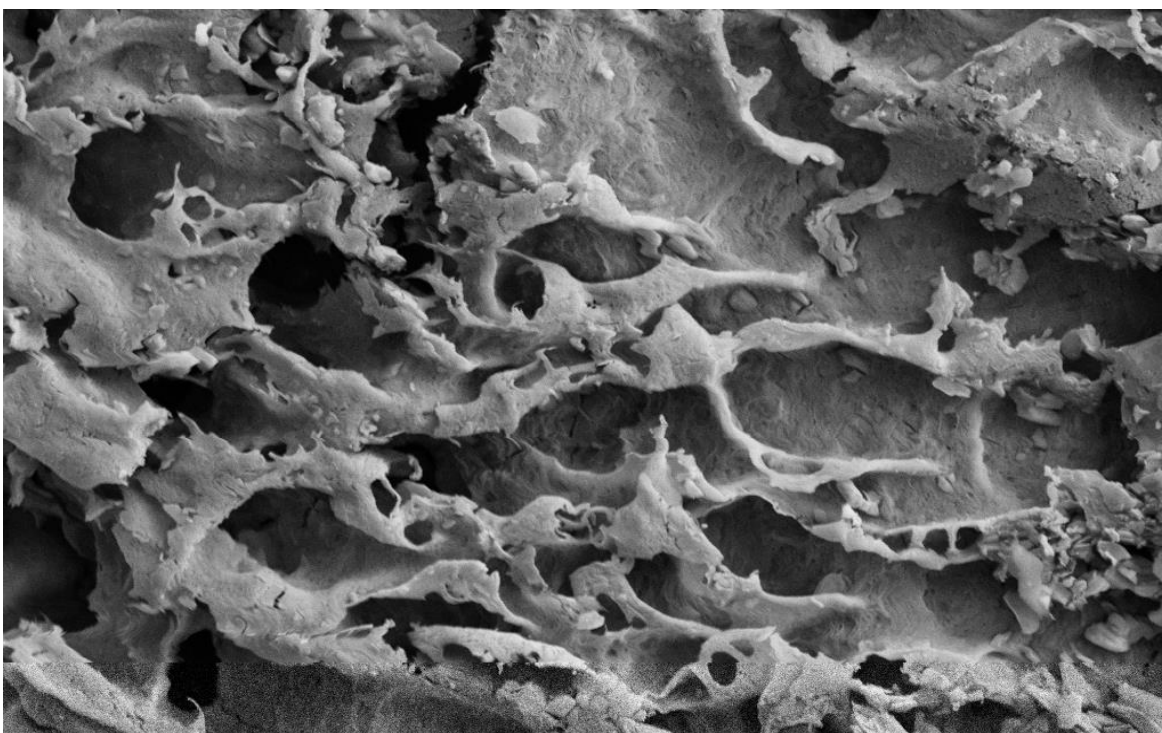


Figure 4.20: SEM image of the surface morphology of a hybrid chitosan- β -glycerophosphate-cellulose formulation at 37°C, formulated using a 5% methylcellulose (formulation 5MC-CHT).

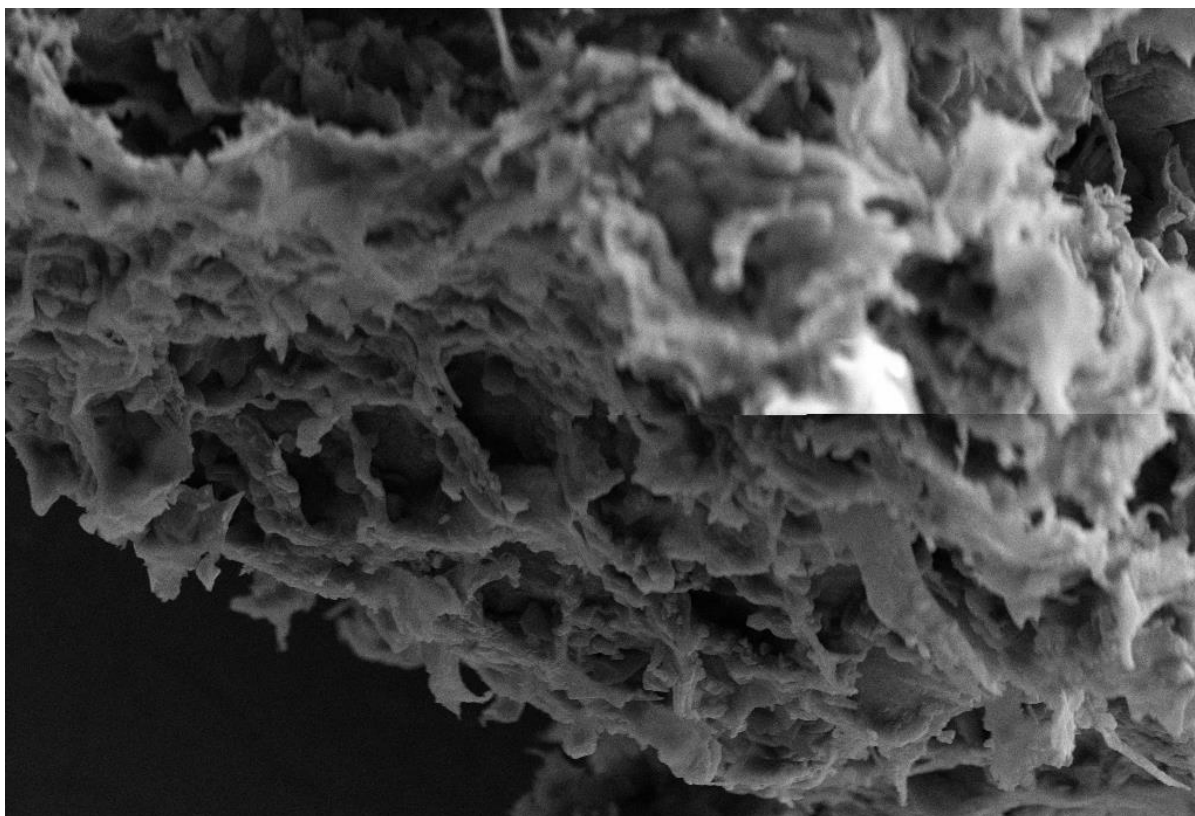


Figure 4.21: SEM image of the surface morphology of a hybrid chitosan- β -glycerophosphate-cellulose formulation at 37°C, formulated using a 7% methylcellulose (formulation 7MC-CHT).

The pluronic-cellulose based formulations depict more solid-like walls even at 4°C (Figure 4.22), as opposed to the particulate-like walls in the chitosan-cellulose formulations (Figure 4.23). This can possibly be attributed to the presence of nanocellulose within the pluronic based formulations. However, it can still be seen that a more loose structure is present at 4°C (Figure 4.22), as compared to the tightly-knit structure seen at 37°C (Figure 4.23). Once again, this indicates that the formulation is in a sol state at storage temperature and forms a gel at physiological temperature.

In addition, the greater surface area (seen in the micrograph in Figure 4.23) provided by the incorporation of nanocellulose into the pluronic based formulations can be linked to the fact that P20NC exhibited swelling behaviour (Figure 4.25), by allowing a greater possibility of fluid exchange with the environment. (Kankala et al., 2018). In contrast, the chitosan-based formulation (5MC-2CHT) had a much smaller surface area (Figure

4.18), and therefore did not display the same swelling behaviour as P20NC (Figure 4.24).

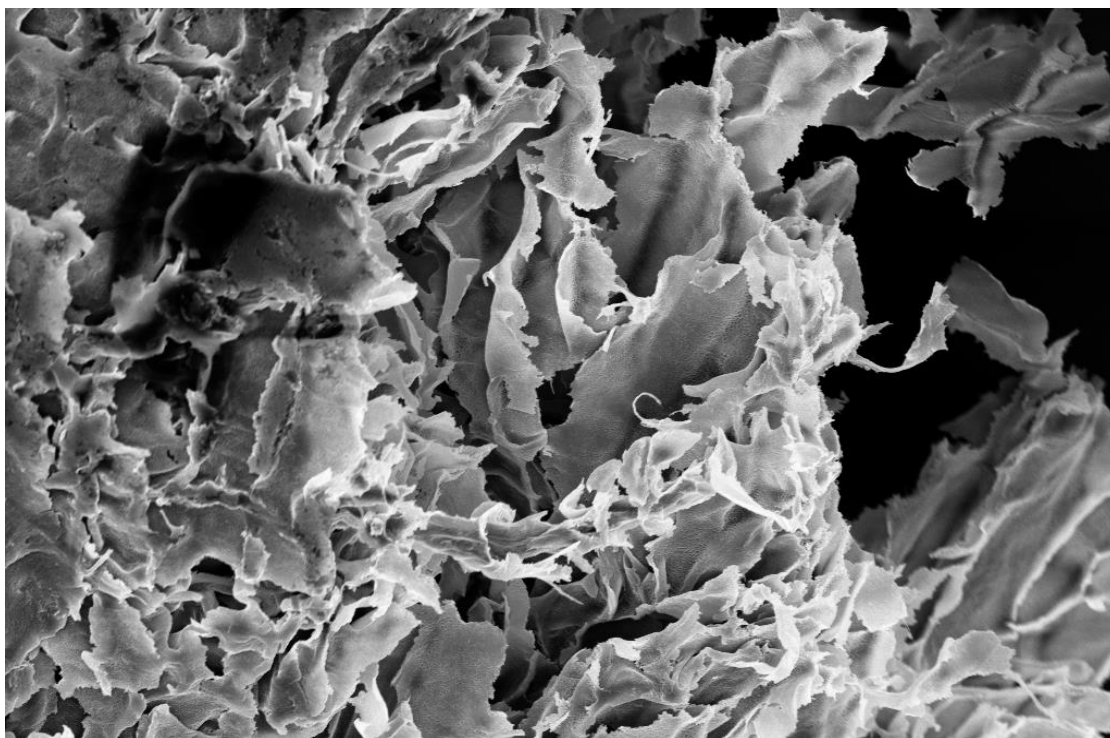


Figure 4.22: SEM image of the surface morphology of a hybrid pluronic-cellulose formulation (P20NC) at 4°C.

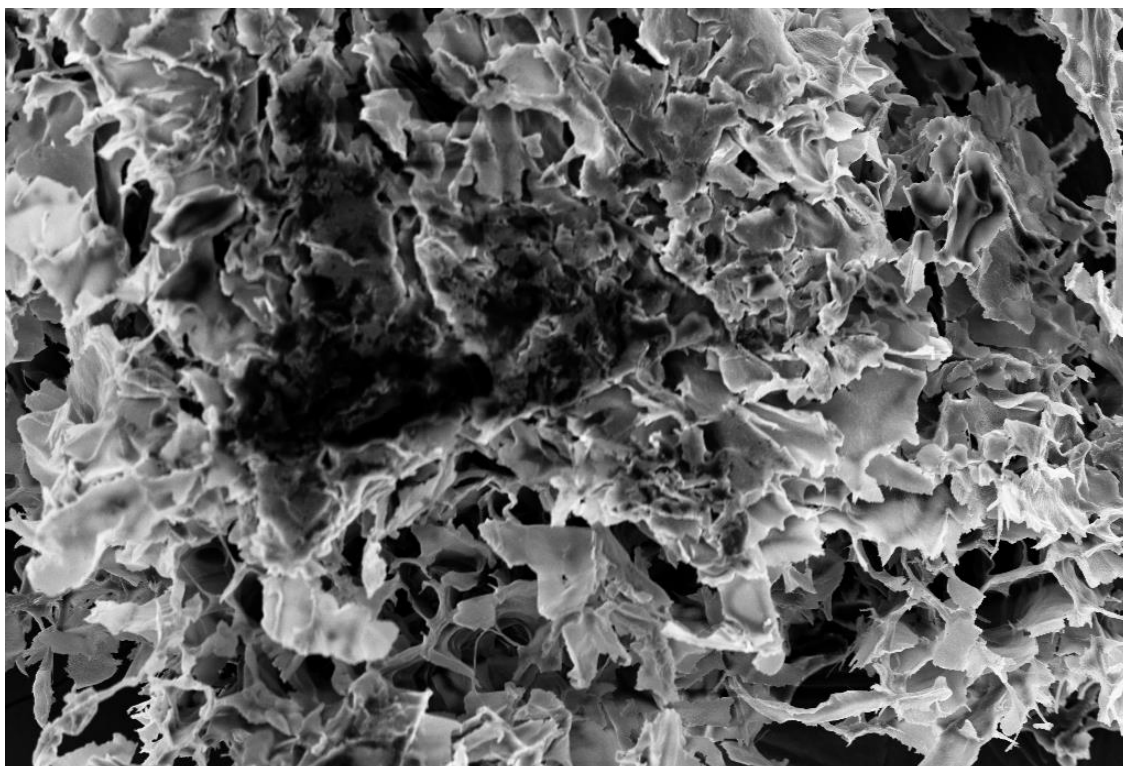


Figure 4.23: SEM image of the surface morphology of a hybrid pluronic-cellulose formulation (P20NC H) at 37°C.

4.3.5 In vitro swelling and erosion of gels

Both systems display bio-erodible behaviour, which is favourable to the proposed system and its application. The chitosan-cellulose based gel showed a 31% erosion over a 28-day period, with no swelling behaviour displayed. In contrast, the pluronic-cellulose based gel initially displayed swelling behaviour, increasing the formulation in weight by 81% over the first ten days, after which erosion took place for the remainder of the 28-day period, and a 27% erosion was seen after the initial swelling had occurred. This could be due to the incorporation of nano-cellulose into the pluronic-based formulation, which has been known to improve the swelling properties of gels, and the greater surface area and porosity of the pluronic-cellulose gels (Figure 4.23), allowing more fluid to be absorbed. The chitosan-cellulose based gel also displays a greater gel strength than the pluronic-cellulose gel, evidenced by a greater elastic modulus at 37°C when a gel is formed (Table 3.3.1; Table 3.3.2), as well as an extended viscoelastic region (Figure 3.8.1; Figure 3.14). This difference in gel strength could also account for the difference in the swelling behaviour of the gels, as it is known that an increase in gel strength often results in a decrease in the degree to which the gels are able to swell (Günter et al., 2020).

Furthermore, the differences in swelling behaviour of the formulations provides us with insight regarding the crosslinking density of the formulations. Formulations with a greater degree of crosslinking or a higher crosslinking density display lower swelling properties (Park et al., 2009). Thus it can be deduced that the chitosan-cellulose hybrid was crosslinked to a greater degree than the pluronic-cellulose hybrid system, with β -glycerophosphate acting as a crosslinking agent by inducing thermal gelation via the formation of physical crosslinks.

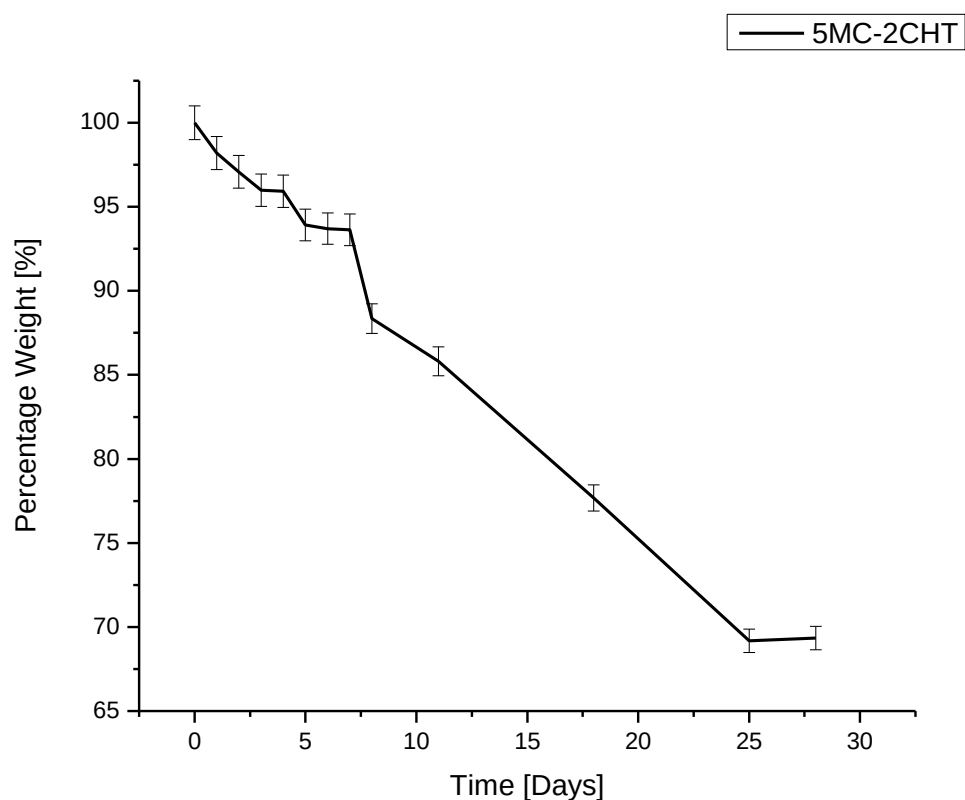


Figure 4.24: Graph depicting the erosion behaviour of the chitosan- β -glycerophosphate-cellulose gel (formulation 5MC-2CHT) with time in an in-vitro simulation of physiological conditions.

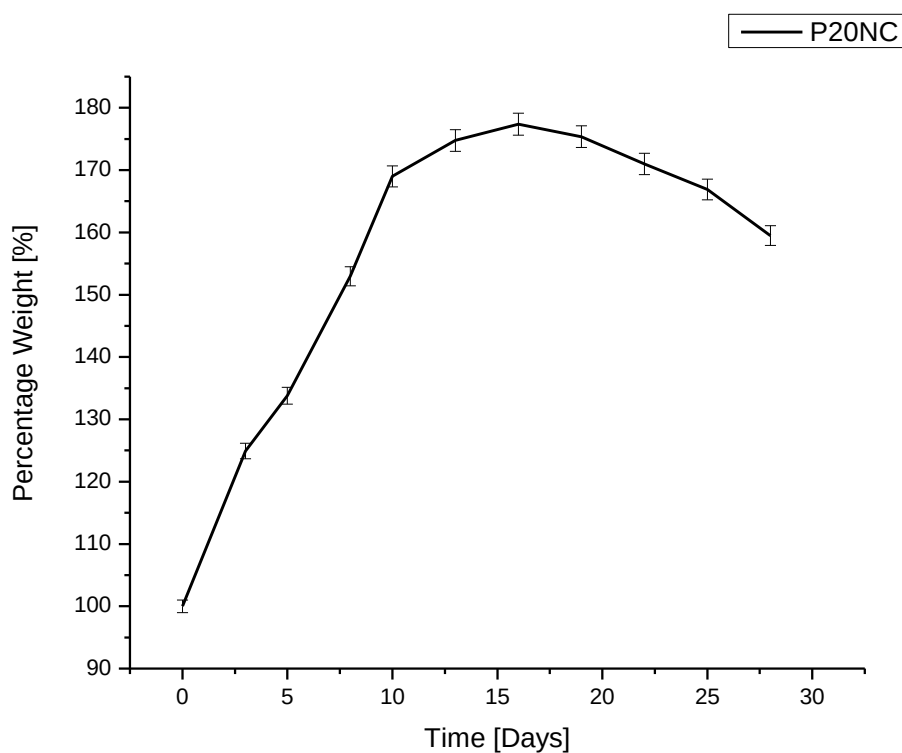


Figure 4.25: Graph depicting the swelling and erosion behaviour of the pluronic-cellulose gel with time in an in-vitro simulation of physiological conditions.

4.3.6 In vitro drug release

While both formulations show zero order release kinetics, the rates of release differ, with the chitosan-cellulose based formulation reaching completion at the six-hour mark with a total of 87% released, and the pluronic-cellulose based formulation showing a slightly more sustained release over a 24h period, achieving a cumulative release of 72%. This could be attributed to the fact that the pluronic-based formulation displays swelling behaviour, thereby slowing down the rate of release(Wan, Heng and Wong, 1993), whereas the chitosan-based formulation only displays erosion.

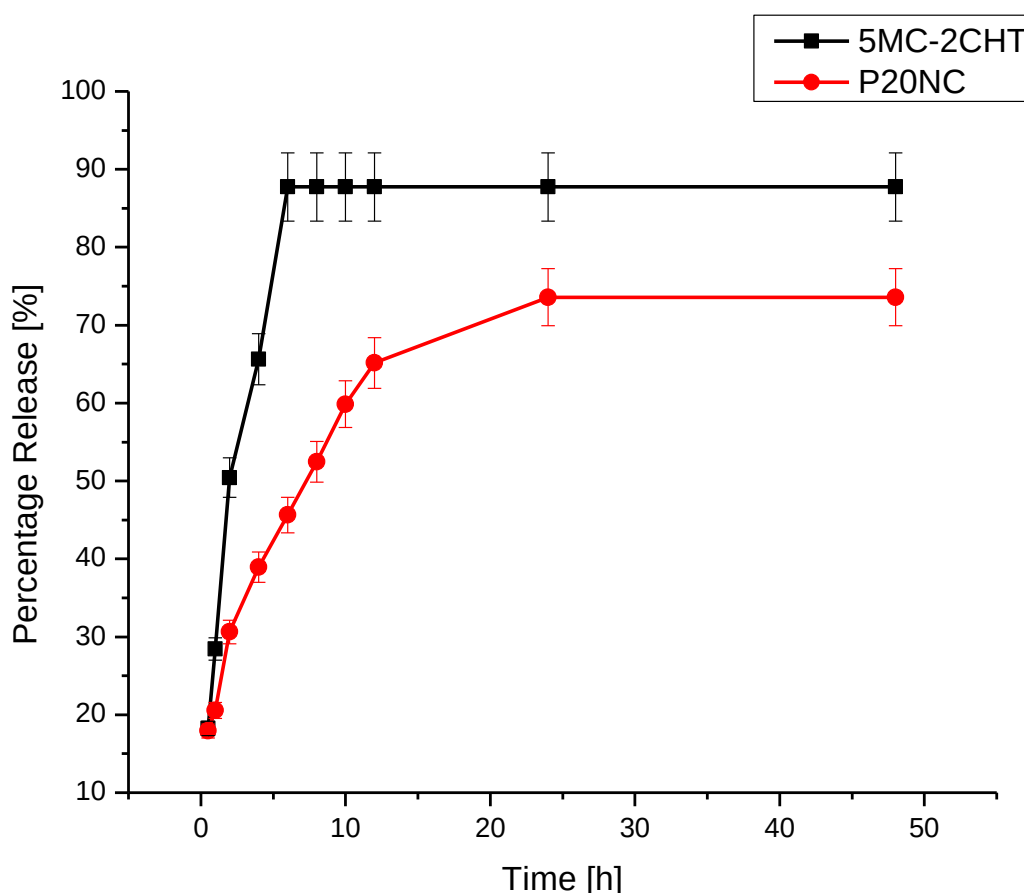


Figure 4.26: Graph depicting the release kinetics of the hybrid polymer systems.

4.4 Concluding remarks

The occurrence of physical crosslinking within the formulations at physiological temperature was confirmed by the increase in the intensity of the peaks noted in the FTIR analysis. This correlated with the resulting improvement in gel strength, as seen

in the rightward shift of peaks towards higher temperatures during differential scanning calorimetry. Morphological analysis of the formulation depicted a clear correlation and direct proportionality between the degree of physical crosslinking and structural arrangement of the formulation with increasing concentrations of cellulose. The difference in structural arrangement of the formulations at storage and physiological temperatures further confirm the thermo-responsive nature of the system and the occurrence of physical crosslinking within the formulations in response to increased temperatures. These differences in the morphology and gel strength of the formulations led to the differences in the swelling and erosion behaviour of the formulations, with swelling behaviour depicted in the pluronic-cellulose formulation, which had a greater surface area than the chitosan-cellulose formulation, which displayed primarily erosion behaviour. The swelling and erosion behaviour of the formulations provided further insight towards the degree of physical crosslinking within the formulations, with the chitosan-cellulose formulation exhibiting a greater degree of physical crosslinking than the pluronic-cellulose formulation. The swelling behaviour of the pluronic-cellulose formulation also affected the release kinetics of the formulation, by slowing down the release of TFA from the system due to the opposing direction of flow of the uptake of fluid into the system versus the release of the bioactive out of the system. Both formulations however reached completion in terms of release within the initial stages of wound healing, rendering them suitable for use in the current application, with the chitosan-cellulose formulation releasing a greater amount of trans ferulic acid and reaching completion within a quicker timeframe compared to the pluronic-cellulose formulation. Each of these results was in agreement with the rheological findings in chapter three of this dissertation. Once again, the effect of polymer concentration proved to be significant with regards to the degree of physical crosslinking, gel strength and morphology.

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CHAPTER FIVE

IN VITRO CELL STUDIES AND IN VIVO ANALYSIS IN A RAT MODEL

5.1 Introduction

Chitosan has been used widely in wound healing due its' believed antibacterial (Dai et al., 2011; Abazari et al., 2021) , haemostatic, and mucoadhesive properties (Patrulea et al., 2015) as well as its ability to promote granulation and organization and accelerate wound healing. Enhanced activity of inflammatory cells such as polymorphonuclear leukocytes, macrophages, and fibroblasts, which are responsible for phagocytosis, the production of various interleukins as well as growth factors, have been demonstrated in veterinary wound studies (Ueno et al., 2001). Aside from its biocompatibility and ease of availability, the ability of cellulose to absorb exudates and retain moisture make it suitable for use in wound healing systems (Schuhladen et al., 2021). In addition, cellulose has been shown to promote granulation and wound healing by fibrogenesis (Portela et al., 2019; Vijayakumar et al., 2019). Cellulose derivatives such as cellulose acetate, carboxymethyl cellulose and bacterial cellulose have been widely used in wound healing applications due to their tunable physiochemical properties, low cost, and ability to deliver various bioactive molecules to the wound site (Abazari et al., 2021). These cellulose derivatives have displayed potential antioxidant properties (Fan et al., 2014), good cell adhesion and promotion of cell proliferation with minimal interference to the formation of new tissue (Wiegand and Hipler, 2010), thus making them ideal candidates as biomaterials in wound healing applications.

To evaluate the wound healing potential of the formulations, in vitro and in vivo analysis needs to be conducted. Cell viability tests and cell migration assays are common techniques that are used to determine the cytotoxicity and to an extent, the wound healing potential of the system. Due to the differences between the physio-chemical nature in vivo and the dilute aqueous solutions that are used in in vitro studies, results often vary (McConnell E., 1995). In vitro tests alone are therefore not enough to prove the efficacy of the formulation, and in-vivo veterinary studies must be conducted. The supplementation of in vitro experimentation with in vivo studies allows researchers to

gain more insight as to the behaviour elicited by the system on a cellular level and the mechanisms which occur in vivo. In vivo studies further provide insight with regards to the potential side effects and risks associated with the use of the system.

Histopathological and observational data obtained from animal studies allow a more realistic clinical extrapolation of the data and a better prediction of how the system might behave in the human model. The physiological similarities between animals and humans allow us to explore potential therapeutic treatments using quantifiable methods in a controlled environment (Nyanzu et al., 2017). Rat models have been commonly used to evaluate wound healing potential due to their ease of availability, simplicity to work with given their size, cost effectiveness, and low demand in terms of post-surgical needs (Nyanzu et al., 2017; Marklund, 2016). The Sprague Dawley strain, in particular, has been extensively used in wound studies and has displayed effective results in enabling researchers to progress their understanding of wound healing and skin tissue regeneration (Yuvarani et al., 2012; Lin, Lin and Hong, 2017).

The purpose of this research was to design a smart responsive sol-gel system that could be used to deliver bio-actives evenly throughout the wound site, no matter the size and shape of the wound. The materials in the system, however, have been carefully selected in an aim to create a system that would inherently promote wound healing by creating an optimal environment for the wound healing process to occur. Whether this goal has been achieved will be determined by the analysis conducted in this chapter.

5.2 Materials and Methods

5.2.1 Materials

Human epithelial keratinocyte cell line (HaCat) was purchased from Cellonex (Johannesburg, South Africa). Phosphate buffered saline, Dulbecco's Modified Eagle Media (DMEM), Foetal Bovine Serum (FBS) and Penicillin/Streptomycin, and the Alamar blue reagent kit were purchased from Sigma Aldrich (St. Louis, MO, USA). Male Sprague Dawley rats were obtained from the Central Animal Services (CAS) unit at the University of the Witwatersrand, Johannesburg, as per protocol in accordance with the University's animal services guidelines. All surgical instruments and syringes

were ultrapure and of elementary grade and obtained from the Central Animal Services (CAS) unit at the University of the Witwatersrand, Johannesburg.

5.2.2.1 Cell Studies

5.2.2.1.1 Preparation of the cellulose-based gels for invitro analysis

Formulations 5MC-2CHT and P20NC were prepared as described in section 3.2.2 of this dissertation, and a concentration of 50 µg/mL of trans ferulic acid was incorporated into the gel as described in section 4.2.2.5. This concentration of trans ferulic acid was used to facilitate the dilution with cell media during in vitro cell studies, and to establish the toxicity and migration ability of the formulation at a concentration of 5 µg/mL, which is the final concentration of trans ferulic acid used in in-vivo analysis. The formulations were sterilized using UV light and allowed to rest overnight before use.

5.2.2.1.2 In-vitro cytotoxicity analysis of thermogels

The cytotoxicity of the gels and their pristine polymers was determined by measuring the absorbance after an assay conducted with the Alamar Blue reagent kit. Alamar Blue is a resazurin based reagent that is blue in colour. Resazurin is permeable to cells and when it enters living cells, it is reduced to a red fluorescent compound called resorufurin. This change in colour and increased fluorescence was detected by measuring absorbance using a Victor X3 microplate reader (Perkin Elmer, MA, USA) at wavelengths of 570 nm and 600 nm. Cultured Human Keratinocyte Cells (HaCat) were used as this cell line is frequently employed when investigating the skin as well as for studies involving inflammatory and immunological responses, making them ideal for use in this wound healing study (Columbo et al., 2017)(Columbo et al., 2017). The cells were plated at a density of 1.5×10^4 cells/mL in 96 well plates and then incubated in Dulbecco's Modified Eagle Medium (DMEM) with 10% Foetal Bovine Serum (FBS) and 1% Penicillin/Streptomycin at 37°C and 5% CO₂ (NuAire 5710, MN, USA) for 24 h. The media was then removed, and the cells were washed twice with phosphate buffered saline. The wells were inoculated with 10 µL of the various pristine polymer and hybrid gel treatment solutions that were premixed with 90 µL of growth media and incubated at 37°C 5% CO₂ for 24h. DMSO (10%) and the untreated cells were used as positive and negative controls respectively. After 24h, 10 µL alamar blue reagent

was added to the wells and the plates were incubated at 37°C 5% CO₂ for four hours before the absorbance was measured using a microplate reader. The percentage viability was then calculated using the following equation:

$$\text{Percentage Viability} = \frac{\text{Average absorbance of treated wells-blank}}{\text{Average absorbance of untreated wells}} \times 100$$

Equation 5.1

5.2.2.1.3 Migration Assay

The wound healing potential of the formulations was investigated in vitro by conducting a scratch test using human keratinocyte cells (HaCat). The cells were propagated in Dulbecco's Modified Eagle Media (DMEM) with 10% Foetal Bovine Serum (FBS) and 1% Penicillin/Streptomycin in an incubator at 37°C and 5% CO₂. For the migration assay, cells were seeded in a 12 well plate at a density of 1x10⁵ cells/mL. The cells were incubated for 24 h after which a single scratch was created in the middle of each well using a 200 µL pipette tip. The wells were then washed twice with phosphate buffered saline to remove any cellular debris created during the scratch (AijazAyesha et al., 2015). The cells were then treated with the respective formulations: (1) MC/CHT/BGP+TFA solution, (2) MC/CHT/BGP solution, (3) MC/P/NC+TFA solution, and (4) MC/P/NC solution and incubated for a further 24 h. Images of the scratch were taken using an inverted light microscope (Olympus CKX53, Tokyo, Japan) at 0 h, and at 24 h after replacing the growth media with phosphate buffered saline. Images were analysed using the NIH ImageJ software and the percentage closure using the following equation:

$$\text{Percentage closure} = \frac{\text{Area of scratch at 24h}}{\text{Area of scratch at 0h}} \times 100$$

Equation 5.2

5.2.2.1.4 Statistical analysis of in vitro wound closure

One way analysis of variance (ANOVA) was used to determine statistical significance of the data acquired in the in vitro study. A Bonferroni test was also conducted to

determine statistical significance between various pairs of datasets. STATA 14 software was used for analysis, and a $p < 0.05$ was considered statistically significant.

5.2.2.2 Animal Studies

5.2.2.2.1 Preparation of chitosan-cellulose based formulation for in vivo analysis

Formulation 5MC-2CHT was prepared as detailed in section 3.2.2 of this dissertation. In addition, another batch was prepared with the incorporation of trans ferulic acid at a concentration of 5 $\mu\text{g/mL}$ (Truzzi et al., 2020), as described in section 4.2.2.6 of this dissertation. Truzzi and co-workers (2020) found that a concentration of 10 $\mu\text{g/mL}$ was too high and proved to be unfavourable in terms of cell migration and proliferation, however a concentration of 5 $\mu\text{g/mL}$ produced favourable results, and thus 5 $\mu\text{g/mL}$ was used in this study. The formulations were sterilized using UV light and allowed to rest overnight before use.

5.2.2.2.2 Animal Husbandry

Male Sprague–Dawley rats (250 g) Rats were housed individually in cages at room temperature ($\pm 25^\circ\text{C}$), maintained under a 12 h light: dark cycle. The animals were fed and given water ad libitum as per CAS protocol/SOP. Rats were allowed to acclimatize to the environment for a week prior to surgery. Food intake and body mass were measured every three days. Food intake was quantified by subtracting the food remaining in the container from the amount of food placed in the container, and the food pellet was left close to the rat to observe if it will feed on them. These animals were monitored according to prescribed CAS protocol. Animals were weighed every three days and the weight was used as an indicator of the general health of the animals, with the ruling that any animal exhibiting a loss of 15% or more of its expected body weight be removed from the study (note: none of the animals had to be removed from the current study as none of the animals displayed a 15% weight loss over the course of the study). All procedures were done in accordance with the Animal Ethics Control Committee of the University of the Witwatersrand's animal care regulations following approval by the Animal Ethics Screening Committee of the University of the Witwatersrand.

5.2.2.2.3. Study Design and methods

A total of 30 Sprague–Dawley rats (250 g) were used in this study. Ethics clearance was obtained from the Animal research Ethics Committee (Clearance number 2020/08/06/C). Rats were anesthetized with Anaketa 100 mg/ml and isoflurane 1-2% (morphine will be given for pain). The skin over the dorsal area was shaved, application fields were outlined with a marking pen just before skin excisions, and the surgical area was disinfected before surgery as per CAS aseptic techniques. A full thickness (3 mm thick) wound of 6 mm diameter was created on the dorsal area of the rat employing the skin punch biopsy apparatus. A total of two wound areas were created on the dorsal area of the rat, one on the right side and one on the left side. The wound diameter was measured for each rat and photographed. The rats were divided into a total of 3 groups of 10 rats each, based on the days when the rat's skin will be harvested for histological analyses. Rats were randomly assigned to each group. The first group (Group 1) of animals was only allowed to heal for 3 days followed by euthanasia and skin harvesting for histology analyses. Group 2 animals were allowed to heal for 7 days followed by euthanasia skin harvesting. The last group (Group 3 animals) was allowed to heal for 14 days before skin harvesting for histological analyses. Of these ten rats in each group, five were treated with a comparative formulation, burn shield hydrogel, on the left-hand side wound, and with a trans ferulic acid (TFA) loaded chitosan-methylcellulose thermogel on the right-hand side wound. While on the other five rats the left wound area was treated with simple gauze to serve as a control, and the right wound area was treated with a pristine (no TFA) chitosan-methylcellulose thermogel. The dressing (1 x 50 mg) was covered with a sterile compress secured with a bandage. Throughout the preparation and surgical procedure, the blood pressure and temperature were monitored using a BP monitor attached to the foot of the rats to ensure normality during experimentation.

5.2.2.2.4 In vivo wound closure

The wounds were examined after euthanasia and photographed to measure the reduction in wound size on days 3, 7, and 14. Image J software was then used to calculate the area of the wound at each time point, as well as the initial area of the wound using the images that were taken of each rat during the surgery. The

percentage wound closure was then calculated for each rat using the following equation:

Percentage wound closure=

$$\frac{\text{Final area of wound at the end of the wound healing period}}{\text{Initial area of the wound at day 0}} \times 100$$

Equation 5.3

5.2.2.2.5 Statistical analysis of in vivo wound closure

One way analysis of variance (ANOVA) was used to determine statistical significance of the data acquired in the in vivo study. A box and whisker plot was used to provide a visual summary of the data. A Bonferroni test was also conducted to determine statistical significance between various pairs of datasets. STATA 14 software was used for analysis, and a $p < 0.05$ was considered statistically significant.

5.2.2.2.6 Histopathological evaluation of tissue

After sufficient fixation of the collected specimens in 10% buffered formalin, the organs were cut according to our standard operating procedure (IdexxSA-AP-SOP-26) and processed according to routine histological tissue processing in an automated tissue processor with standard operating procedures IdexxSA-AP-SOP-27. Following tissue processing, sections were cut of 5-6 μm (IdexxSA- AP-SOP-30) and the slides produced were obtained in an automated Hematoxylin and Eosin tissue stainer (IdexxSA AP-SOP-205) before histological evaluation. Scoring was done according to 5 features as seen in the table below:

Table 5.1 Scoring system for histological analysis of wound healing

SCORING SYSTEM FOR HISTOLOGICAL ASSESSMENT OF WOUND HEALING		
Mononuclear leukocytes	0	No leukocytes evident
	1	Mild presence
	2	Moderate number of cells
	3	Prominent feature
Angiogenesis	0	Absent
	1	Mild in granulation tissue and/or subcutis
	2	Moderate in granulation tissue and/or subcutis
	3	Severe in granulation tissue and/or subcutis
Fibroblasts/granulation tissue	0	No evidence
	1	Mild presence, in specific locations
	2	Present in <50% of defect examined
	3	Present in >50% of defect examined
Epithelization	0	No evidence
	1	Epidermal thickening and cell migration at wound margins, less than 50% of lesion
	2	>50% of wound epithelialized
	3	Epithelialization complete

5.3. Results and Discussion

5.3.1 Cell Studies

5.3.1.1 In-vitro cytotoxicity analysis of thermogels

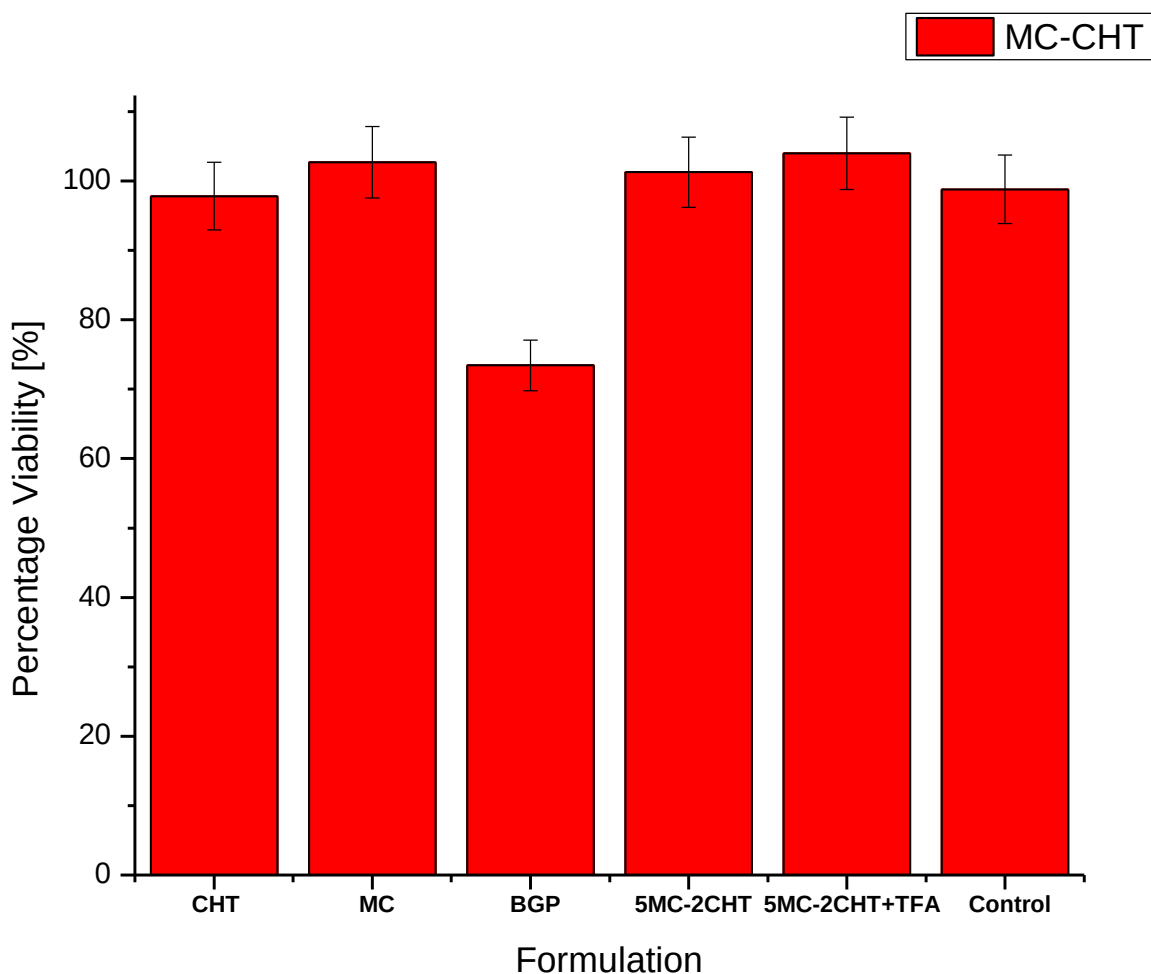


Figure 5.1: Graph depicting the cell viability of HaCat cells over a 24-h period when treated with various pristine polymers as well as hybrid cellulose-chitosan based polymer solutions. Where ‘CHT’ refers to pristine chitosan; ‘MC’ refers to pristine methylcellulose; ‘BGP’ refers to β -Glycerophosphate, ‘5MC-2CHT’ refers to the formulation formulated using an initial 5% concentration of methylcellulose and combined in a 1:2 ratio with chitosan, and ‘+TFA’ refers to the same with the addition of the bioactive trans ferulic acid.

While chitosan and methylcellulose are considered non-cytotoxic, β -glycerophosphate is cytotoxic as can be seen in the graph above, therefore, a very minimal amount was used in formulating the chitosan-cellulose thermogel. As can be seen, when combined with chitosan and hybridized with cellulose, the cytotoxicity of β -glycerophosphate was

neutralized, and the formulation showed a viability of 101% without the TFA, and 104% with TFA, compared to a viability of 98% in the control. Therefore, we can see that the chitosan-cellulose based gels are not only non-cytotoxic, but they promote cell growth as well.

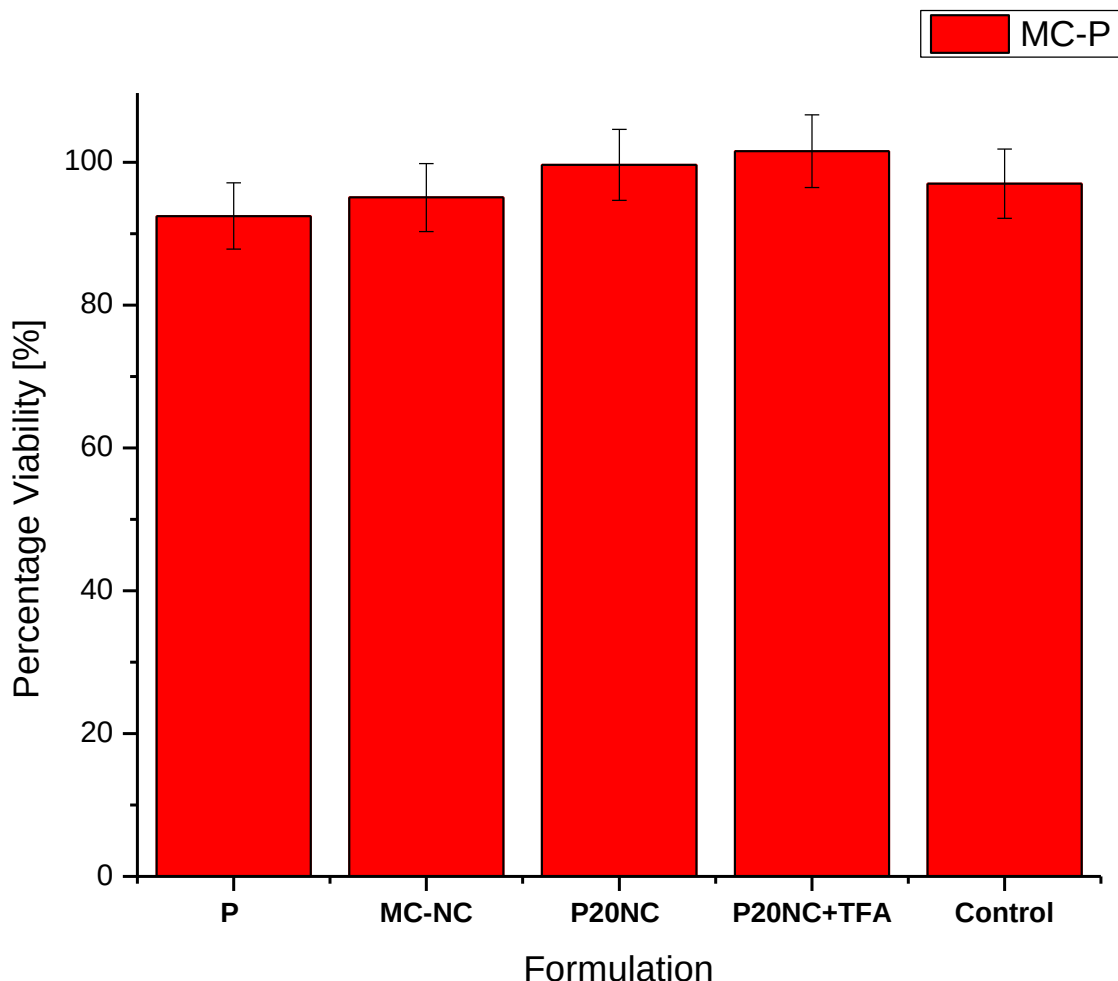


Figure 5.2: Graph depicting the cell viability of HaCat cells over a 24-h period when treated with various pristine polymers as well as hybrid cellulose-pluronic based polymer solutions. Where ‘P’ refers to pristine pluronic,; ‘MC-NC’ refers to the methylcellulose-nanocellulose blend; ‘P20NC’ refers to the hybrid of pluronic and the methylcellulose-nanocellulose blend in a 20:80 ratio, and ‘+TFA’ refers to the same with the incorporation of the bioactive trans ferulic acid.

The pluronic-cellulose based gels also proved to be non-cytotoxic, and promoting cell growth, with cell viability percentages of 99% in the gel without the addition of TFA, and 101% in the gel with TFA, compared to a 96% viability in the control. In both formulations, it is the cellulose component that seemed to have boosted the cell

viability, with pristine cellulose displaying a better percentage viability than either pristine chitosan or pristine pluronic. The stimulation of cell proliferation by cellulose plays a role in the acceleration of wound healing(Lin and Boehnke, 2009).

5.3.1.2 Migration Assay

The greatest percentage closure in the migration assay was seen in the chitosan-cellulose based gel, with almost comparable results between the pristine gel showing $82\pm10.98\%$ closure and the gel containing TFA showing an $83\pm8.64\%$ closure. The pluronic-cellulose based gels also fared well relative to the control, with the pristine pluronic-cellulose based gel showing a $79\pm7.91\%$ closure, and the gel containing TFA showing a $76\pm2.89\%$ closure, compared to the untreated control which showed only $56\pm20.34\%$ closure. Representative images of each formulation in the migration assay can be seen in figure 5.4.

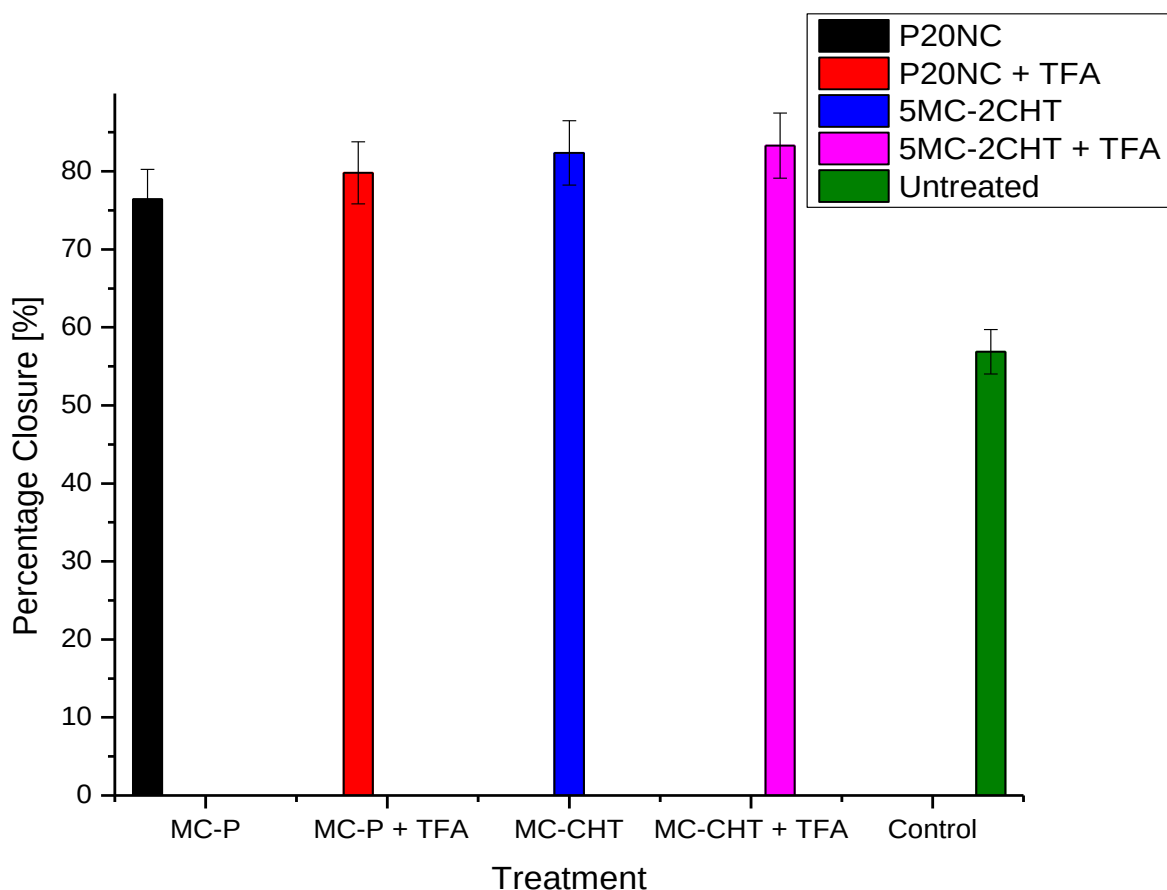
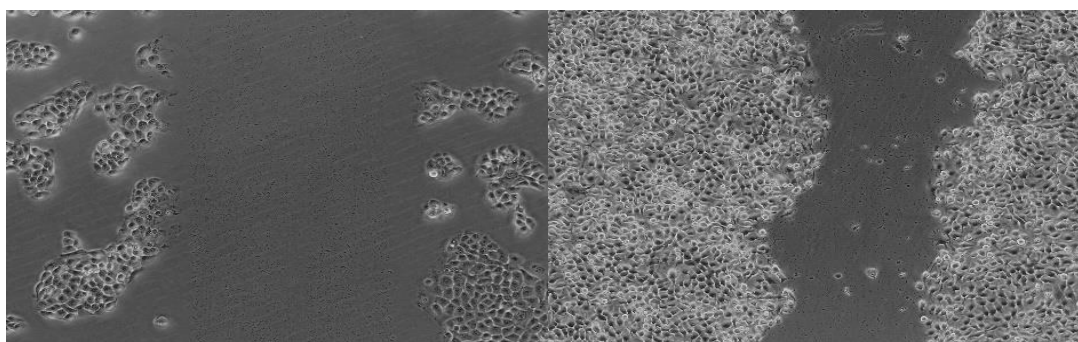
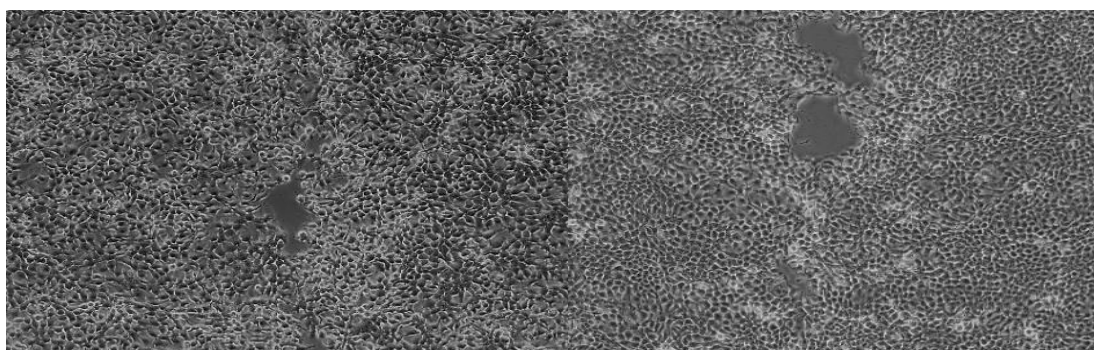


Figure 5.3: Graph depicting the percentage closure of a wound in a migration assay after treatment with hybrid chitosan-cellulose and pluronic-cellulose based systems.



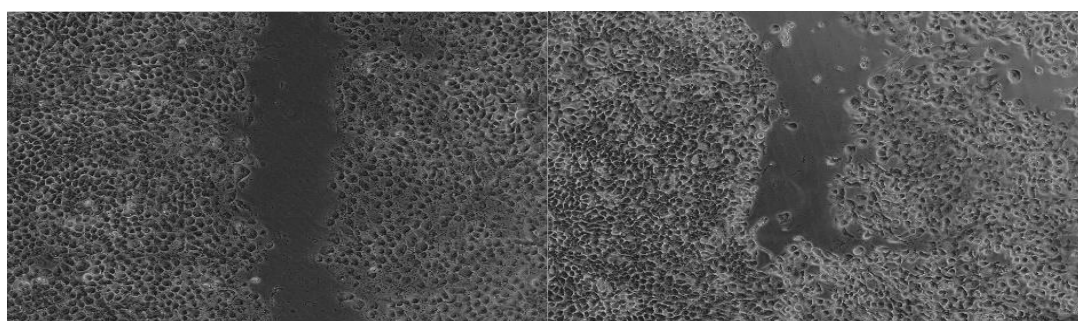
0H Untreated

24H Untreated Control



24H MC-CHT

24H MC-CHT + TFA



24H MC-P

24H MC-P + TFA

Figure 5.4: Migration of HaCat cells after treatment with chitosan-cellulose and pluronic-cellulose based formulations, as seen with an inverted light microscope at a magnification of 4x.

5.3.1.3 Statistical analysis of in vitro wound closure

A one-way analysis of variance depicted that the differences in the in vitro wound closure were highly significant ($p < 0.0001$). In addition, pair-wise comparisons of the data sets in a Bonferroni test suggested that all data sets were significant compared to the control ($p < 0.0001$), but the difference in the percentage closure between each

chitosan-cellulose based formulation and pluronic-cellulose based formulation respectively was not significant ($p=1.000$).

5.3.2 Animal Studies

5.3.2.1. In vivo wound closure

The greatest percentage closure at each time point was exhibited by the pristine chitosan-cellulose based gel treatment group, with a 61% closure on day three of healing, 88% closure on day seven of healing, and a 98% closure rate at the end of fourteen days. In comparison, the control group showed only a 23% closure on day three of healing, 53% closure on day seven, and an 85% closure rate on day fourteen of healing. Thus, it can be said the chitosan-cellulose based gel substantially increased the rate of healing. The chitosan-cellulose based gel containing TFA displayed the second fastest rate of healing with a 46% closure on day three, 85% closure on day seven, and 98% on day fourteen in keeping with the pristine chitosan-cellulose based gel. While the pristine chitosan-cellulose gel showed a much higher percentage closure on day three, indicating a faster rate of wound healing and suggesting inherent wound healing activity in the pristine chitosan-cellulose gel, the gel containing TFA showed a comparable percentage closure to the gel without the bioactive TFA at the end of the wound healing period, and the difference in the percentage wound closure between these two groups was statistically insignificant overall ($p=1.000$). The comparator fared the worst overall, with only 13% of closure after day three, and a 79% wound closure at the end of fourteen days.

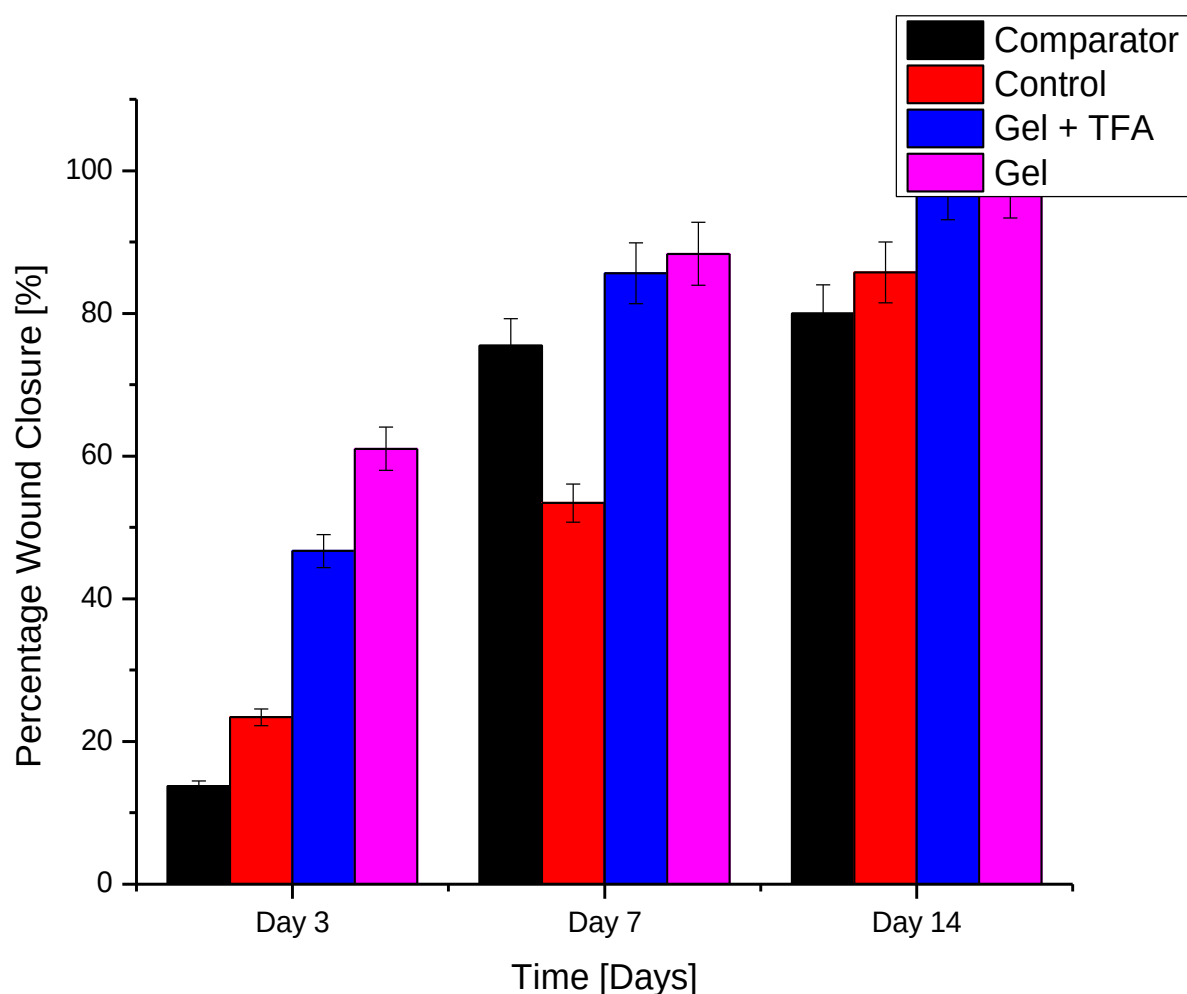


Figure 5.5: Graph depicting the percentage wound closure over time of wounds treated with a chitosan-cellulose based thermo-responsive gel, a comparator on the market, and a gauze control.

5.3.2.2 Statistical analysis of in vivo data

A one-way analysis of variance showed that the mean percentage wound closure differed significantly between the treatment groups ($p=0.0105$). The mean percentage closure (across all timepoints) for the various formulations were as follows: $82.56 \pm 18.79\%$ for the pristine chitosan-cellulose gel, $77.14 \pm 25.54\%$ for the chitosan-cellulose gel containing TFA, $56.40 \pm 32.68\%$ for the comparator, and $54.19 \pm 30.73\%$ for the control group. Pair-wise comparisons from a Bonferroni analysis showed a statistically significant difference in wound closure between the pristine chitosan-cellulose gel and the control treatment groups ($p=0.039$), with no statically significant difference noted between the pristine chitosan-cellulose gel and the gel + TFA ($p=1.000$).

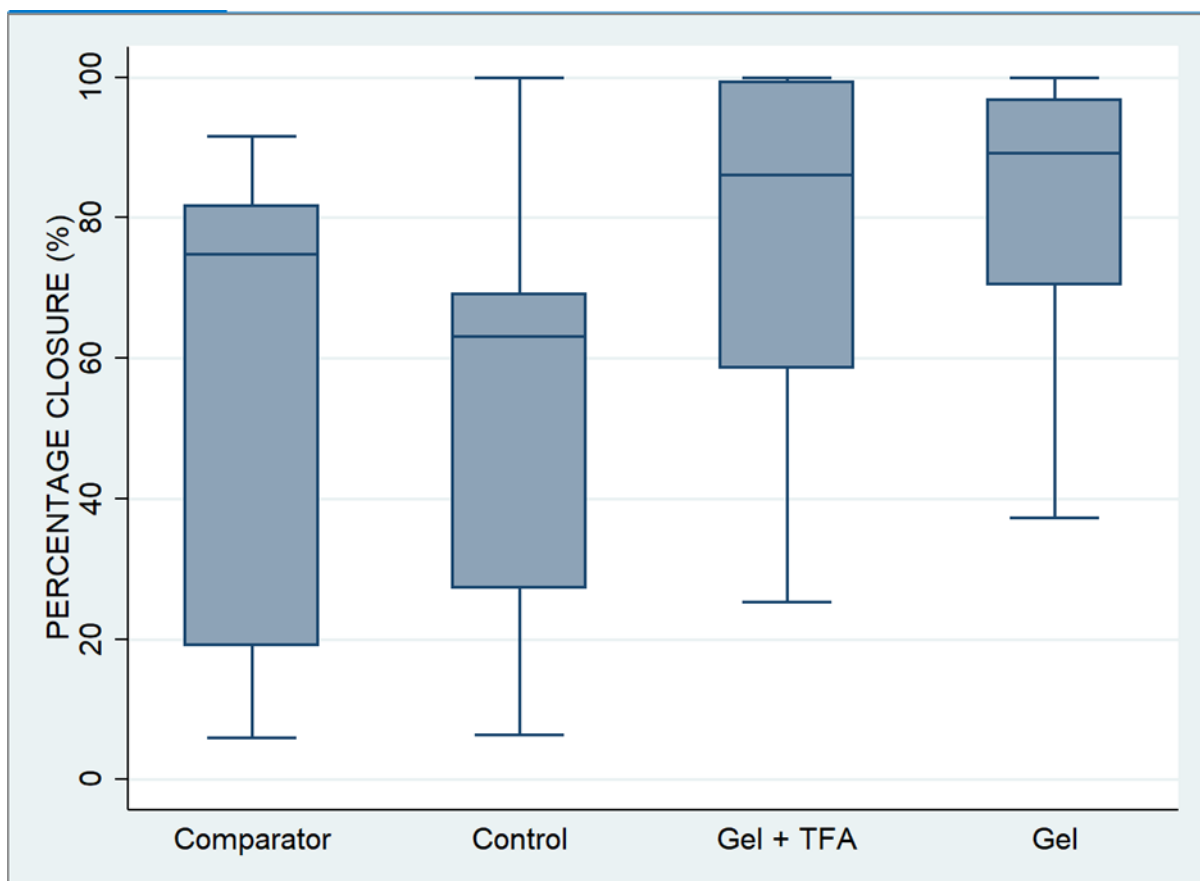


Figure 5.6: A box and whisker plot showing the distribution of the wound healing potential of various treatments.

In the box and whisker plot above, while all datasets are negatively skewed, the median values of the gel + TFA and the pristine gel datasets lie outside the boxes of the control and comparator datasets respectively, indicating that the chitosan-based gels showed superior performance to the control and comparator groups. The minimum percentage closure in the pristine gel plot is greater than the minimum closure in any other group, with the gel+TFA treatment group ranking second best. In addition, the range and interquartile range are smallest in the pristine gel group, signifying that the data is the least dispersed in this group and all wounds in this group have relatively high percentage closures.

5.3.2.3 Histopathological analysis of wounds

Table 5.2 Histopathological grading of wounds on day 3 of healing

Wound Treatment	Inflammation	Angiogenesis	Fibrosis	Epithelialisation
Medicated gel	2	0-1	1	0
Medicated gel	1	1-2	1	0
Medicated gel	2	2	2	1
Burn shield	2	2	2	0
Burn shield	1	1-2	1	0
Burn shield	1	0-1	0	0
Unmedicated gel	2	2	2	1
Unmedicated gel	1-2	1	2	1
Unmedicated gel	1-2	2-3	1-2	1
Control	2	2	2	0
Control	2	2-3	2	1
Control	2	2	1	1

Of note on day three of wound healing is that rats treated with the pristine chitosan-cellulose based gel showed the greatest progress in wound healing by being the only category in which all rats in the group already showed epithelialisation (Table 5.2), as can be seen in figure 5.7. This group also showed the greatest degree of fibrosis at this stage, with the fibroblastic cells being moderate in number and arranged in a slightly haphazard to somewhat liner pattern. Fibroblasts are essential in wound contraction as they provide the contractile forces, otherwise known as cell traction

forces, on the extracellular matrix, which bring the wound edges closer together (Harris, Stopak and Wild, 1981; Porter Sue, 2007). This explains why the greatest percentage of wound closure was seen in the pristine chitosan-cellulose based gel on day three of healing. While fibrosis and epithelialisation are not typically expected as early as day three, it can be said that the chitosan-cellulose based gel promotes wound healing thereby facilitating fibrosis and promoting epithelialisation from the very beginning. The process of epithelialization requires an optimal environment that is moist and has adequate nutrition and bacteriological control (Enoch and Leaper, 2008), therefore, it can be said that the presence of epithelialization indicates that the system provides an optimal environment for wound healing to occur. Moderate Inflammation and angiogenesis were also seen in this group of rats as expected on day three, and this was comparable to the control group and the group treated with the gel containing TFA.

Interestingly, while the chitosan-cellulose based gel containing trans ferulic acid showed a moderate inflammatory response in keeping with the control and the pristine chitosan-cellulose based gel, it showed a slightly lower degree of angiogenesis, fibrosis, and epithelialisation than the pristine gel, with no epithelialisation seen in 66.66% of rats in this treatment group on day three as can be seen in figure 5.8, however it still fared better than the comparator in all categories.

In contrast, the group of rats treated with the comparator, burn shield hydrogel, fared the worst from all the treatment groups with the lowest degree of inflammation, angiogenesis, and fibrosis on day three. Only mild infiltration of lymphocytic plasma cells and macrophages were seen in this group compared to a moderate presence in all other groups, including the control (Figure 5.10). In addition, no epithelialisation was seen in this any of the rats within this treatment group (Table 5.2) as can be seen in figure 5.9, suggesting that burn shield hydrogel did little to promote wound healing. Macroscopically however, not much difference can be seen in all treatment groups on day three, aside from the reduction in the size of the wounds (Figure 5.11).



Figure 5.7: Wound area of a rat treated with the pristine chitosan-cellulose based gel on day three of wound healing.

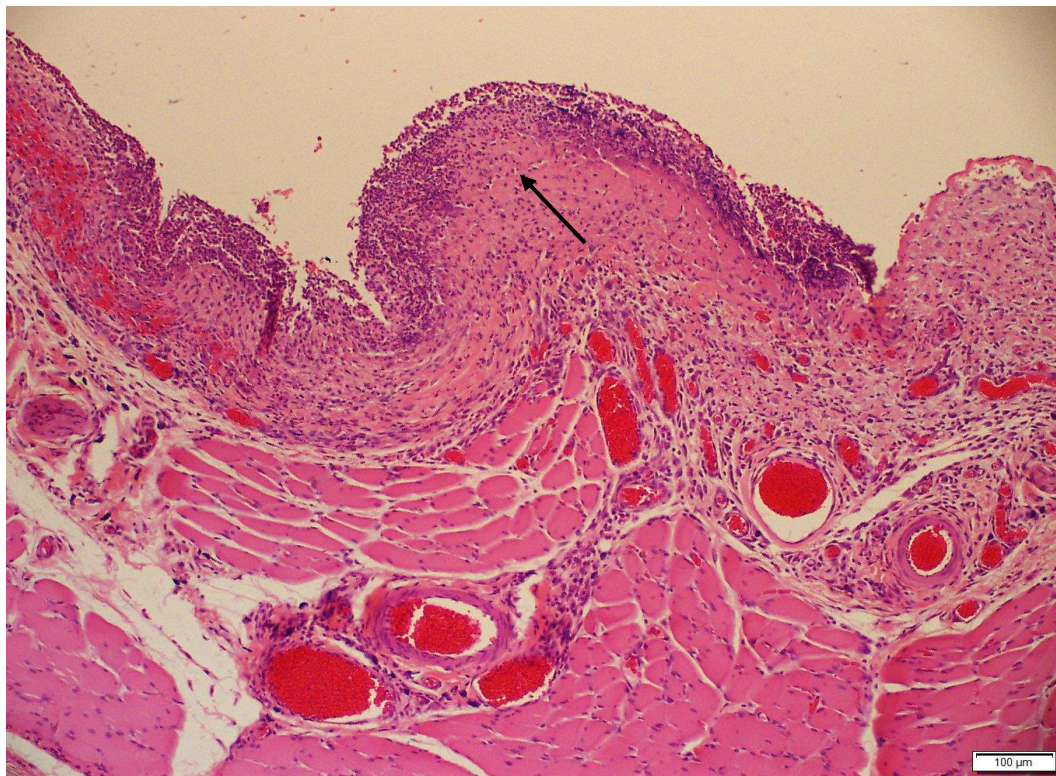


Figure 5.8: Wound area of a rat treated with the chitosan-cellulose based gel containing TFA on day three of wound healing.

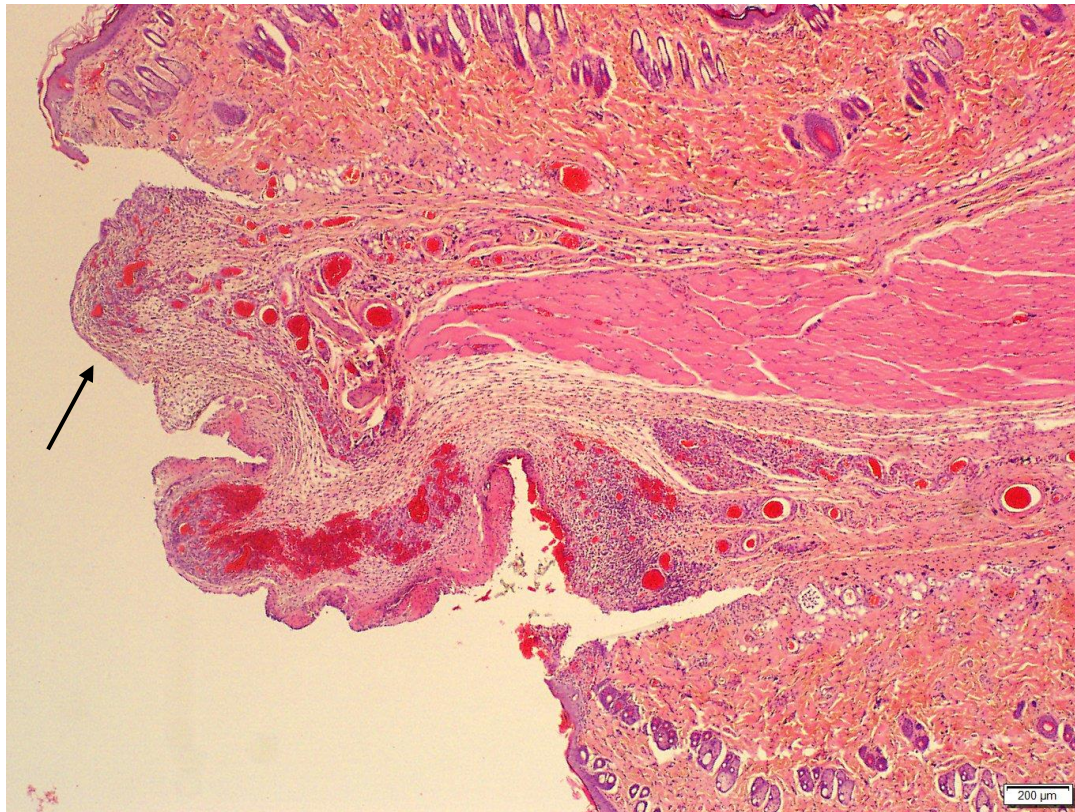


Figure 5.9: Wound area of a rat treated with the comparator, burn shield hydrogel, on day three of wound healing.

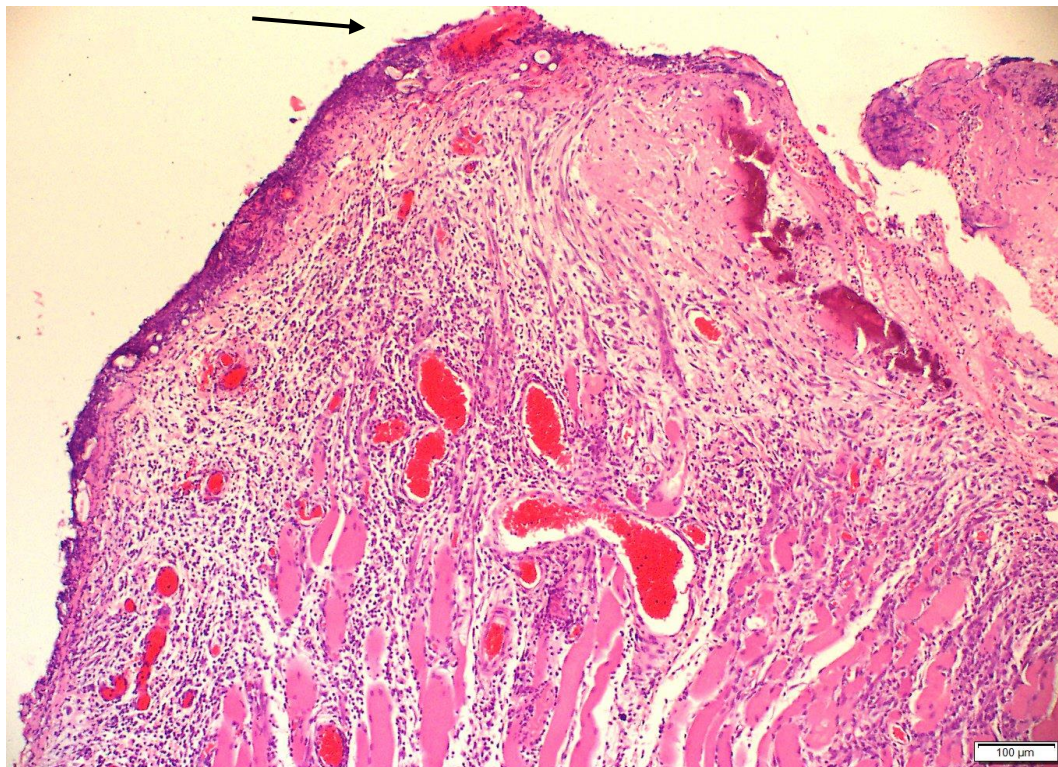


Figure 5.10: Wound area of a rat treated with gauze, serving as a control, on day three of wound healing.



Comparator

Gel + TFA



Control

Gel

Day 3

Figure 5.11: Wounds from various treatment groups after three days of healing.

Table 5.3 Histopathological grading of wounds on day 7 of healing

Wound Treatment	Inflammation	Angiogenesis	Fibrosis	Epithelialisation
Medicated gel	1	1	3	2
Medicated gel	2	1-2	3	3
Medicated gel	1	1-2	2-3	3
Burn shield	1-2	1-2	3	2
Burn shield	2	2	3	2
Burn shield	2	1	3	2
Unmedicated gel	1-2	1-2	3	1
Unmedicated gel	1	1-2	3	3
Unmedicated gel	0	0	1	3
Control	1	1-2	3	1
Control	2	1	3	3
Control	2	2	3	3

On day seven, the group of rats treated with the pristine chitosan-cellulose based gel was the only category with markedly reduced inflammation (Table 5.3) and angiogenesis compared to day three (Table 5.2), as expected. The hypothesis for this is that this group of rats had the greatest degree of epithelialisation on day three, resulting in the wound being covered and not exposed to irritants from the environment, thus reducing inflammation. This suggests that the rate of healing was significantly improved in this treatment group and the proliferative phase of healing had already begun at day three, thereby signalling the end of the inflammatory phase and resulting

in decreased inflammation seen at day seven (Bainbridge, 2013). Epithelialisation also increased in this treatment group from day three up to day seven as expected, with the defect being completely covered with epidermis and level with the surrounding skin. The epithelial layer was more differentiated in this treatment group on day 7 (Figure 5.12), than in all other treatment groups, including the chitosan-cellulose based gel containing TFA. Fibrosis was lower in this treatment group than any other treatment group as can be seen in figure 5.12 and table 5.3, with no granulation tissue and the presence of resident cells such as adipocytes, as well as the migration of pilosebaceous units from the dermis. This suggests that the healing process occurred by regeneration (indicated by the arrow in Figure 5.16) in this treatment group, as opposed to repair with the deposition of scar tissue. The group of rats treated with the chitosan-cellulose based gel containing TFA showed moderate epithelialisation at this stage, with the defect completely covered by the epidermis. A great degree of fibrosis was still seen in this treatment group however, with numerous collagen fibres interspersed between the fibroblasts, as seen in figure 5.13. Wounds in this treatment group showed healing with scab formation on day seven of wound healing, as can be seen in figure 5.16.

Only a slight decrease was seen in inflammation in the control group as well as in the group of rats treated with the chitosan-cellulose based gel containing TFA, and an increase in inflammation was seen in the group of rats treated with burn shield (Table 5.3), contrary to what would be expected on day seven of wound healing, as seen in figure 5.14. While fibrosis and epithelialisation increased across all treatment groups from day three up to day seven, the degree of epithelialisation was the lowest in the group of rats treated with the comparator, with all other groups showing complete epithelialisation in at least 66.66% of rats at this stage, and not a single rat showing complete epithelialisation in the comparator group (Table 5.3). Fibrinopurulent exudate was also still seen in the burn shield treatment group on day seven. In contrast, while incomplete epithelialisation was still seen in some rats in the control group at this time point, as can be seen in figure 5.15, a decrease in the fibrinopurulent exudate was seen even in those rats with incomplete epithelialisation in the control group.

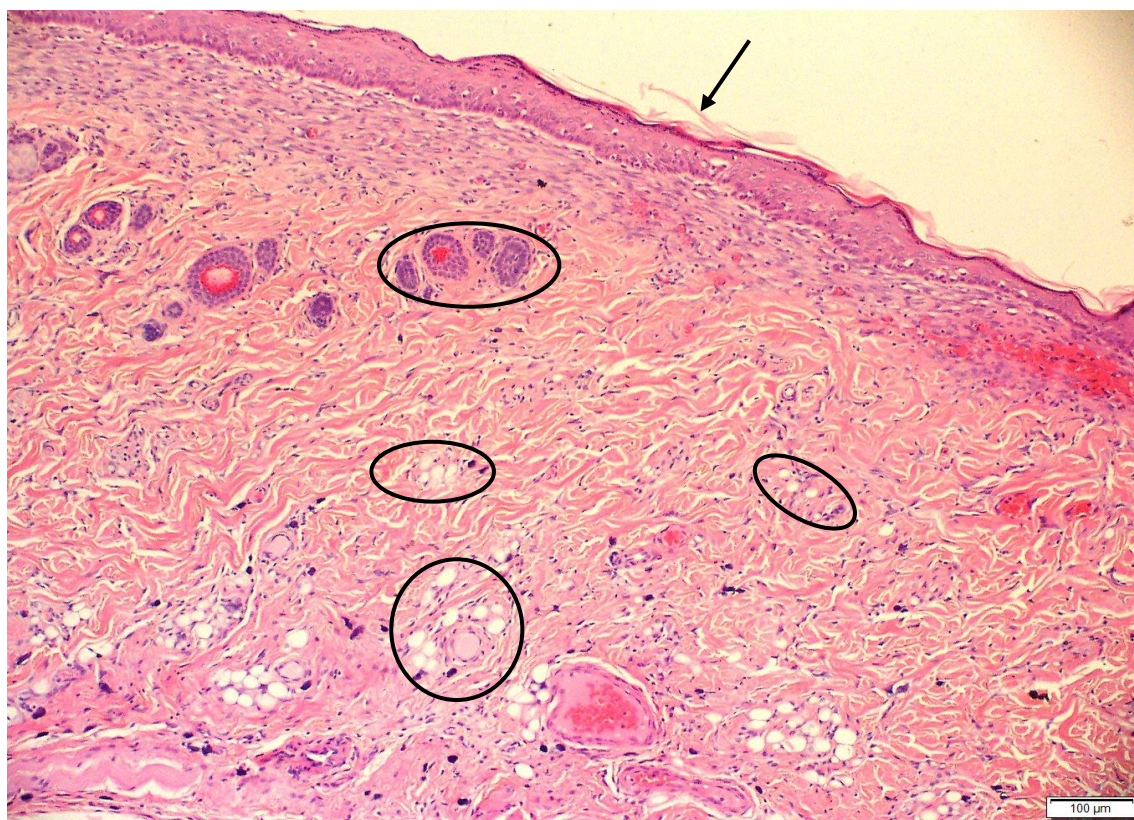


Figure 5.12: Wound area of a rat treated with the pristine chitosan-cellulose based gel on day seven of wound healing.

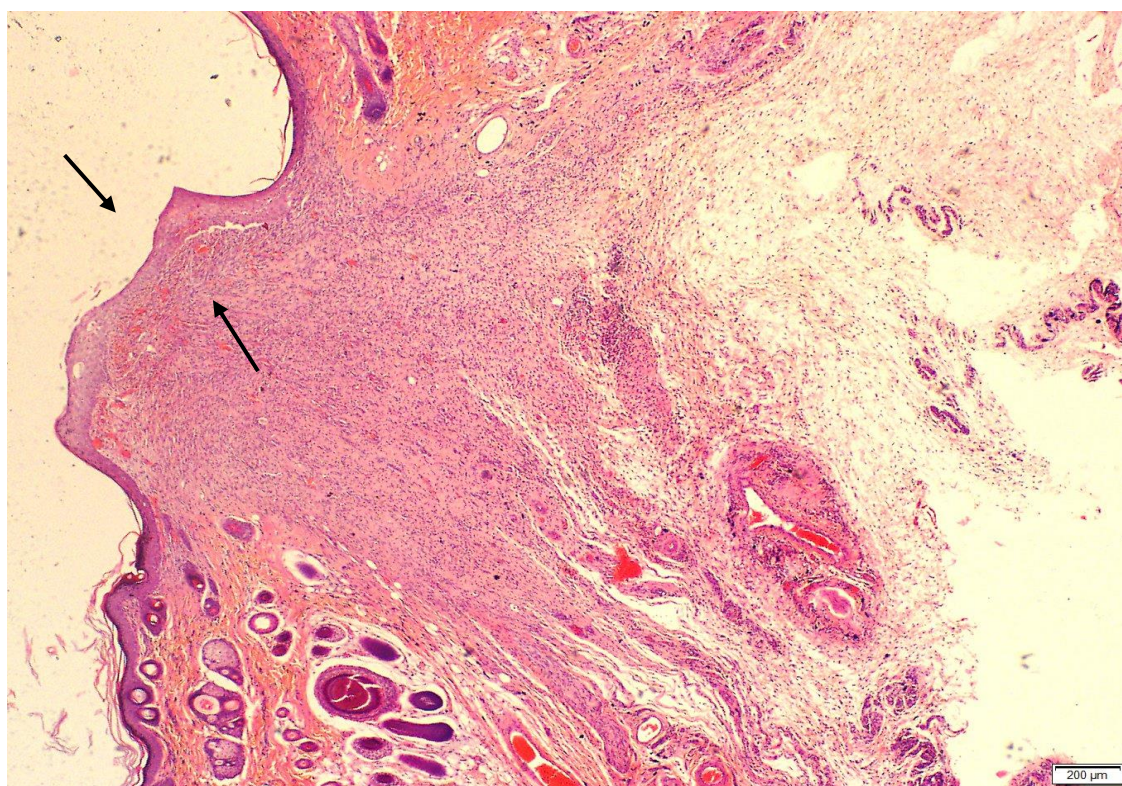


Figure 5.13: Wound area of a rat treated with the chitosan-cellulose based gel containing TFA on day seven of wound healing.

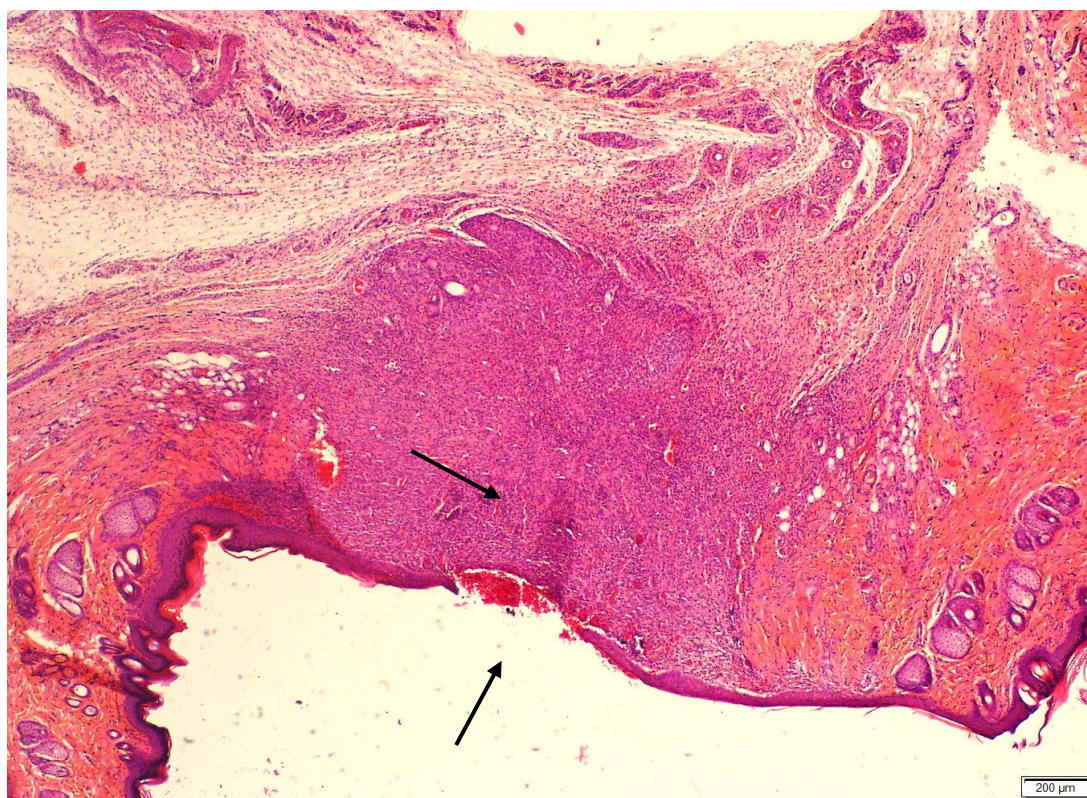


Figure 5.14: Wound area of a rat treated with the comparator, burn shield hydrogel, on day seven of wound healing.

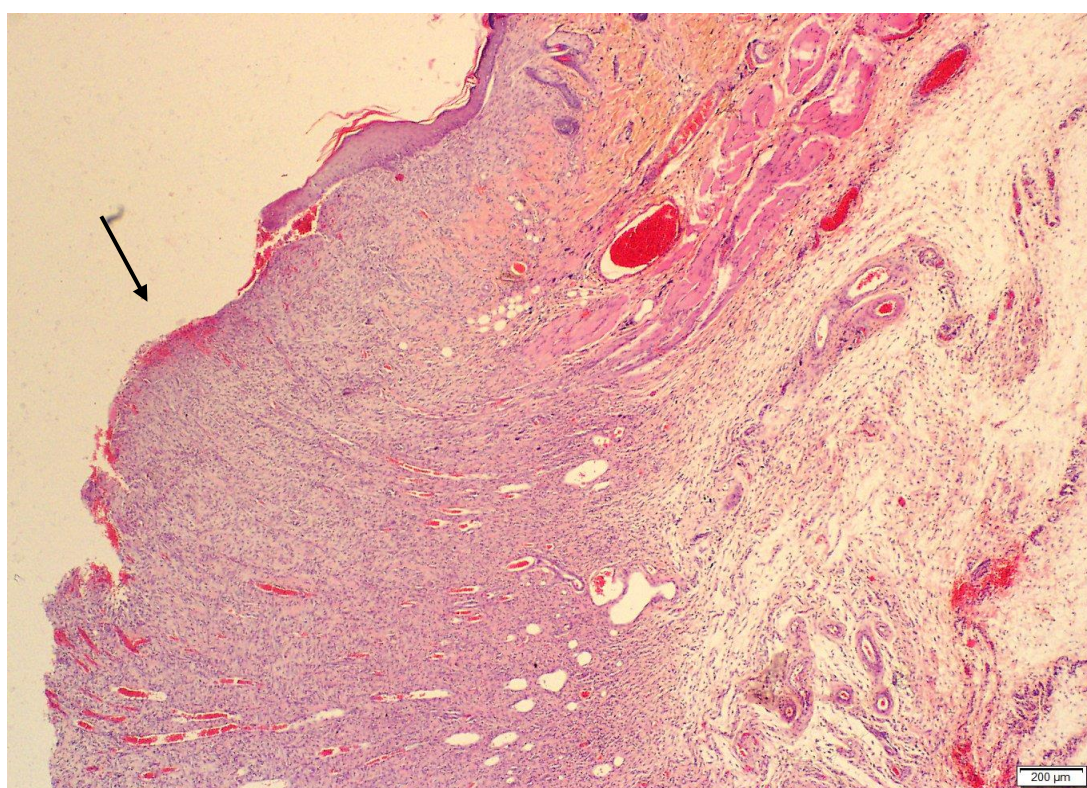


Figure 5.15: Wound area of a rat treated with gauze, serving as a control, on day seven of wound healing.



Comparator

Gel + TFA



Control

Gel

Day 7

Figure 5.16: Wounds after seven days of exposure to the various treatments.

Table 5.4 Histopathological grading of wounds on day 14 of healing

Wound Treatment	Fibrosis	Epithelialisation
Medicated gel	3	3
Medicated gel	3	3
Medicated gel	3	3
Burn shield	3	1
Burn shield	2	3
Burn shield	3	3
Unmedicated gel	2	3
Unmedicated gel	0	3-Normal epidermis
Unmedicated gel	2	3
Control	2	1
Control	1	3
Control	3	3

*Only fibrosis and epithelialisation are shown at day 14 as these are the most significant categories at this time point, given the wound healing process.

By day 14, the group of rats treated with the pristine chitosan-cellulose based gel showed complete wound healing with a notable normal epidermis displayed (Figure

5.17; Table 5.4). Hair follicles together with pilosebaceous glands can clearly be seen at this point, together with a thick mature epidermis. Fibrosis had also decreased from day seven (Table 5.3) to day 14 (Table 5.4) in this treatment group, indicating that healing occurred by tissue regeneration as opposed to mere tissue repair with the deposition of scar tissue and the formation of scabs at the wound site. Fibrosis requires a delicate balance in the wound healing cascade, as without fibroblast activity, there will be too little granulation tissue and the wound will not heal (Gabbiani et al., 1956), however excess fibroblastic activity and fibrosis can result in hypertrophic scar tissue, and the formation of keloids (McAnulty, 2007). Fibroblasts generally decrease in number around day 14, towards the end of the wound healing process, when the tensile strength of the newly formed extracellular matrix is similar to that of surrounding healthy tissue (Hinz et al., 2007). The group of rats treated with the chitosan-cellulose based gel containing TFA showed the second highest degree of epithelialisation at the end of the wound healing period (Table 5.4), with the epidermis completely filling the entire defect and the presence of mature granulation tissue that is horizontally arranged and regularly interspersed by hair follicles (Figure 5.18). While fibrosis had not decreased in this treatment group, it still showed the highest degree of wound healing with mature granulation tissue and the least amount of scarring, second only to the pristine chitosan-cellulose based gel (Figure 5.21).

In contrast, in both the control group and the comparator groups, not all rats showed complete epithelialisation (Table 5.3; Figure 5.19; Figure 5.20), even at day 14, with granulation tissue more haphazardly arranged in the wounds associated with these treatment groups. Mild inflammation and angiogenesis can still be seen in the comparator treatment group even at day 14, while none can be seen in the control group, as expected at this time point. Pilosebaceous units can be seen in the control at day fourteen, and none can be seen in the comparator treatment group, which could suggest that the control healed even better than the comparator.

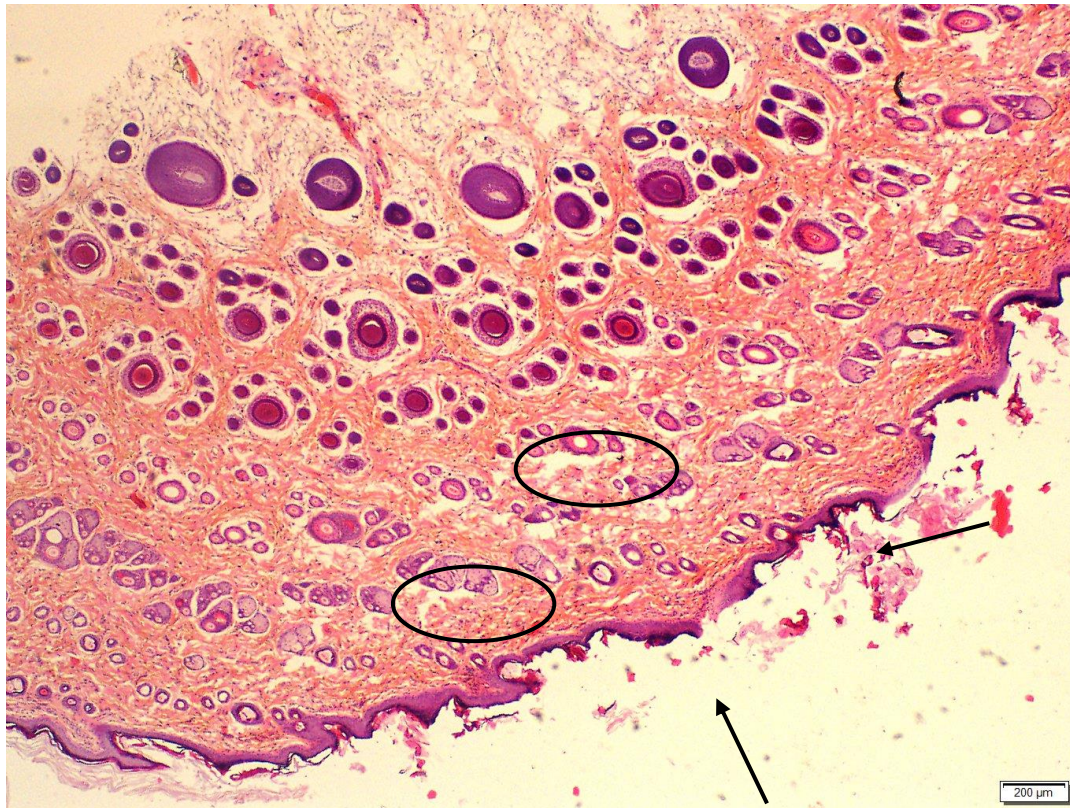


Figure 5.17: Wound area of a rat treated with the pristine chitosan-cellulose based gel on day fourteen of wound healing.

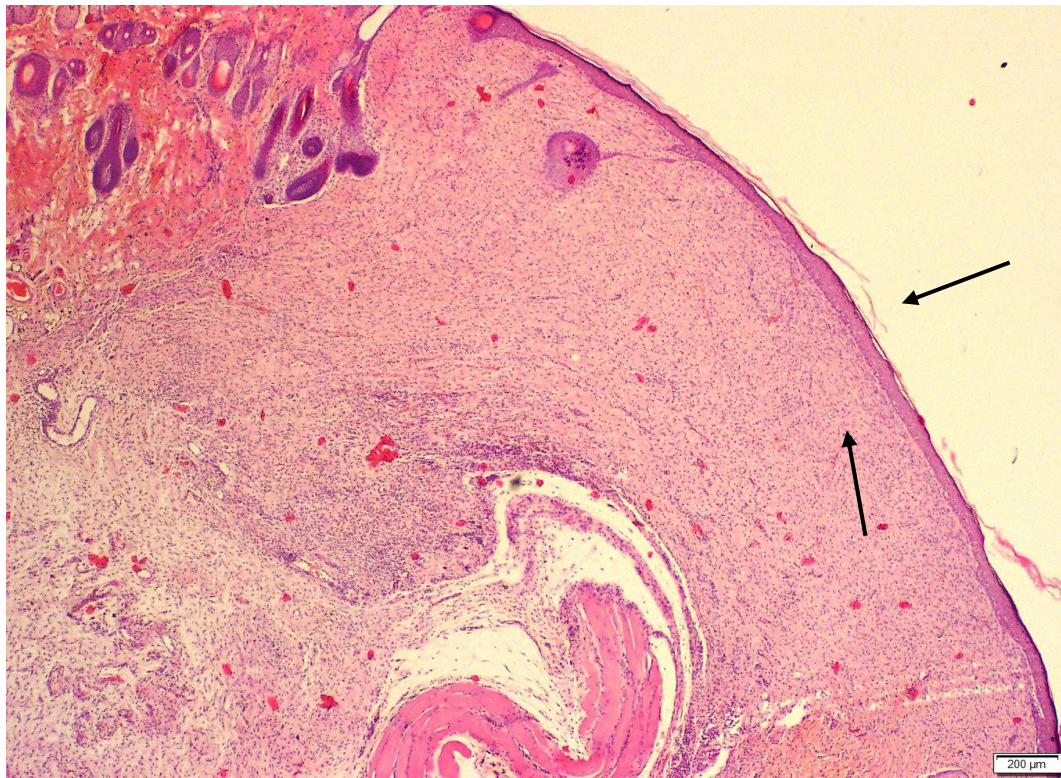


Figure 5.18: Wound area of a rat treated with the chitosan-cellulose based gel containing TFA on day fourteen of wound healing.



Figure 5.19: Wound area of a rat treated with the comparator, burn shield hydrogel, on day fourteen of wound healing.

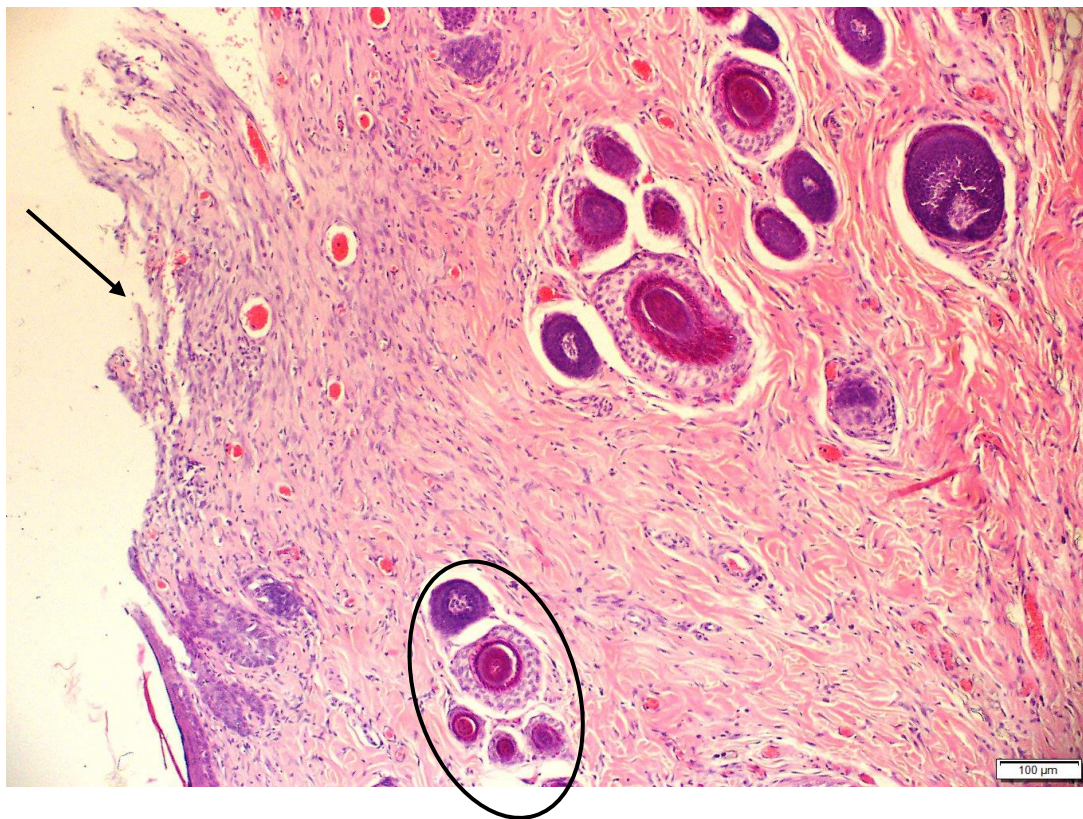
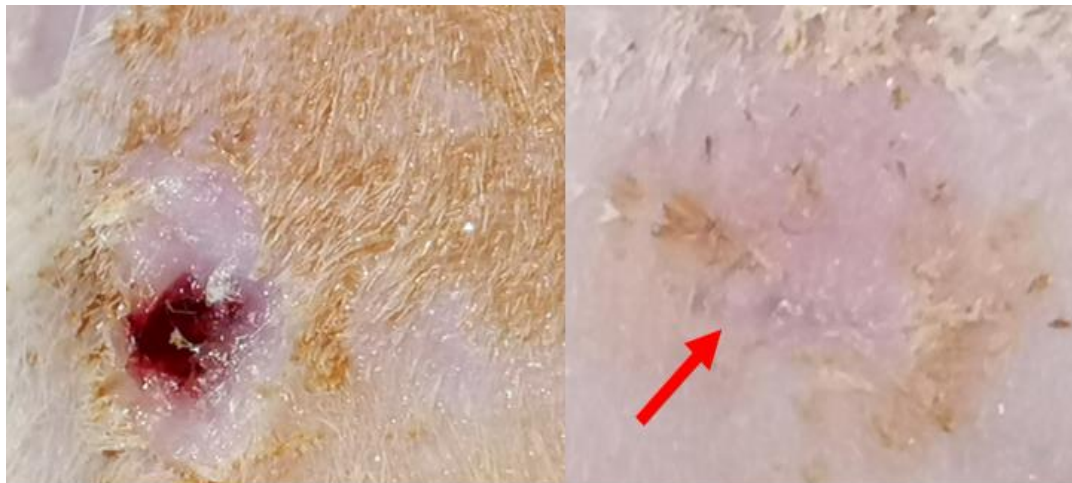


Figure 5.20: Wound area of a rat treated with gauze, serving as a control, on day three of wound healing.



Comparator

Gel + TFA



Control

Gel

Day 14

Figure 5.21: Wounds after fourteen days of healing with the various treatments.

5.4 Concluding remarks

In conclusion, none of the treatments altered the normal phases of wound healing as wound healing progressed as expected at each time point. However, the rate and degree to which the wounds healed differed with each treatment group, as can be seen in the histology images and wound closure figures at each time point. Both the fastest

rate of wound healing and the greatest degree of wound healing with tissue regeneration can be seen in the group of rats treated with the pristine chitosan-cellulose based gel, with the second highest degree of wound healing and maturation of granulation tissue seen in the group of rats treated with the chitosan-cellulose based gel containing TFA. Both chitosan-cellulose based treatment groups showed favourable results and fared better in terms of the rate and degree of wound healing than the comparator and the control groups. Therefore, the chitosan-cellulose based thermogel shows great potential for application in wound healing, with or without the incorporation of a bioactive. Thus, the aim of this research has been achieved, as the system created an optimal environment for the wound healing process to occur and the chitosan-cellulose polymer system showed inherent wound healing activity.

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6.1 Concluding remarks

Both hybrid systems were able to form a stiff gel at physiological temperature, while remaining in a sol state at ambient temperature. The relatively low viscosity and elastic modulus of the chitosan-cellulose formulation at ambient temperature allows for ease of application via a syringe into the area, without having to worry about the formulation forming a gel during the time that it takes to apply the system to the affected area. Both hybrid systems displayed good thermal stability and were bio-erodible, however there were notable differences in their morphology, fluid uptake and release kinetics. The chitosan-cellulose system reached completion in terms of crosslinking with β -glycerophosphate acting as a crosslinking agent to induce thermal gelation. This resulted in the system displaying primarily erosion behaviour as opposed to swelling behaviour and resulted in a quicker release of the bioactive from the system. Contrarily, the pluronic-cellulose system was crosslinked to a lesser degree and thus initially displayed swelling behaviour over erosion behaviour, which in turn retarded the release of the bioactive from the system, allowing for release over 24 h.

Both hybrid systems proved to be non-toxic on the HaCat cell line, with greater percentage viabilities than their respective controls, thereby suggesting that they in fact stimulate cell growth. While the chitosan-cellulose polymer system was more suitable for the current application, hybridization of cellulose with pluronic could be employed in the formulation of a suitable system for other applications. The chitosan-cellulose hybrid system displayed superior results in in-vitro analysis of its wound healing potential and was thus selected for further analysis in-vivo.

Interestingly, the chitosan-cellulose hybrid polymer system performed remarkably well in vivo, both with and without the incorporation of trans ferulic acid. The chitosan-cellulose system sans the incorporation of trans ferulic acid exhibited the fastest rate and greatest progress in terms of wound healing at each time point, and it was found to promote healing with tissue regeneration (as opposed to merely healing by repair

with the formation of scar tissue), with a notable normal epidermis being formed at the end of the wound healing period. This positive outcome will have a tremendous impact in the management of wounds, resulting in quicker healing times and an aesthetically pleasing wound which will improve the patient's quality of life, should the system ever reach the stage of commercialization. Thus, it can be concluded that the polymer system is inherently bioactive and plays an active role in facilitating and promoting the wound healing process. Commercially, the fact that the polymer system displays inherent wound healing activity and a bioactive does not necessarily have to be incorporated is a favourable prospect, allowing for a simpler and more cost-effective manufacturing process.

6.2 Limitations of the study and recommendations

Among the limitations of the study is that the formulation was only tested in one animal model, i.e. a small rat model. Further studies could be undertaken in a larger animal model such as a pig to determine the behaviour of the pristine chitosan-cellulose gel as well as the gel containing TFA. While both chitosan-cellulose formulations performed better than the control and the comparator in the current study, they performed comparably well to one another, rendering the incorporation of trans ferulic acid unnecessary. It would be beneficial to ascertain if this translated to a larger animal model or if the pharmacokinetics and pharmacodynamics of the trans ferulic acid differed in a larger model.

In addition, only one concentration of TFA was tested in-vivo, i.e. 5 µg/mL. Further studies to evaluate different concentrations such as 7.5 µg/mL or 10 µg/mL would be beneficial to determine whether greater concentrations of trans ferulic acid would have a more marked effect on the wound healing process as compared to the formulation sans TFA.

Due to ethical and budgetary constraints, only one of the hybrid formulations developed was selected for in-vivo analysis during this study. While the chitosan-cellulose based formulation proved to be the more suitable choice of the two for numerous reasons mentioned throughout the dissertation, the pluronic-cellulose based

formulation displayed satisfactory results in terms of in-vitro analysis, and it would be interesting to see its performance in-vivo.

6.3 Future Outlook

The novel hybrid thermo-responsive polymer system opens a plethora of opportunities for future innovation in the development of drug delivery devices. The possibilities of creating targeted drug delivery systems with tailored specifications are endless with the use of stimuli responsive polymers. Thermo-responsive polymers such as cellulose, pluronic and chitosan are ideal candidates due to their favourable properties in terms of providing the system with strength, tuneable gelation characteristics, their ease of availability and cost effectiveness. By hybridizing stimuli-responsive polymers, the rheological, physico-mechanical and chemical properties of the formulation can be altered to suit the needs of any system thereby allowing these systems to be employed in a wide variety of applications. The release kinetics of the system can also be altered, making the use of stimuli responsive systems ideal for in-situ depot formulations as well, especially when natural and biocompatible thermo-responsive polymers, such as cellulose, are incorporated. By altering the gelation temperatures, the use of thermo-responsive polymer systems in cryotherapy for the treatment of wounds could also be explored. In addition, while the polymers reviewed within this chapter largely display LCST behavior, further research into polymers that display UCST, and consequently the hybridization and exploitation of these polymers, is needed.

The inception of layer by layer dually or multi stimuli responsive polymer systems via the hybridization of polymers that are responsive to different stimuli is an intriguing prospect for future research and will undoubtedly pave the way for the development of innovative hybrid polymer systems for a broad spectrum of applications. While the scope of this dissertation entailed thermo-responsive polymers and their applications in the biomedical field, many other stimuli responsive polymers exist, with a plethora of applications that have proven to be beneficial in a variety of other fields such as environmental remediation, oil adsorption, marine and textile applications (Li et al., 2018; Wang et al., 2018; Chatterjee and Chi-leung Hui, 2019; Tian et al., 2019; Gu et al., 2020).

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Hybrid Thermo-Responsive Polymer Systems and Their Biomedical Applications

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OPEN ACCESS

Edited by:

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University of Parma, Italy

Reviewed by:

Xufeng Dong,
Dalian University of Technology, China
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University of Tennessee, Knoxville,
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Specialty section:

This article was submitted to
Smart Materials,
a section of the journal
Frontiers in Materials

Received: 25 November 2019

Accepted: 10 March 2020

Published: 31 March 2020

Citation:

Sarwan T, Kumar P, Choonara YE
and Pillay V (2020) Hybrid
Thermo-Responsive Polymer
Systems and Their Biomedical
Applications. *Front. Mater.* 7:73.
doi: 10.3389/fmats.2020.00073

Targeted and controlled drug delivery employing “smart materials” is a widely investigated field, within which stimuli-responsive polymers, particularly those which are thermo-responsive, have received considerable attention. Thermo-responsive polymers have facilitated the formulation of *in situ* gel forming systems which undergo a sol-gel transition at physiological body temperature, and have revolutionized the fields of tissue engineering, cell encapsulation, and controlled, sustained delivery of both drugs and genes. However, the use of single thermo-responsive polymers in the creation of these systems has posed numerous problems in terms of physico-mechanical properties, such as poor mechanical strength, high critical gelation concentrations (CGC) resulting in increased production costs and solutions that are too viscous, toxicity, as well as gelation temperatures that are incompatible with physiological body temperatures. Hybridization of these thermo-responsive polymers with other polymers has therefore been employed, resulting in the creation of tailor-made drug delivery systems that have optimal gelation temperatures and concentrations, ideal viscosities and improved gel strengths. This article reviews various thermo-responsive polymers that have been employed in the formulation of thermo-gelling systems. Special attention has been given to the hybridization of each of these polymers, the resulting systems that have been created, and their biomedical applications.

Keywords: thermo-responsive polymers, hybrid, chitosan- β -glycerophosphate, pNIPAAm, pluronic, cellulose, PEG-PCL

INTRODUCTION

Stimuli responsive drug delivery systems have been widely investigated due to their role in facilitating targeted and controlled drug delivery (Thambi and Lee, 2019). Among the stimuli responsive systems, thermo-responsive drug delivery systems have received considerable attention, and will thus be the focal point of this article (Mura et al., 2013; Huang et al., 2019). Thermogels are gels that undergo an *in situ* sol-gel transition by virtue of the difference in their ambient storage temperature and the physiological temperature of the body (Kumar et al., 2018; Figure 1). Ideally, thermogels should be free flowing solutions at room temperature, and only form gels once administered into the body (Ruel-Gariépy and Leroux, 2004). This thermo-responsive behavior is attributed to a change in the hydrophilic and hydrophobic interactions among the polymer molecules and water molecules that make up the solution (Bajpai et al., 2008; Shah et al., 2018).



In-situ thermo-gelling systems for the treatment of wounds

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Wits Advanced Drug Delivery Platform

17 November 2020

Global Young Academy Young Scientists Ambassadors Programme

South Africa-Italy 2020

Abstract

Stimuli responsive polymer systems have received considerable attention in recent years to their tuneable properties and ability to act “intelligently” in response to certain stimuli. Thermo-responsive polymers in particular have enabled the formulation of in-situ sol gel systems that act as a solution at ambient temperature but are able to form a gel once exposed to physiological temperature. These systems are ideal for application in wounds due to their ease of application and their ability to completely fill the wound defect and ensure even distribution of incorporated bio-actives. However, there are numerous shortfalls when creating a system composed of only a single thermo-responsive polymers. Hybridization of these polymers has therefore allowed for the creation of thermo-responsive polymer systems with tailored rheological, chemical and mechanical properties. This presentation aims to highlight the hybridization of a natural thermo-responsive polymer, Cellulose, with a synthetic thermo-responsive polymer Pluronic F127, and a semi-synthetic thermo-responsive polymer, Chitosan- β -glycerophosphate, respectively.

In-situ thermo-gelling systems for the treatment of wounds



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BRICS STI Multilateral Project. 2021



4 November 2021



Abstract



The burden of wounds is a global one, with the incidence steadily increasing. Adequate wound care is imperative to mitigate further complications that threaten the potential loss of limbs or the development of systemic infection. In recent years, the aim of wound care has shifted from merely covering the wound and preventing infection, to actively encouraging the wound healing process. This has been achieved by moving away from the sole use of traditional dressings such as medicated gauze, to combining these with formulations such as hydrogels which create an optimal environment for the wound healing process to occur by controlling the moisture of the wound, enabling gaseous exchange and enhancing proliferation of cells and angiogenesis. Further developments in the pharmaceutical field have introduced in-situ gel forming systems, formulated with the use of stimuli responsive polymers. This presentation details the development of a hybrid thermo-responsive polymer system with inherent wound healing activity.



4 November 2021



ANIMALS RESEARCH ETHICS COMMITTEE (AREC)



STRICTLY CONFIDENTIAL

CLEARANCE CERTIFICATE NUMBER: 2020/08/06/C

APPLICANT: Ms T Sarwan

School: Department: Pharmacy and Pharmacology; Location: CAS

PROJECT TITLE: Observing the effects of an injectable thermo-responsive polymer system on Sprague Dawley rat model with surface cut wound.

Category: C; Species and Numbers involved: 30X 6-8 weeks old, male Sprague Dawley Rats

Approval is hereby given for the use of animals for the research project named above and described in the application reviewed by a quorate meeting of the AREC held on 25 Aug 2020. This approval remains valid until 1 Nov 2022 and is conditional to the following (if blank there are no special conditions):

Condition 1	Condition 2	Condition 3	Condition 4

All material changes to the approved research must be reported to the AREC before they are implemented. Failure to do so will invalidate this clearance certificate.

An annual progress report must be provided to the AREC.

The use of these animals is subject to AREC guidelines on the use and care of laboratory animals, is limited to the procedures described in the application and is subject to additional conditions listed below:

I, the Chair of the AREC (or my designated representative) am satisfied that the proposed research is ethical as judged by local law, international standards and University policy.

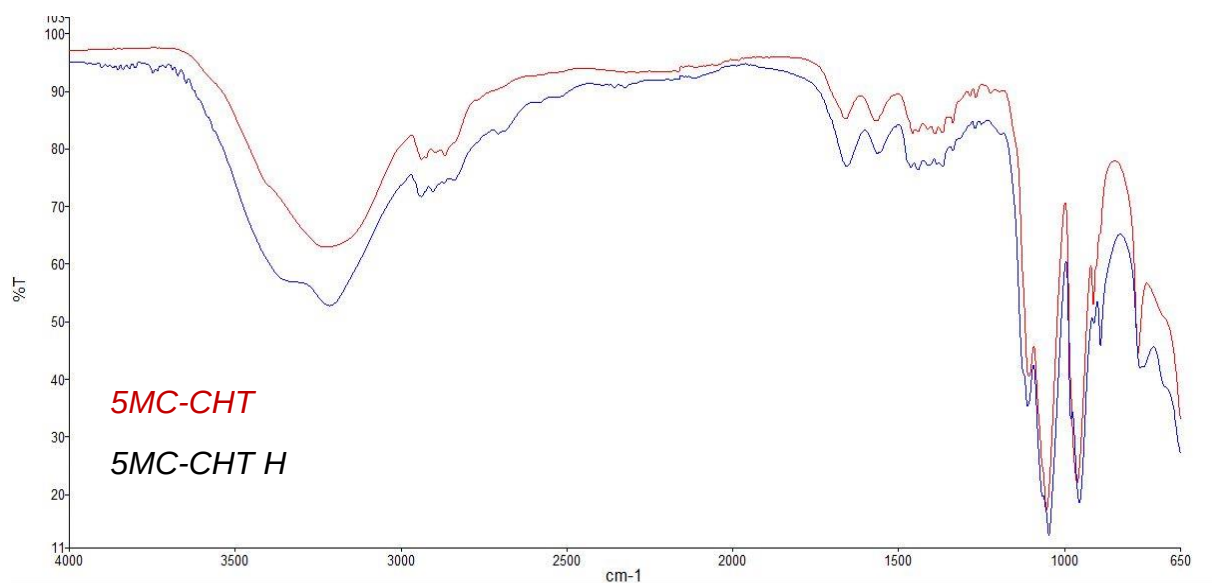
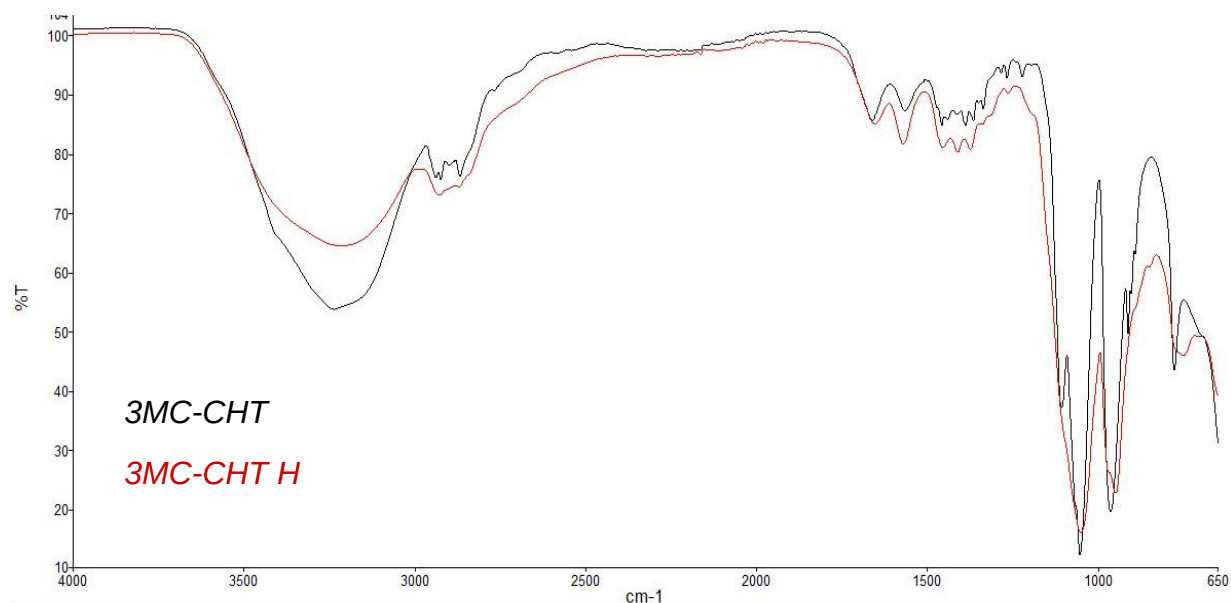
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(Chairperson of the AREC)

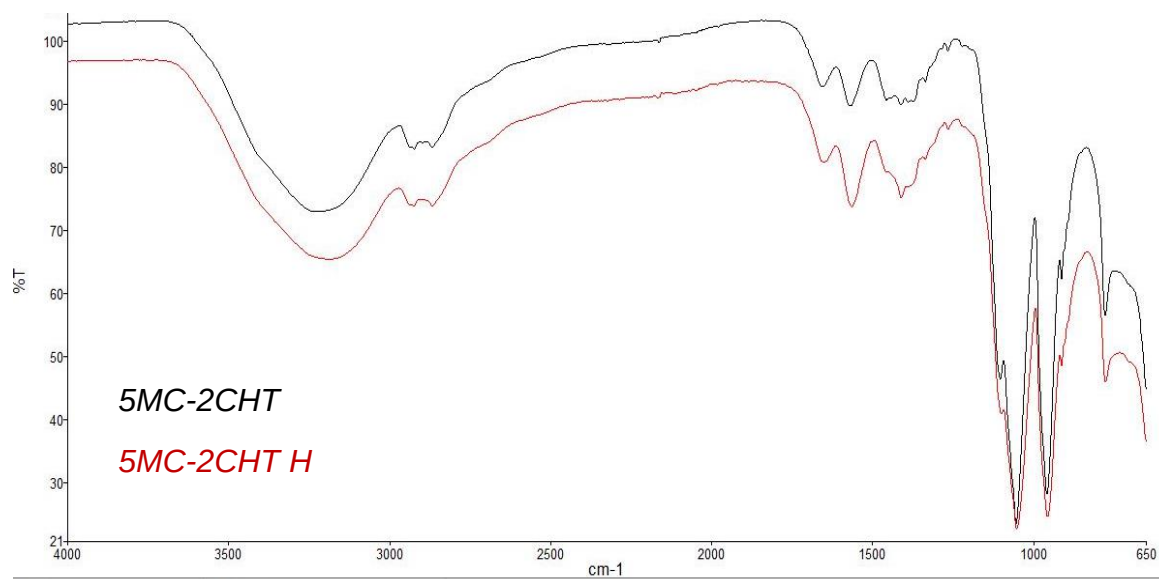
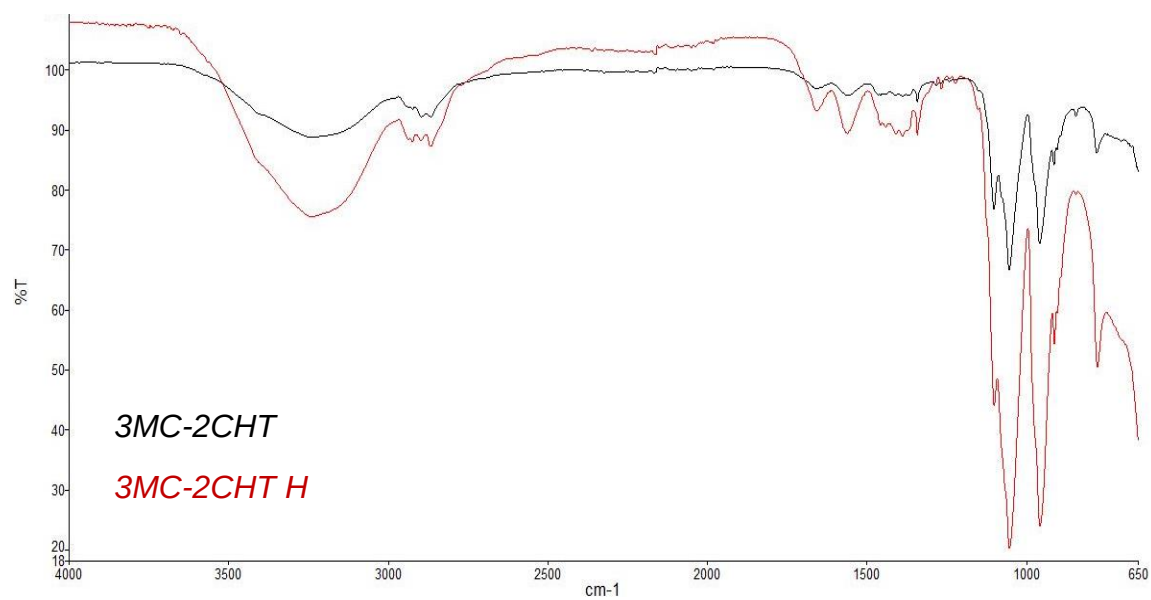
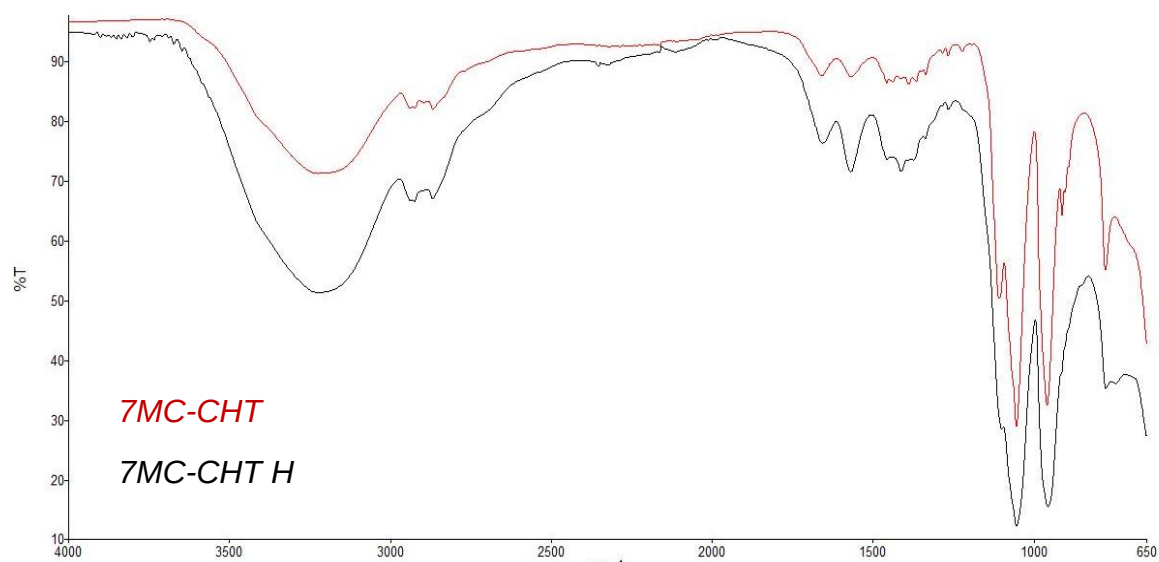
I am satisfied that the persons listed in this application are competent to perform the procedures described in the application, in the context of Section 23 (1) (c) of the veterinary and Para-veterinary Professions Act (19 of 1982).

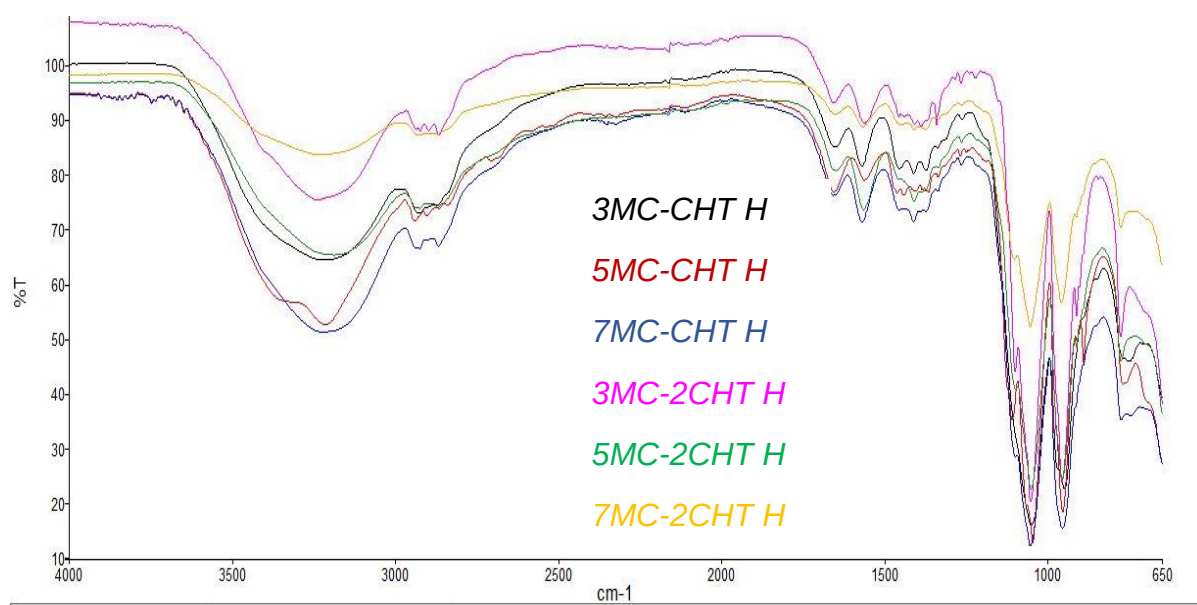
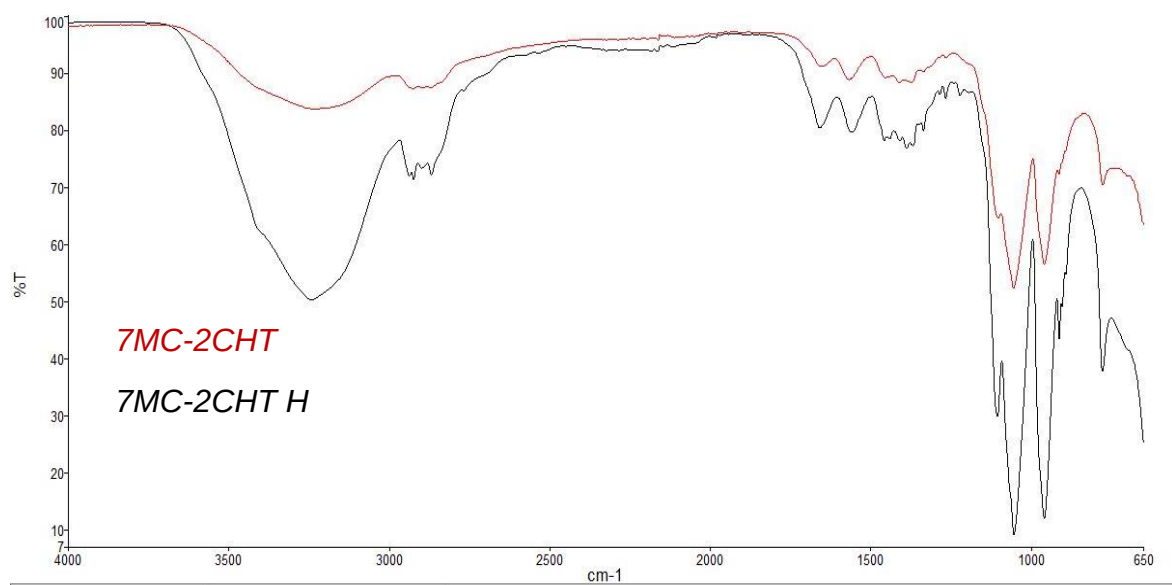
Signed: _____  _____ Date: 04 November 2020
(Registered Veterinarian)

CC: Student supervisor: «Title1» «Initials1» «Supervisor_surname»
Director Central Animals Service: Dr Kim Jardine

Appendix E: Stacked FTIR spectra of the chitosan-cellulose formulations at ambient and physiological (formulations labelled with an “H” to signify heated) temperatures







Appendix F: Stacked FTIR spectra of the pluronic-cellulose formulations at ambient and physiological (formulations labelled with an “H” to signify heated) temperatures

