UNIVERSITY OF BIRMINGHAM

INVESTIGATION INTO A THERMO-SENSITIVE CHITOSAN/β-GLYCEROPHOSPHATE HYDROGEL FOR OCULAR WOUND-HEALING

by

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ABSTRACT

The chitosan/β-glycerophosphate (CGP) hydrogel is a reasonably well understood thermo-sensitive system that has been widely used as the basis for successful tissue engineering and drug delivery systems. The aim of this project was to characterise an optimal CGP gel composition for a novel ocular wound-management system. Whilst much research has been done into the potential applications of the CGP system over the past few decades, no literature is currently available on the CGP-decorin combination which was the focus of this project. More specifically, the potential for this system to prevent serious visual impairments by way of delivering corneal-healing drugs is yet to be investigated. Rheological measurements of various gel compositions and release profiling experiments were performed to characterise the effectiveness of the system. The gel was also tested for cytotoxicity and for its effectiveness against a range of clinically isolated multi-resistant bacteria to determine its potential for biomedical application. Results show that a successful thermo-sensitive CGP system, that is liquid at room temperature and which gels at around physiological pH, has been produced and is able to release both antimicrobial peptide and anti-scarring drugs. The gel has no cytotoxic effect on corneal fibroblast cells and is effective in limiting the colony growth of a number of multi-resistant bacteria. The results show that this system has positive potential for ocular wound-healing.

ACKNOWLEDGEMENTS

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CGP	Chitosan-β-glycerolphosphate	**CF**	Corneal fibroblast
DD	Degree of deacetylation	**AMP**	Antimicrobial peptide

1 INTRODUCTION

The chitosan/β-glycerophosphate (CGP) hydrogel is a reasonably well understood thermo-sensitive system and has been widely used as the basis for successful tissue engineering systems. Medical application of the CGP system stems from the addition of therapeutic agents and polymeric additives. The effect of the addition of these agents on the thermo-gelation process and gel properties is not yet known and is the focus of this project. The long-term aim is to develop an injectable system which forms a robust gel *in vitro* and is able to deliver therapeutic agents to the wound site.

1.1 Introduction to gels

Polymer gels consist of a cross-linked network of polymer chains that are able to inflate upon absorption of a solvent, such as water (Derossi et al., 1991). Because of their ability to swell, gels have received much attention for use in drug delivery (Kwon, Han and Kim, 1991), tissue scaffolds (Gutowska, Jeong and Jasionowski, 2001) and other biomedical applications. Gels are described as a soft material, being neither completely solid nor liquid, although they do exhibit mechanical properties similar to those of an elastic solid. All gels contain two phases – the continuous phase and the solid phase, which refer to the solvent and dispersed solid respectively. Depending on the nature of interaction between the polymer chains, the gel can be described as being either chemical or physical (Larson, 1999). A chemical gel is one in which covalent bonds form between the polymer chains – permanent interactions which prevent reversibility of the gel. Chemical gel networks cannot be thermally broken and as such there is no transition from gel to solution (gel-sol transition) upon heating. By contrast, a

physical gel exhibits thermally reversible properties. Weak hydrogen bonds stabilise the polymer network at low temperatures and are relatively easy to break upon heating. A physical gel undergoes a gel-sol transition when heated (Larson, 1999).

1.2 Hydrogels

Hydrogels currently receive much attention for their potential use in novel biomedical applications such as tissue engineering, bio adhesives and as drug delivery systems (Hoffman, 2002). Hydrogels are three-dimensional polymer networks containing mostly water (>90%). The swelling behaviour of conventional hydrogels is minimally affected by environmental changes; however recent developments have resulted in the production of stimuli-responsive hydrogels which may swell or de-swell with alterations to pH or temperature (Alarcon, Pennadam and Alexander, 2004). Sensitivity to environmental changes makes these hydrogels particularly useful, especially for biomedical applications (Park and Shalaby, 1993; Drury and Mooney, 2003; Masteikova, Chalupova and Sklubalova, 2005; Supper et al., 2014).

1.3 Chitosan hydrogels

Chitosan is an aminopolysaccharide derived from the partial depolymerisation and deacetylation of chitin, a component found in the exoskeletons of crustacean shells (Muzzarelli, 1977). Chitosan has proven to be advantageous for medical applications due to its biocompatibility (Molinaro et al., 2002) biodegradability, low cytotoxicity (Rao, 1997) and in recent years has received much attention as the basis of drug delivery systems (Bhattarai et al., 2010; Bernkop-Schurch and Dunnhaupt, 2012). In 2000, Chenite et al. developed an injectable, thermosensitive, pH-dependent solution based on the neutralization of chitosan by

addition of β-glycerophosphate (GP). The resulting CGP solution is liquid at physiological pH and room temperature, and becomes a gel if heated to body temperature (Chenite et al., 2000).

1.3.1 Production of chitosan by the deacetylation of chitin

Chitin can be chemically deacetylated by different mechanisms (acid hydrolysis, oxidative-reductive and nitrous acid depolymerization). The physicochemical properties and functionality of the resulting chitosan depend on two main factors: the degree of deacetylation and the degree of polymerization. In concentrated alkaline conditions acetyl groups in chitin are split (Figure 1) and form chitosan when the proportion of free NH_2 groups in the polymer chain exceeds 75% (Muzzarelli, 1977). The resulting chitosan is a linear polysaccharide comprised of randomly distributed β-(1,4)-linked D-glucosamine and N-acetyl-D-glucosamine units. The proportion of deacetylated D-glucosamine units relative to remaining N-acetyl-D-glucosamine units dictates the degree of deacetylation (DD).

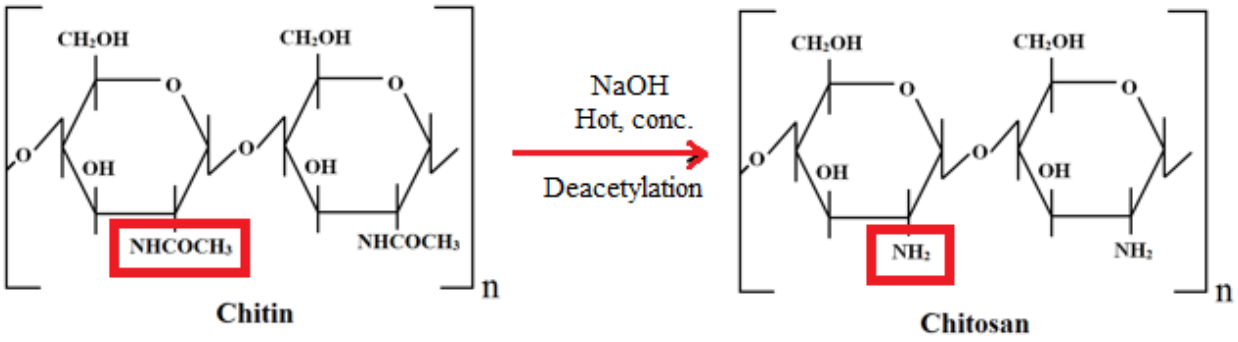

***Figure 1**: Mechanism of deacetylation of chitin to form chitosan using NaOH.*

1.3.2 Solubility of chitosan

Chitosan is insoluble in solution above pH 6 due to the presence of positively charged, hydrophobic amino groups (Sogias, Khutoryanskiy and Williams, 2010). In acidic conditions, the conversion of NH_2 groups on the chitosan to NH_3^+ allows for ionic interactions between the polymer chains and water molecules to form. Previous investigation into the CGP gel system has found chitosan to be insoluble above pH 2, as noted in the literature by use of 0.1 M HCl solutions for dissolution prior to GP addition (Chenite et al., 2000).

1.3.3 Role of glycerophosphate in CGP hydrogel formation

The presence of cationic amine groups on chitosan enables the formulation of polyelectrolye complexes upon exposure to anionic molecules, such as GP. It is generally accepted that the mechanism for CGP gelation involves a number of interactions, including; (i) ionic cross-link formation between oppositely charged amine and phosphate groups on chitosan and GP respectively, (ii) hydrogen-bond formation due to neutralisation of chitosan by proton-absorbing GP or (iii) hydrophobic interactions between chitosan chains (Garipey et al., 2007; Ganji et al., 2007; Bhattarai et al., 2010).

The theory that GP is directly incorporated into the gel system was confirmed by Cho et al (2005) who found that the cationic charge on chitosan was decreased with the addition of GP and that the amount of decrease was directly proportional to GP concentration. However, recently GP was found to be freely diffusible out of the gel system (Filion and Buschmann, 2013) which suggests that GP serves only as a proton acceptor for chitosan and does not electrostatically interact with the polymer.

More specifically, the recent work of Supper et al (2013) suggests that it is the polyol moiety of GP that is responsible for the thermal sensitivity for the gel system. High GP concentrations lead to faster gelation time (Ahmadi and Bruijn, 2008). It is believed that the glycerol moiety forms a protective shell of weak hydrogen bonds around the chitosan chain which shields the polymer from thermal degradation, allowing it to remain in solution at low temperatures. As the temperature is increased, the protective layer is disrupted and the chitosan chains become free to form a strong network through hydrophobic interactions. The higher the concentration of GP, the higher the sol/gel transition temperature (Supper et al., 2013).

1.3.4 Mechanical properties of CGP hydrogel

Hydrogels are self-supporting networks with the ability to respond to external forces and which display complex rheological behaviour in the form of viscoelasticity. The storage modulus of a hydrogel is dependent on a number of factors, including; molecular structure and concentration, ionic elastic modulus, pH and temperature. The shear modulus (G*) of a gel, comprising storage (G') and loss (G") moduli describes the ratio of shear stress to shear strain and can be used to indicate the proportion of solid to liquid properties of the gel (Chenite et al., 2001; Cho et al., 2006). The storage, or elastic, modulus measures the stored energy of the gel, representing the elastic portion, and the loss modulus measures the energy that is dissipated as heat and represents the viscous portion (Meyers and Chala, 1999). A comparison of the relationship between elastic and viscous moduli with temperature can be used to characterise the sol-gel transition of CGP gel (Chenite et al., 2001). Investigation into the rheological properties of CGP hydrogel has identified three distinct regions of shear modulus behaviour as a function of temperature (Chenite et al., 2001; Cho et al., 2005; Sa-

Lima et al., 2010) (Figure 2). Region one represents the viscoelastic fluid-like behaviour of the CGP solution, where both elastic and viscous moduli decrease with increasing temperature (Graessley, 1974). This decrease in elastic modulus can be explained by a reduction in molecular size, due to increased chitosan chain flexibility, and dehydration of the network which leads to a reduction in rheological properties (Cho et al., 2005). Region two is the temperature range in which both moduli suddenly increase as a result of the three-dimensional network formation. In this region, G'>G" due to the increase in elasticity in the system. Finally, region three shows a slowing of gel formation caused by an increase in viscosity of the newly formed network.

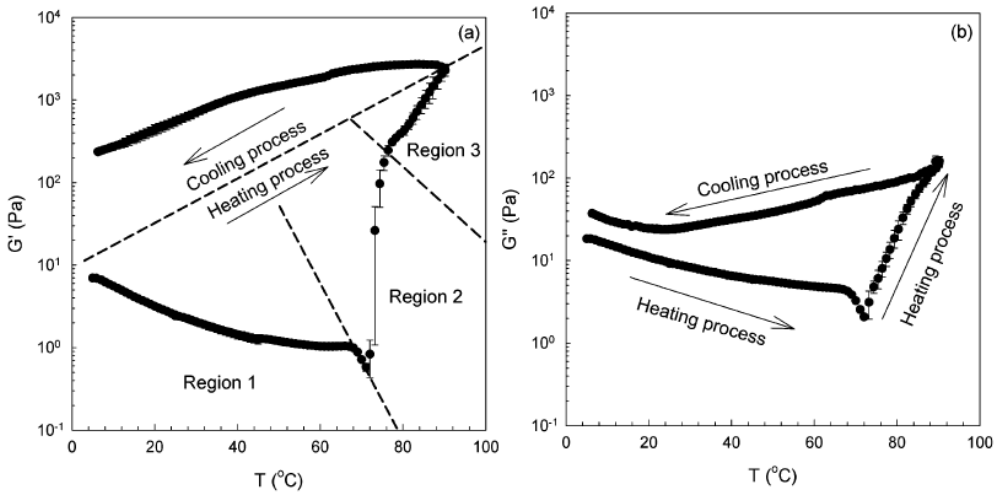

Figure 2: *The variation in G' and G" with increasing temperature (Cho et al., 2005).*

1.3.5 Effect of the degree of deacetylation of chitosan on gel properties

The availability of free NH_2 groups in chitosan has been found to effect the CGP gelation process and gel properties. Gel turbidity is determined by both DD and the homogeneity of the re-acetylated chitosan medium. It was found that homogenously re-acetylated chitosan with DD between 35 – 50% is necessary for the production of a clear gel (Berger et al., 2005).

Increased cross-link formation resulting from a higher DD has been found to reduce gelation time (Ganji et al., 2006). 88% DD produced a more uniform, porous and connective gel than 80% DD (Chang et al., 2012) and 84% DD was able to be stored as a stable liquid at 4°C for 3 months with no apparent change in viscosity (Ruel-Gariepy et al., 2000).

1.3.6 Role of pH

Gelation time and temperature are modulated by the pH of the CGP solution (Kim et al., 2010). At low pH values, the free amine groups on chitosan are protonated which results in electrostatic repulsions between the polymer chains and solubility of the molecule. At higher pH values (> 6.2), the neutralisation of the chitosan chains leads to the formation of gel-like precipitates (Chenite et al., 2000). Upon neutralization of chitosan, electrostatic interactions which previously predominated between oppositely charged chitosan and GP are overcome. The pH sensitivity of the system is dependent on electrostatic interactions between ionic groups on chitosan and GP (Li et al., 2014).

1.3.7 Effect of the molecular weight of chitosan on gel properties

The appearance of the CGP hydrogel was found to become more compact and regular with increasing molecular weight (Zhou et al., 2008). Cho et al. (2006) suggested that higher molecular weight chitosan could result in gelation, even without the use of an additional gelling agent. It was found that drug encapsulation efficiency was highest in medium molecular weight chitosan-based gels (Kouchak et al., 2012) and that stronger hydrogen and electrostatic bonds form in formulations with higher molecular weight chitosan than in other formulations (Honary, Maleki and Karami, 2009).

1.3.8 Effect of temperature on the gelation process

The transition of the CGP system from solution to gel is known to be temperature dependent (Chenite et al., 2000) and there are a number of factors contributing to this thermosensitive behaviour. The apparent proton dissociation constant (pK_{ap}) of chitosan is known to be highly temperature dependant and reduces as temperature increases (Filion, Lavertu, & Buschmann, 2007). Proton transfer from chitosan to the GP increases with temperature which was confirmed by a measured reduction in pH of the gel system during the thermo-gelation process (Supper et al., 2013). The reduction in the ionization of chitosan, as well as increased thermal agitation of water molecules, contribute to a reduction in both the number of hydrogen bonds and cohesion of the glycerol protective shells around the chitosan molecules allowing gel precipitates to form (Supper et al., 2013).

1.3.9 Thermoreversibility

Ganji et al. (2006) discussed the effect of GP concentration on the thermoreversibility of the gel, attributing the extent of reversibility to the nature of gelation mechanism which is determined by the amount of GP present. At low concentrations of GP, gelation is mainly by the formation of temperature-dependent hydrophobic interactions which result in a thermally reversible gel system. However, at higher concentrations of GP (> 0.5 mol/l), hydrogen bonds between the chitosan and chitosan-water molecules predominate the gelation process due to the increased neutralising effect of GP. These interactions are independent of temperature and are non-reversible upon cooling.

1.4 Chitosan-based hydrogels for wound healing

Chitosan hydrogels can be classified as being either chemical or physical hydrogels. Chemical hydrogels are formed by covalent crosslinking of chitosan with another molecule. Physical hydrogels are formed by reversible bonding, such as ionic interactions, secondary interactions, grafted chitosan hydrogels and entangled hydrogels (Berger et al., 2004). The application of entangled chitosan hydrogels is limited by their lack of mechanical properties and tendency to dissolve, whereas all other variations of chitosan hydrogels exhibit characteristics that make them potential stimuli-responsive drug delivery vehicles. As a natural polymer, chitosan is widely investigated as an antimicrobial agent for preventing and treating infections owing to both its intrinsic antimicrobial properties and its ability to deliver extrinsic antimicrobial compounds into the infection site (Dai et al., 2011). Chitosan hydrogels have been successfully used for drug delivery (Bhatterai, Gunn and Zhang, 2010) and have provided vehicles for various therapeutic agents in treatments for, tissue regrowth (Mattioli-Belmonte et al., 1999), burn care (Dai et al., 2011) and cancer (Wang et al., 2013).

1.4.1 Bacterial infections in the eye

As an external surface, the eye is an organ that is at continual risk of exposure to airborne infectious bacteria in the external environment. Bacteria invading the surface of the eye can lead to bacterial infections effecting the surface and/or interior of the eye. Bacterial keratitis is a potentially blinding infection that results in corneal ulceration and which poses a serious threat to vision if left untreated. Ulceration to the cornea can cause scarring which interferes with vision by blocking or distorting light entering the eye (WebMD, 2014). The two major predisposing factors to bacterial keratitis in working age population have been found to be

ocular trauma and contact lens wear. Contact lens wear has been found to be the main risk factor for developing the condition in the developed world where standards of contact lens hygiene in are such that the risk is greater than in a developed setting (Musa et al., 2010). A retrospective review of contact lens-related microbial keratitis in British Defence personnel in Iraq found an incidence level of 35 per 10,000 people. 17 eyes (63%) lost visual acuity (Musa et al., 2010). Other influencing factors include ocular surface disease, systemic disease and ocular surgery (Bourcier et al., 2003). A study by Mahajan (1983) found *Staphylococci*, *S. pneumoniae*, and *P. aeruginosa* to be the main pathogens in 823 eyes with infections such as acute bacterial conjunctivitis, corneal ulceration and postoperative infections.

1.5 Antimicrobial peptides

Antimicrobial peptides (AMPs) are low molecular weight proteins capable of opposing activity of bacteria, viruses and fungi in the body. Most AMPs are both cationic and hydrophobic at the surface which makes them effective at interacting with microbial membranes that often exhibit anionic surfaces (Wimley, 2010). It is this opposition in charge that results in high selectivity of AMPs to microbes, due to the formation of strong electrostatic bonds between the molecules. Upon entry into the lipid-rich membranes, AMPs contribute to wound healing by killing target cells (Izadpanah and Gallo, 2005). Differences in the susceptibility of a single microorganism to a panel of antimicrobial peptides has been shown to indicate that the size, sequence, conformation, charge and overall hydrophobicity of the AMP are all important factors in determining its effectiveness (Brogden, 2005). Similarly, cell structural components specific to the strain of bacteria, such as the integrity of their cell walls, affects the resistance of a strain to antimicrobials. Gram-positive bacteria, those which

retain the colour of the crystal violet stain in the Gram stain, have a cell wall composed of a thick layer of peptidoglycan. By contrast, bacteria with a thin layer of peptidoglycan in their cell wall are classified as gram-negative and are generally found to be more resistant to antimicrobials due to the presence of a second outer membrane layer.

1.5.1 Corneal scarring

The cornea is a clear, dome shaped structure that is the outermost surface of the eye and serves to both protect the eye from external matter and focus the light that enters the eye. The epithelium is the cornea's external layer that blocks foreign and harmful material from entering the eye; it is highly sensitive to pain and so is able to quickly detect a scratch to its surface. Corneal disorders can occur as a result of infection, trauma or mechanical damage and may result in distortion to light entering the eye. This can cause a decrease in overall vision, visual quality and, in the case of corneal scarring, complete sight loss (Whitcher, Srinivasan and Upadhyay, 2001). Where corneal infections have caused significant structural damage to the cornea, the most common treatment options are eye-drop administration or corneal transplantation.

1.5.2 Decorin

Decorin is a small leucine-rich proteoglycan found naturally occurring in the extracellular matrix. It is a TGF-β1/2 antagonist and is therefore able to regulate various cellular functions through interactions with components of the extracellular matrix (Davies et al., 2004, Hocking et al., 1998 and Logan et al., 1999a). There are a number of factors which make decorin potentially suitable to treat corneal scarring. Firstly, decorin has anti-scarring properties due

to its ability to decrease both TGF-β1/2 receptor activation and signalling which results in an interruption of the transcriptional activation of extracellular matrix production (Akhurst, 2006 and Yamaguchi et al., 1990). Secondly, decorin is able to modify fibrogenesis of collagen and the resulting fibre (Reese et al., 2013) and form an activity-blocking complex with connective tissue growth factor (CTGF) (Vial et al., 2011). A study into corneal wound healing found that decorin gene therapy delivered to the stroma inhibited corneal fibrosis in vivo (Mohan et al., 2011).

1.6 Scope of the work

The aim of this project is to investigate the potential to combine antimicrobial peptides and anti-scarring drugs with an existing CGP hydrogel for use as an ocular wound healing system. Although much research into the CGP system has been conducted, at present no studies have focused on the potential for this system as an eye dressing. The project will focus on mechanical and medicinal properties of the gel required to ensure its success in application.

2 EXPERIMENTAL (MATERIALS AND METHODS)

2.1 Materials

All materials used in this project were purchased from Sigma (Poole, UK) unless stated otherwise. A number of molecular weight grades of chitosan were purchased from Heppe and are detailed in Table 1 below.

Table 1: *Varieties of chitosan used to create CGP hydrogel systems throughout the project.*

Material name	Mw Grade (appr. kDa)	Degree of Deacetylation (DD) (%)
Chitosan 95/20	90	95
Chitosan 95/500	350	95
Chitosan 95/1000	400	95
Chitosan 95/2500	500	95
Chitosan 95/3000	600	95

2.2 Sample preparation

Following a method described by Chenite et al. (2000), a typical chitosan solution was obtained by dissolving chitosan (200 mg) in HCl solution (9 ml, 0.1 M) and stirring for 3 hours using a magnetic stirrer. The resulting solution was then chilled at 4°C for 3 hours. GP (560 mg) dissolved in deionised water (1 ml) was added drop-wise to the chilled chitosan solution whilst stirring to produce a clear, homogenous liquid solution. The solution was incubated at 37 °C to form a gel. For certain experiments described, different concentrations

of antimicrobial peptide (AMP) and decorin were added to the CGP liquid solution, vortexed for 30 seconds and incubated at 37 °C to form a gel.

2.3 Characterisation of CGP hydrogel system

Mechanical, visual and cytotoxic properties were investigated in order to characterise both the structure and function of the CGP system.

2.3.1 Rheological Characterisation

The rheological characterisation of hydrogel samples was performed in an ARES rheometer from TA Instruments, fitted with parallel plate geometry (diameter of 35 mm). The rheometer used is a strain controlled instrument that consists of applying a torque and measuring the resultant displacement. Measurement parameters for each test are described the in the relevant sections below.

2.3.2 Effect of molecular weight of chitosan on gel elastic modulus

CGP gels were made as described in section 2.2. Dynamic frequency sweeps were performed on five different molecular weight CGP pre-gelled samples (0.2 ml, 90, 350, 400, 500, 600 kDa). The tests were performed at room temperate and the elastic modulus (G') was measured as a function of frequency under an oscillation range of 0.031 – 31Hz.

2.3.3 Imaging of optical properties

Hydrogels were prepared as described in section 2.2 and applied to glass marbles that had been incubated at 37 °C and laid over text to demonstrate physical and optical properties of the system.

2.3.4 Effect of temperature on gel elastic modulus

CGP solutions were made as described in section 2.2. Temperature ramps were performed on CGP solutions (0.2 ml, 500 kDa) to measure the thermosensitive gelation process as indicated by a change in hydrogel elastic modulus. Elastic modulus (G') was measured as a function of temperature under oscillation measurement of 1Hz (1 rad s^{-1}).

2.3.5 Cytotoxicity assay

Cell line corneal fibroblast cells from a confluent flask were trypsinised, incubated for 5 minutes to detach cells and blocked with media (RPMI) to inhibit trypsin action of the cell. Cell counts were performed and a combination of suspension (125 µl) and media (375 µl) wasplaced in 16 wells of a 24 well plate. Cells were then left overnight in an incubator to allow for attachment. CGP hydrogels (450, 500, 600 kDa) were prepared following the method in section 2.2 then autoclaved to form a block of each gel which were cut into 0.4 cm^3 units. The gels were added to the well plate and incubated for 24 hours before being removed. All cells were then incubated with trypsin (250 µl) and then blocked with media (250 µl). Cells were counted on a haemocytometer with n=6 technical repeats and n=3 biological repeats.

2.3.6 Effect of shear rate on gel viscosity

CGP gels were made as described in section 2.2. The viscosity of CGP hydrogel (500 kDa) was measured by a strain-controlled steady rate sweep tests across a shear rate range of 10 – 450 sec^{-1}.

2.3.7 Effect of solution on gel elastic modulus

CGP hydrogels were made as described in section 2.2 and stored in PBS (2 ml) for one week. Dynamic frequency sweeps were performed at room temperate and the elastic modulus (G') was measured as a function of frequency under an oscillation range of 0.031 – 31Hz to determine gel elastic modulus compared to a control gel.

2.4 Addition of therapeutic agents to CGP gel for wound-healing

2.4.1 Effect of AMP concentrations on gel elastic modulus

CGP gels were made as described in section 2.2. Dynamic frequency sweeps were performed on pre-gelled CGP hydrogels (0.2 ml, 500 kDa) containing AMP at 0.9 % v/v, 1.9 % v/v and 3.8 % v/v. The tests were performed at room temperate and the elastic modulus (G') was measured as a function of frequency under an oscillation range of 0.031 – 31Hz.

2.4.2 Release profiling of AMP

AMP (2.9 % v/v) was added to CGP solutions (0.5ml 500 kDa) made using the method described in section 2.2, covered in aluminium foil and left to gel in an incubator at 37 °C. Upon complete gelation, any excess water was removed. PBS (2 ml) was added on top of

each 1 ml AMP-hydrogel sample. Plates were covered in foil and 100 µl aliquots taken at t=0, 1, 2, 3, 4, 5, 6, 7, 24 hours. Fluorescence of plates was read at 488 nm using a Glomax multi detection system.

2.4.3 AMP dispersion imaging

Hydrogels were prepared as described in section 2.2. Red food dye (10 µl) was added to the gel and standard camera images taken to demonstrate dispersion behaviour. Separately, AMP (10 µl) was added to gels which were then transferred to slides and imaged at 488 nm with an Axioplan-2 fluorescence microscope and images obtained with AxioVision Microscopy Software (both from Carl Zeiss Ltd., Hertfordshire, UK).

2.4.4 Microbiology of hydrogel

Seven strains of clinically isolated multi-resistant bacteria (Table 2) were provided as a gift by Dr. Beryl Oppenheim for this project.

Table 2: *Varieties of clinically isolated bacterial strains tested.*

Bacteria
Methicillin Sensitive *S. aureus*
Methicillin Resistant *S.aureus*
Coagulase Negative *Staphylococcus*
P. aeruginosa
E. coli
M.catarrhalis
H. influenza

CGP hydrogels (50 µl, 500 kDa) were prepared with AMP (1.5% v/v) from stock solution (15 mg/ml) and exposed to seven different strains of multi-resistant bacteria to evaluate the effectiveness of the antimicrobial properties of the hydrogel. LB broth was used to culture the bacteria on the gel during incubation at 37 °C for 24 hours.

2.4.4.1 Colony forming unit (CFU) solution microbiology

Dilution plates were prepared by adding bacteria solution from around the gel (100 µl) and broth (100 µl) to wells and pipetting 10 µl aliquots into consecutive wells to create a 1 in 10 dilution along each row. From the dilution plates, solutions at each dilution (10 µl) were pipetted onto agar in a dropwise triangle formation. Following incubation overnight at 37°C, cell counts were taken by observing the number of visible units of each bacteria at a particular dilution factor.

2.4.4.2 Hydrogel microbiology

Hydrogel was transferred from the original into a new well plate, maintaining the same arrangement of wells. Alamar blue dye (10 µl) in H_2O (90 µl) was added to each of the wells on top of the hydrogel. The plate was incubated for 1 hour and analysed at 488 nm using a Glomax multi detection system.

2.4.5 Effect of decorin concentration on gel elastic modulus

CGP gels were made as described in section 2.2. Dynamic frequency sweeps were performed on pre-gelled CGP hydrogels (0.2 ml, 500 kDa) containing decorin at 1.9 % v/v, 3.8 % v/v and 7.4 % v/v. The tests were performed at room temperate and the elastic modulus (G') was measured as a function of frequency under an oscillation range of 0.031 – 31Hz.

2.4.6 Release profiling of decorin

Decorin (5.7 % v/v) was added to CGP solutions (0.5 ml, 500 kDa) and left to gel in an incubator at 37 °C. Upon complete gelation, any excess water was added to the 96 well plate. PBS (2 ml) was added on top of each 1 ml decorin-hydrogel sample. 100 µl aliquots were taken at t=0, 1, 2, 3, 4, 5, 6, 7, 24 hours. Decorin ELISA kit was used to analyse according to manufacturer's instructions (R&D Systems, Abingdon, UK).

2.4.6.1 Decorin ELISA

The prepared capture antibody (360 µg/ml of mouse anti-human decorin when reconstituted with 1 ml PBS) was selected and diluted to 60 µL/10 ml in PBS. 100 µL of the diluted capture antibody was used to coat each well of the 96 well plates and incubated overnight at room temperature. The plate was washed three times with PBS Tween-20 (0.05%) and then blocked by adding of reagent diluent (300 µl, 1% BSA in PBS) and incubated for at least one hour. Separately, standard solutions (100 ng/ml of recombinant human decorin in 0.5 ml of reagent diluent) were added to the 96 well dilution plate (as used in section 2.16), diluting 1000 fold with each new row. The blocked plate was then washed a further three times and 100 µl samples from the dilution plate were transferred to the ELISA plate and incubated at room temperature on the shaker for two hours. The wash step was repeated. 100 µl of the detection antibody (60 µl in 10 ml reagent diluent) was added to each well of the ELISA plate and incubated for two hours whilst shaking. The wash step was repeated. 100 µl of strepravidin-HRP (60 µl in 10 ml reagent diluent) was added to each well and incubated in the dark, at room temperature for 20 minutes. The wash step was repeated. 100 µl of substrate solution was added rapidly to each well and incubated as with the strepravidin-HRP. Finally, stop

solution (50 µl) was added to each well and tapped gently to ensure thorough mixing. The plate was covered with foil and read at 488 nm using a Glomax multi detection system.

2.4.6.2 NanoDrop analysis

2 ml aliquots of PBS solution from decorin release study (section 2.4.6) of CGP gels (500 kDa) containing decorin (7.4% v/v) vs. control gel were analysed with a NanoDrop ND-1000 Spectrophotometer. Decorin absorbance was measured over time points 0, 0.5, 1, 2, 3, 4, 5, 6, 7 hours at a wavelength of 220 nm.

2.5 Statistical Analysis

All statistical analysis was carried out using SPSS 17.0 (IBM SPSS Inc., Chicago, IL)and data was presented as mean ± SEM. The Shapiro-Wilk test was used to ensure all data was normally distributed before parametric testing using a one-way ANOVA with Tukey post-hoc test. Statistical significance threshold was $p<0.05$. All data was normally distributed and was analysed using the same method.

3 RESULTS

3.1 Sample Preparation

All CGP systems have shown to be a solution at room temperature and to form gels around physiological temperature and pH. Gelation rates across 450 kDa, 500 kDa and 600 kDa molecular weight gels were all in the range of 30 - 60 secs, with 90 kDa taking a longer time (>120 secs). The solutions changed from being a transparent liquid to an opaque solid upon heating. Although no differences in clarity or colour were observed in the hydrogels' appearance, visual observation indicated that 90 kDa produced a more viscous gel (data not shown). The addition of AMP and decorin to CGP gels had no effect on the characteristics of the gelation process (data not shown).

3.2 Characterisation of CGP gel system

3.2.1 Effect of molecular weight on gel elastic modulus

To establish which chitosan molecular weight would produce the most robust hydrogel, dynamic frequency sweeps were performed on hydrogels of 4 molecular weight varieties (90 kDa, 450 kDa, 500 kDa, 600 kDa) to measure the effect of molecular weight on elastic modulus (G'). The results show that there was no significant difference in G' between the molecular weight grades of CGP hydrogel (Figure 3).

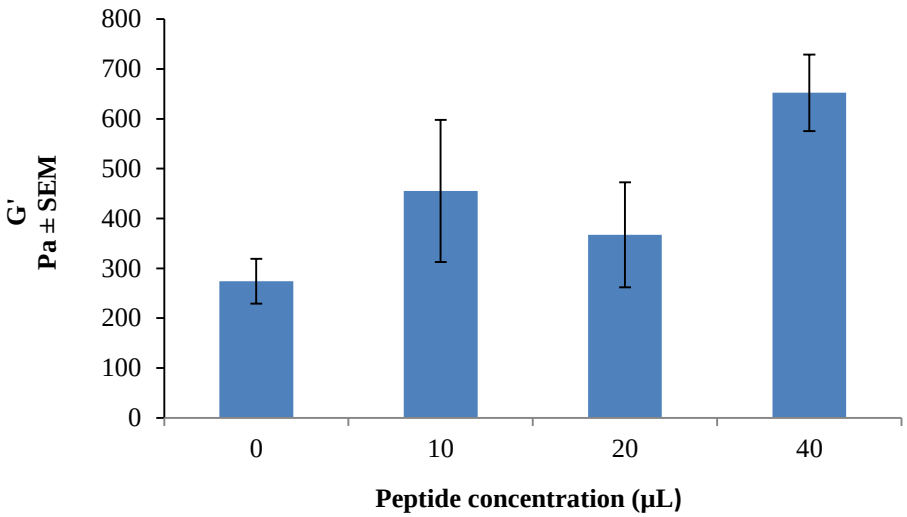

Figure 3: *Variation in G' of hydrogels with increasing molecular weight chitosan. Mean ±SEM, n=6. Differences between gels are not statistically significant.*

3.2.2 Imaging of optical properties of gel

Application of the CGP hydrogel (500 kDa) to the surface of a glass ball at 37 °C (Figure 4) demonstrated the ability of the gel to form a thin (1 mm), self-supporting gel layer at physiological temperature. Optical clarity of the gel was shown by the ability to read text underneath a layer of gel (Figure 4 c).

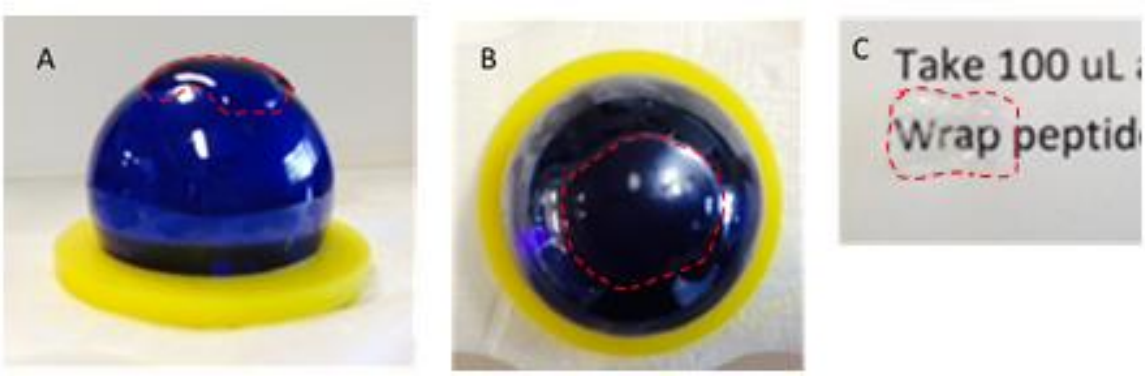

Figure 4: *Images showing a), b) gelation of CGP solution upon contact with glass 'eye' at near body temperature and c) demonstration of gel clarity upon complete gelation.*

3.2.3 Effect of temperature on gel elastic modulus

In order to identify the temperature-dependent gelation behaviour of the CGP solution, gel elastic modulus (G') of CGP gel (500 kDa) was measured as a function of temperature between 25 – 41 °C. The resulting curve can be described in two different regions (Figure 5). The first region shows little change in G' until around 33 °C where there becomes a steady increase in G', indicating a possible gelation onset point. The second region is characterised by an abrupt increase in G' because of the formation of the hydrogel three-dimensional network resulting in the sol-gel transition. The elastic modulus (G') was greater than the loss modulus (G") with increasing temperature which indicates the increase in elasticity of the system. Sol-gel transition temperature, characterised by a sudden increase in G', was found to be near physiological temperature, at 34 °C.

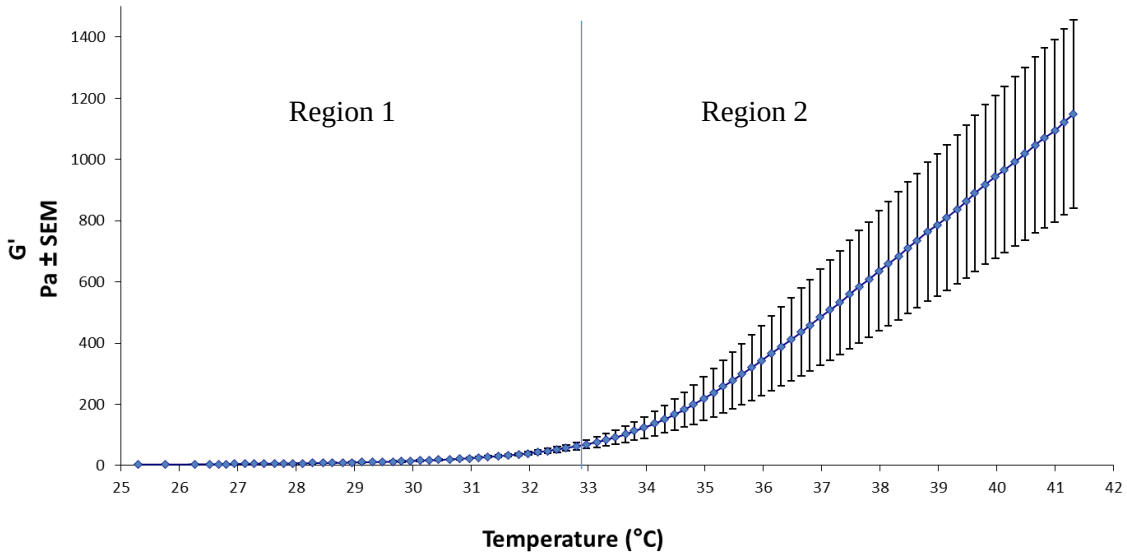

Figure 5 *Elastic modulus of 500 kDa CGP solution as it undergoes gelation between 26 - 42°C at a rate of 1°C/min.*

3.2.4 Hydrogel cytotoxicity assay

CGP hydrogels of three different molecular weights (450, 500, 600 kDa) were tested for toxicity in corneal fibroblast (CF) cells. The number of surviving CF cells after treatment with CGP hydrogels of 450 kDa, 500 kDa and 600 kDa was not significantly different from control (Figure 6), indicating that CGP hydrogels were not toxic to CF cells at the molecular weights tested.

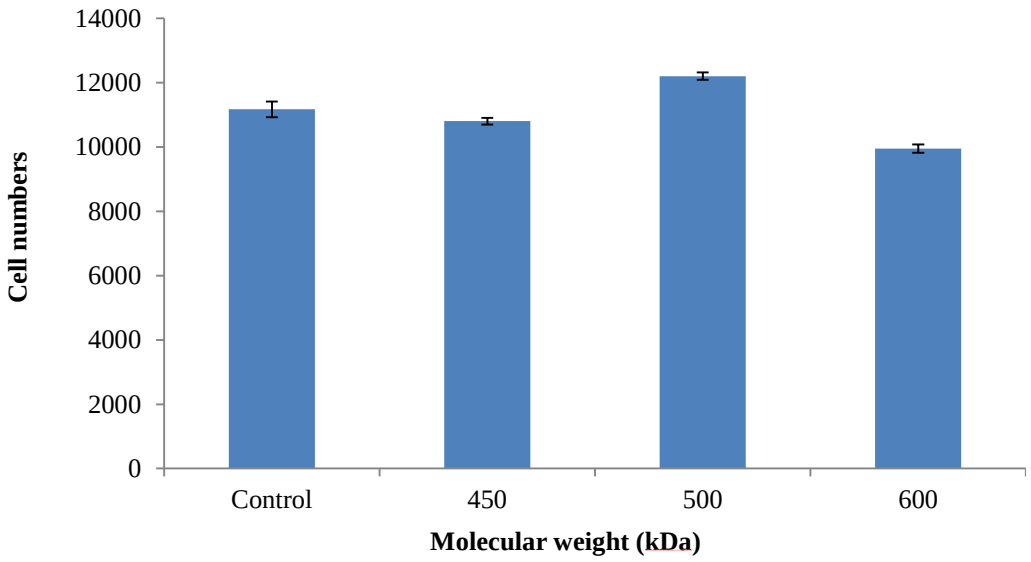

Figure 6: *Number of remaining corneal fibroblast cells following application of different molecular weight CGP hydrogels. Means ± SEM, n=3 for control, n=5 for all hydrogel treatment conditions.*

3.2.5 Effect of shear rate on gel viscosity

Strain-controlled steady shear rate sweep tests were performed on CGP hydrogels (500 kDa) to measure the effect of shear rate on gel viscosity. A clear trend of decreasing viscosity with increasing shear rate between 10 – 50 Hz can be observed, after which point gel viscosity plateaus in the range of 0-20 Pa/s as the shear rate increases (Figure 7).

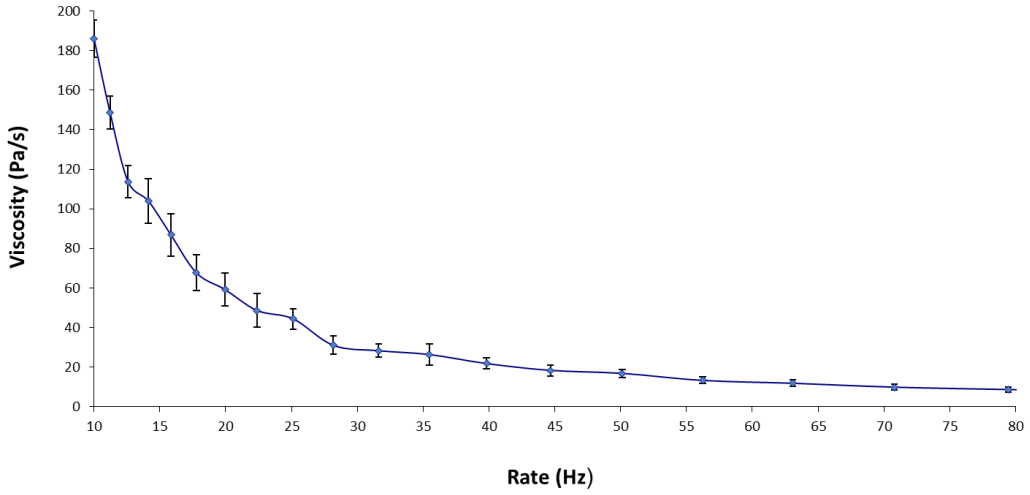

Figure 7: *Viscosity of hydrogel with increasing shear rate (10 – 80Hz). Means ± SEM, n=8.*

3.2.6 Effect of solution on gel elastic modulus

CGP hydrogels (500 kDa) stored in PBS (2 ml) for one week exhibited significantly lower gel elastic modulus (G') than control (Figure 8), indicating a weakening effect of solution on the gel. Comparison between weight measurements of dried and hydrated CGP gels showed swelling to be 7 % the initial, dried weight (data not shown).

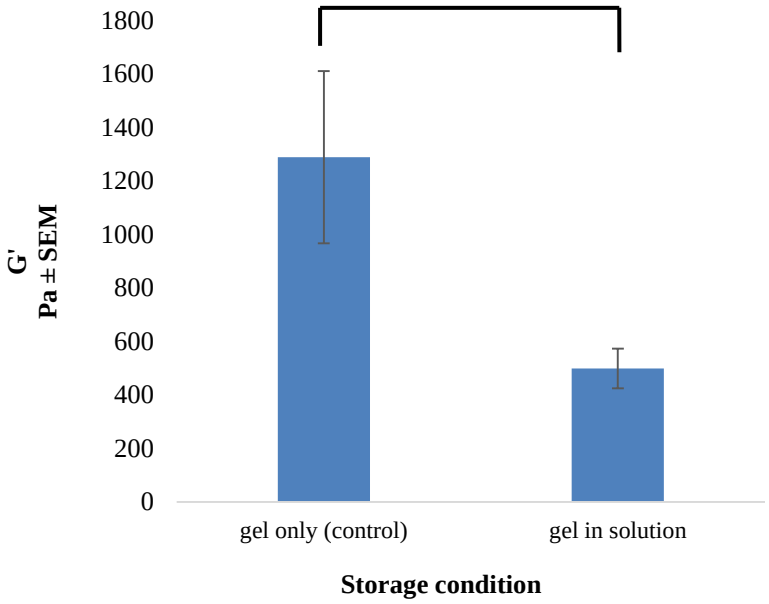

Figure 8: *Strength of 95/2500 grade CGP hydrogel after 1 ml gel was kept in 2 ml PBS solution for 1 week vs. 1 ml of control CGP hydrogel. Mean ± SEM, n=3. Difference between the two conditions is deemed statistically significant, black line indicates P<0.05.*

Addition of therapeutic agents

3.2.7 Effect of AMP concentration on gel elastic modulus

The addition of AMP will provide wound healing potential to the CGP gel system. Dynamic frequency sweeps were performed on CGP hydrogels (500 kDa) containing different concentrations of AMP to measure any effect of the addition on gel elastic modulus. In hydrogels containing AMP at 1.9% v/v and 3.8 % v/v, the elastic modulus was not significantly different from control (Figure 9). There was however a significant difference between hydrogel containing AMP at 3.8 % v/v and control (Figure 9), indicating that addition of AMP at 1.9% v/v and 3.8 % v/v has a negligible effect on gel elastic modulus, but at 3.8 % v/v, AMP may increase the elastic modulus of the hydrogel.

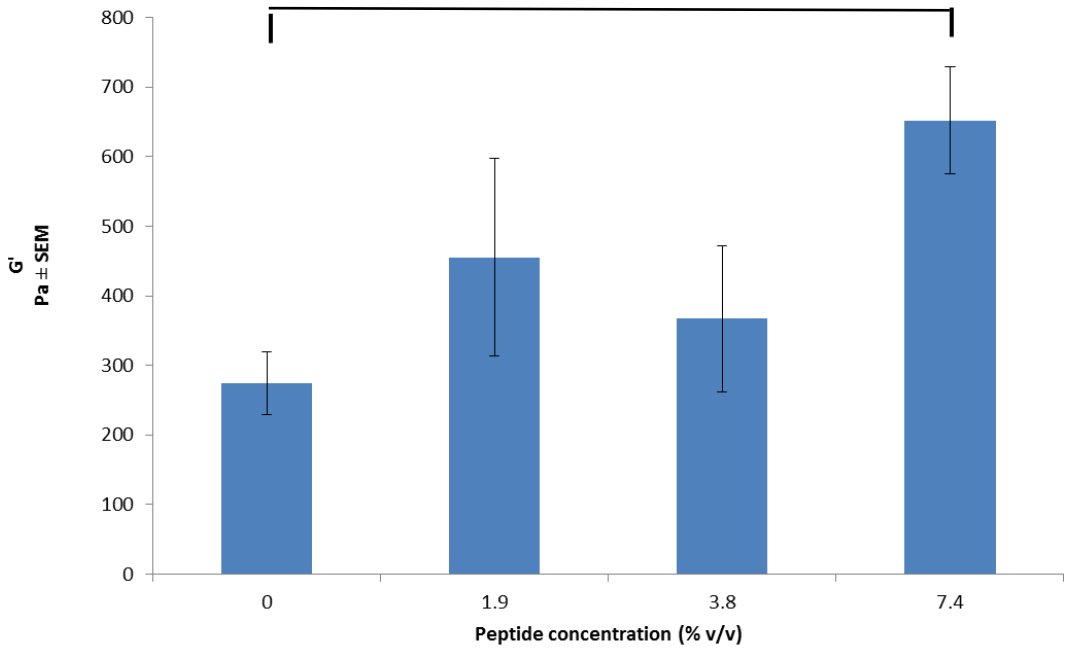

Figure 9: *Gel elastic modulus (G') of 500 kDa CGP hydrogels containing different peptide concentrations. Mean ±SEM, n=6. Differences of release rate with different concentrations are deemed statistically significant (P= 0.013), black lines indicate P<0.05*

3.2.8 Release profiling of AMP

CGP hydrogels of three molecular weights (450, 500, 600 KDa) containing 10 µl AMP were tested over 48 hours to measure their effectiveness at releasing the peptide. An increase in AMP concentration in solution over 48 hours was recorded in all molecular weight hydrogels (Figure 10), indicating that AMP was successfully released from the hydrogel. The release followed a homogeneous profile consisting of similar increases in AMP concentration at each time point which resulted in a uniform curve. There was no significant difference between the release rates of the molecular weights. Percentage release from 450, 500 and 600 kDa CGP gels was 55%, 60% and 49% respectively.

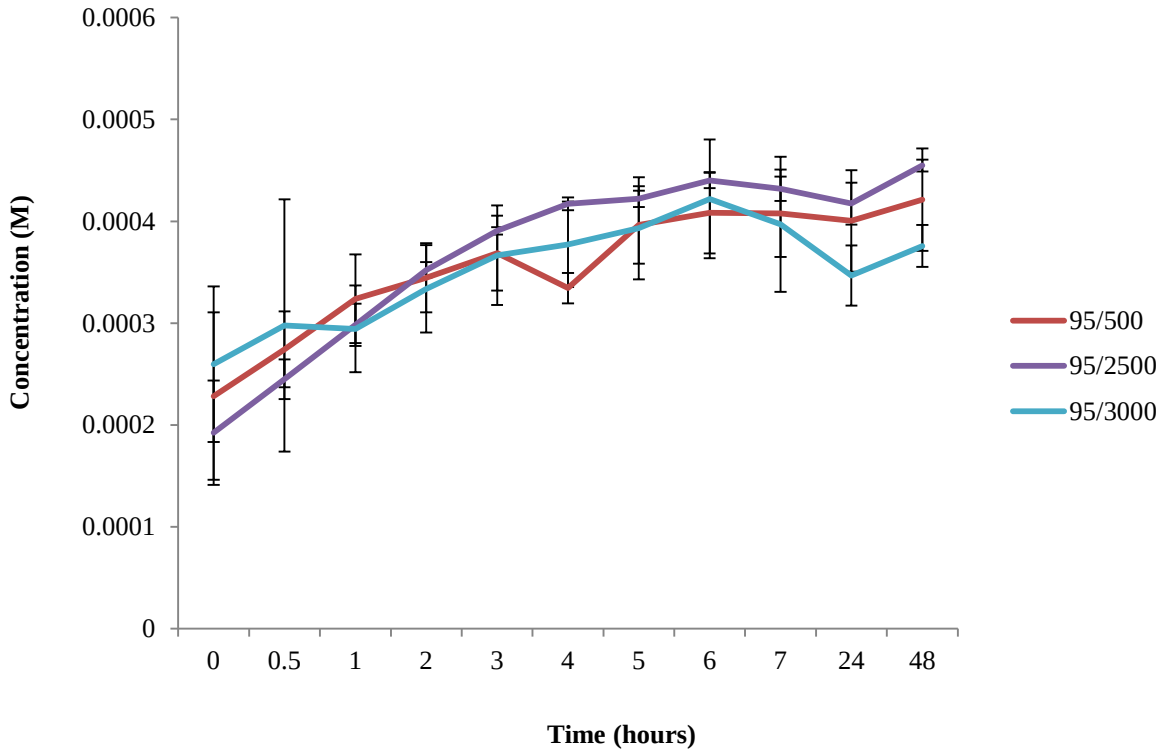

Figure 10: *Release rate of 10 µl peptide from 0.5 ml of different molecular weight CGP hydrogels over 48 hours. Means ± SEM, n=3. There is no significant difference between molecular weights.*

3.2.9 Imaging of peptide dispersion through gel

Images of the dispersion behaviour of red food dye through CGP hydrogel show a non-homogeneous dispersion (Figure 11 a,b,c) and indicate a possible non-uniform dispersion behaviour of AMP in the gel. This food dye model is proved by optical microscopy images of CGP hydrogels (500 kDa) containing antimicrobial peptide (Figure 11 d,e,f) which show areas of high contrast that indicate localised concentrations of AMP within the hydrogel.

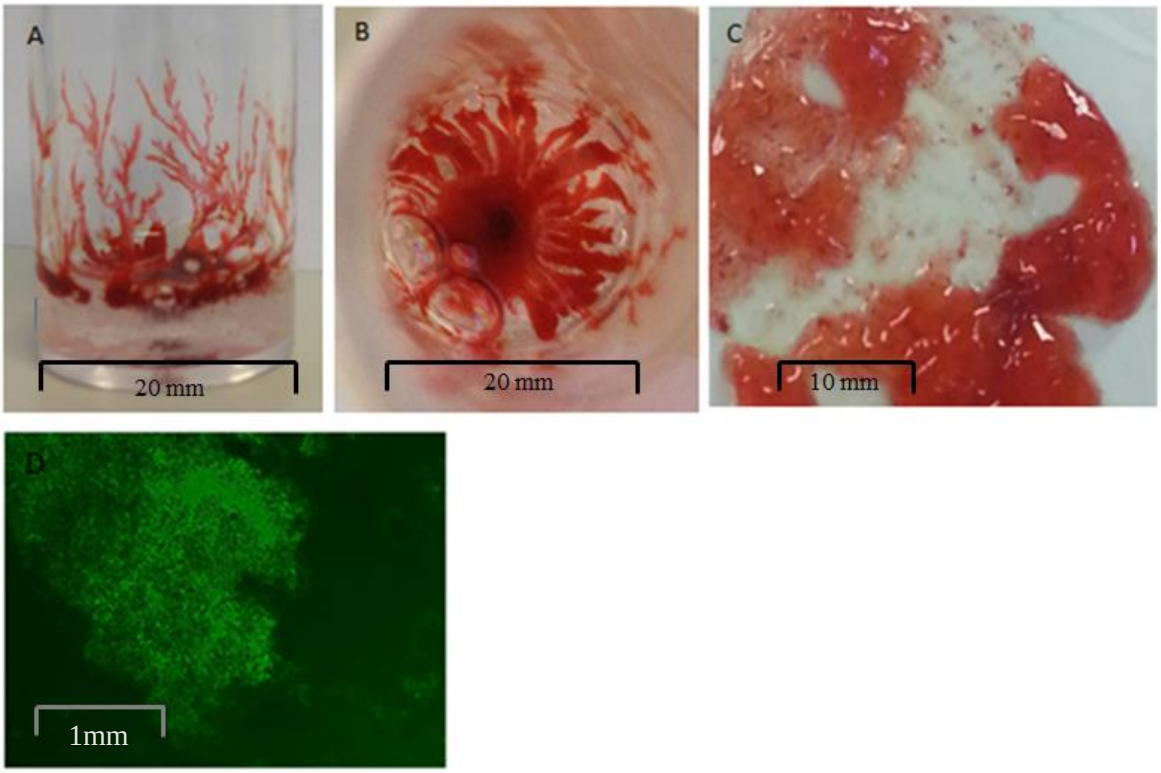

Figure 11: *a), b), c) Dispersion of red food dye through CGP hydrogel, d) Image of peptide dispersion through hydrogel at 5x magnification.*

3.2.10 Microbiology of hydrogel

The effectiveness of CGP hydrogel (500 kDa) containing AMP was tested against seven different strains of multi resistant bacteria. A significant antimicrobial effect was found against *P. aeruginosa*, Methicillin Resistant *S. aureus* and *H. influenza* cells (Figure 12), with no significant effect found against the other strains tested. Results indicate that AMP-containing CGP hydrogels (500 kDa) provide effective antimicrobial action against certain multi resistant strains of bacteria. Findings show significant differences between both Coagulase Negative *Staphylococcus* and *P. aeruginosa* in CFU solution compared to control solution. No significant difference was found between the other strains and control (Figure 13).

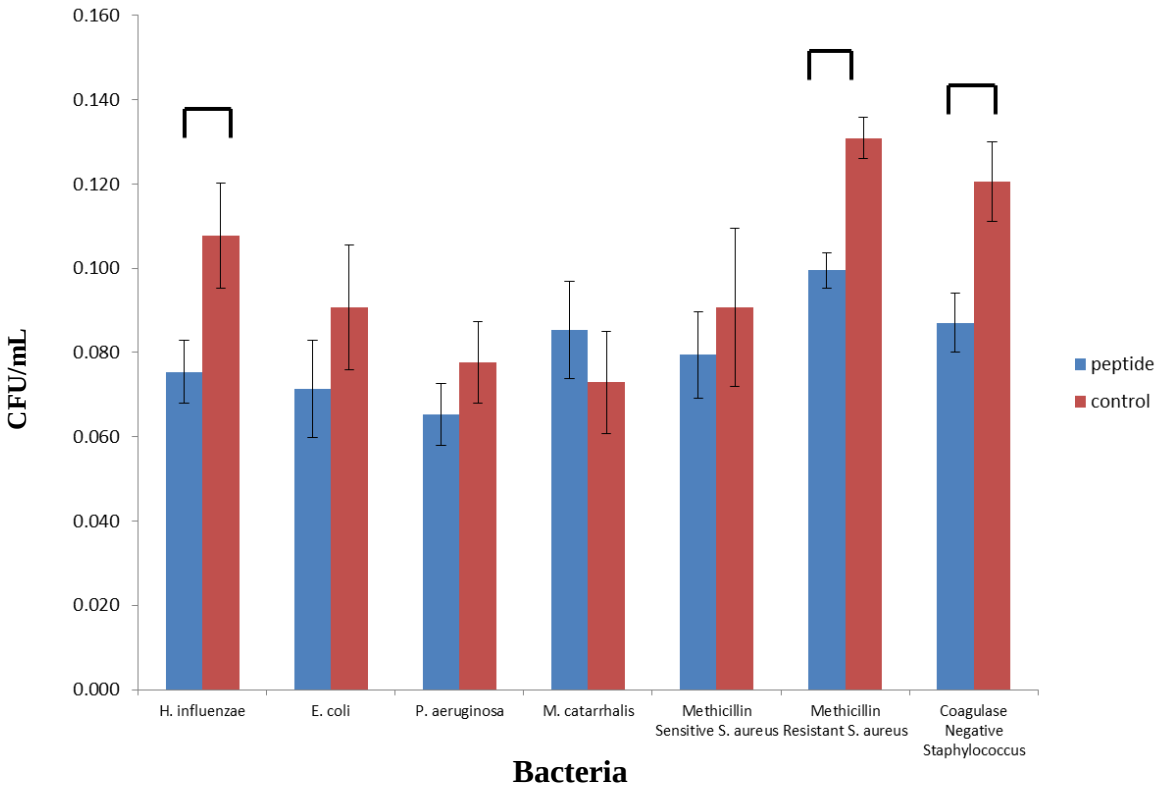

Figure 12: *Surviving concentrations of ten strains of multi resistant bacteria dissolved in antimicrobial peptide-containing CGP hydrogel (500 kDa) solutions compared to control. Means ± SEM, n=6. Black lines indicate $p<0.05$.*

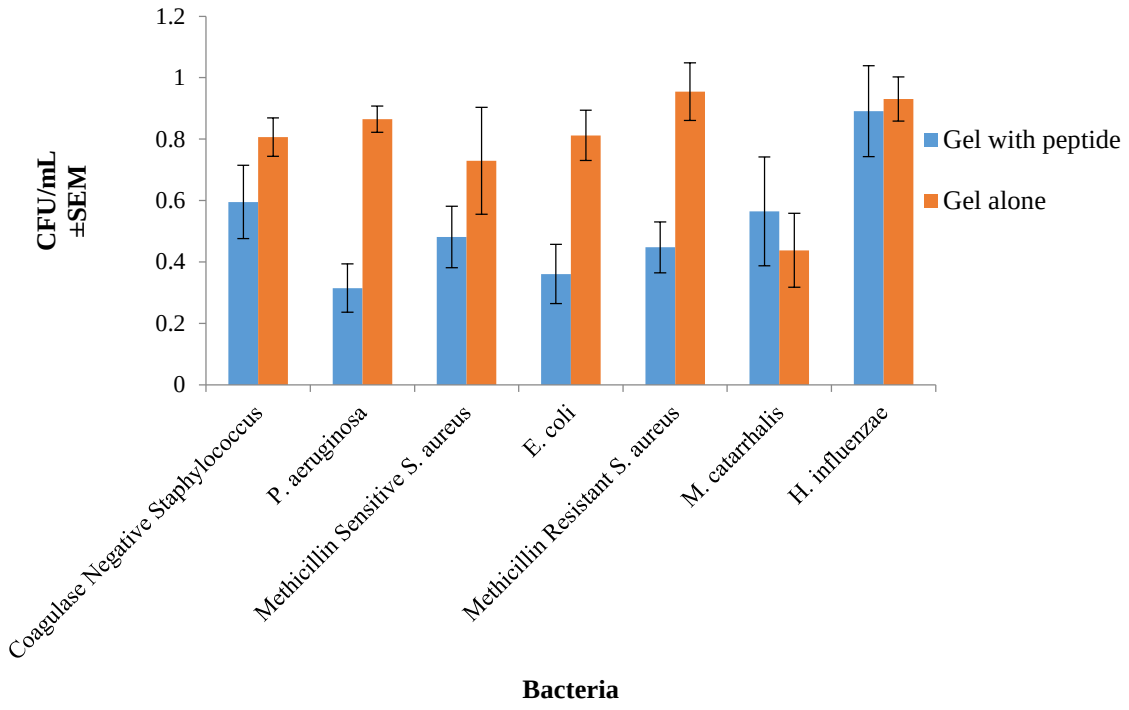

Figure 13: *Remaining colony-forming units (CFU) of multi resistant bacteria in solution following exposure to antimicrobial CGP hydrogel. Means ± SEM, n=3. Black lines indicate p<0.05.*

3.2.11 Effect of decorin concentration on gel elastic modulus

With its anti-scarring properties, the addition of decorin to the CGP system provides potential for its use in effective wound management. Dynamic frequency sweeps were performed on CGP hydrogels (500 kDa) containing different concentrations of decorin to measure any effect of the decorin on gel elastic modulus. The elastic modulus of hydrogels containing decorin at 1.9 % v/v, 3.8 % v/v and 7.4 % v/v was not significantly different to control (Figure 14), indicating that decorin has a negligible effect on gel elastic modulus at the concentrations tested.

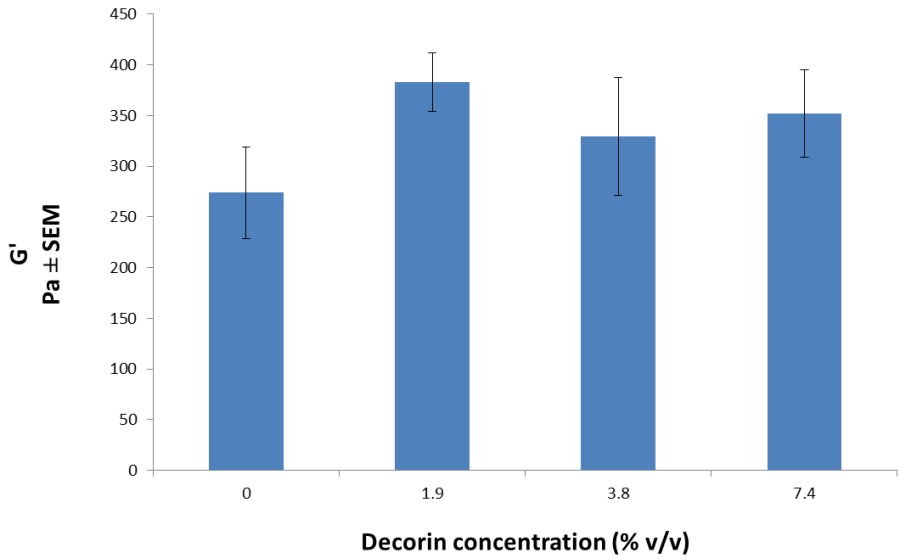

Figure 14: *Gel strength (G') of 95/2500 grade CGP hydrogels containing different decorin concentrations. Mean ±SEM, n=6. Differences not deemed statistically significant (P=0.378)*

3.2.12 NanoDrop analysis

NanoDrop analysis showed an increase in protein absorbance over 7 hours compared to control and indicates a steady release of decorin over this time period (**Figure 15**).

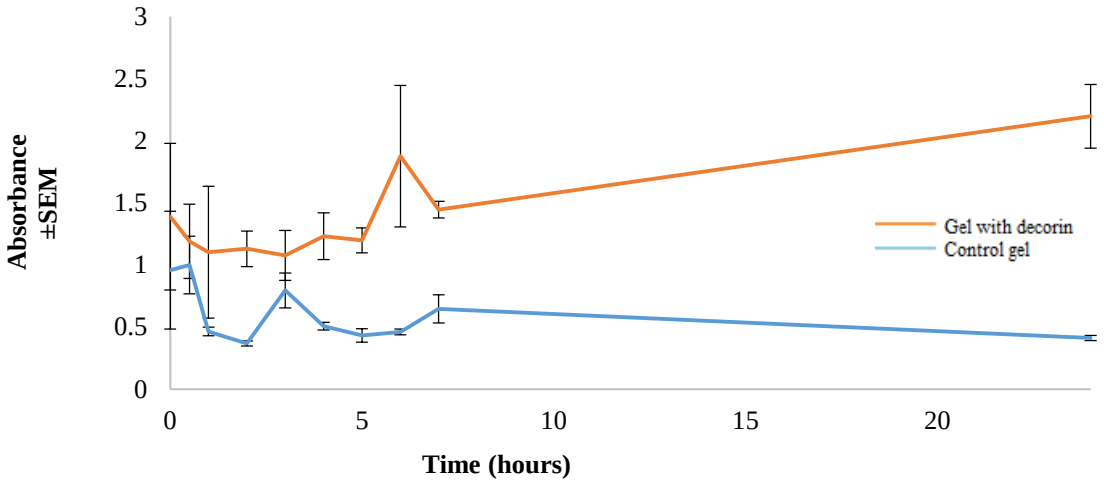

Figure 15: *Absorbance of decorin measured with NanoDrop analysis, showing decorin released from gel over tim compared to control.*

4 DISCUSSION

4.1 Characterisation of CGP gel system

4.1.1 Effect of molecular weight on gel elastic modulus

The majority of ocular medications are administered by topical delivery methods, such as eye-drops and ointments which take advantage of the accessibility of the eye (Kaczmarek et al., 2014). The disadvantage of topical delivery methods is that the eye employs efficient natural defence mechanisms which remove foreign material and therefore mean that frequent repeat administration is necessary. The aim of this project was to develop an eye dressing that enables controlled release of drugs into the eye over a sustained period of time. Ideally, this novel system retains its shape at least until the required drug dosage has been released. To enable this to happen, the gel would be sufficiently solid upon gelation. The results indicate that there was no significant difference in the solidity of gels within the range of molecular weights tested. This result is unexpected based on previous studies which report that chitosan-gelatine blends (Liu et al., 2011), chitosan films (Nunthanid et al., 2001) and chitosan adsorbing polysaccharide droplets (Hou et al., 2012) made with higher molecular weight chitosan all exhibit higher elastic modulus and viscosity than those made with lower molecular weight chitosan. Further studies have shown that chitosan complexes form stronger hydrogen and electrostatic bonds in formulations with higher molecular weight chitosan than in other formulations (Honary, Maleki and Karami, 2009). Although the degree of deacetylation (DD) of chitosan has been found to modulate mechanical properties of the GP gel (Liu et al., 2011), with higher DD giving way to greater mechanical elastic modulus, all chitosan grades used in this project were of ~95% DD so it is unlikely that this was an

influencing factor in this case. The average molecular weight tested in these studies is 290 kDa, compared to 410 kDa in this project and so it is possible that differences in gel elastic modulus are less between higher molecular weight grades of chitosan. It is also possible that in the case of the CGP system, the addition of GP limits any increases in elastic modulus with molecular weight that would arise in chitosan solutions alone. The gel must be sufficiently rigid to hold its shape for long enough to allow the optimal dose of decorin and AMP to be released. Premature degradation of the gel would limit the wound-healing effectiveness of the system.

4.1.2 Imaging of optical properties of gel

The successful gelation of CGP on the surface of a glass sphere at 37 °C was to be expected based on previous reports of gelation of this system at 37 °C (Chenite et al., 2001). Further, the results show optical transparency of the gel which provides exciting potential for the use of CGP as an ocular wound dressing that still allows patient vision. The thickness of the CGP dressing was measured at 1 mm which is similar to that of other current eye treatments, such as soft and hard contact lenses whose centre thickness measures at ~ 30 µm and ~ 100 µm respectively. Overall the results suggest promising evidence that the CGP hydrogel could be used successfully on the surface of the eye without causing significant obstruction.

4.1.3 Effect of temperature on gel elastic modulus

The results show an increase in gel elastic modulus (G') with increasing temperature, as expected based on previous investigation into the thermal properties of the CGP system (Chenite et al., 2001; Cho et al., 2005). Based on previous work, a sigmoidal curve showing

G’ as a function of temperature was expected and although the results do not show a high-temperature plateau, the general shape of the curve is as expected and would likely have plateaued if a broader temperature had been explored. The onset of gelation, as characterised by an abrupt increase in G’, is shown at 34 °C which is slightly below 37 °C which has previously been reported as the gelation temperature of this system (Cho et al., 2005; Ahmadi and Bruijn, 2008). As an external surface, the cornea changes temperature depending on both body and ambient temperatures. Corneal temperature has been found to reach a maximum of 37 °C, and at ambient temperature lies between 32 °C and 34.5 °C (Kessel et al., 2010). Therefore, in the case of corneal applications, it is advantageous for the CGP system to gel at 34 °C or lower.

4.1.4 Cytotoxicity Assay

Corneal fibroblast (CF) cells are specialized cells found within the cornea and which play a major role in allowing transparency, synthesizing corneal components and effecting healing. Cytotoxicty measurements of the CGP gel on CF cells is important in order to determine the feasibility of using this hydrogel successfully for wound healing of the cornea. The cytotoxicity assay conducted showed that there was no significant difference in the number of surviving CF cells that were exposed to different molecular weight CGP gels compared to control. These findings are in line with previous chitosan-toxicity studies which showed low toxicity of chitosan in the case of intravenous, oral and nasal administration (Hirano et al., 1989; Aspden et al., 1997; Knapczyk et al., 1989). Furthermore, Felt et al. (1999) found that there was good ocular tolerance for chitosan in rabbits following topical administration of a chitosan-based eye dressing to the corneal surface.

4.1.5 Effect of shear rate on viscosity

CGP hydrogel viscosity was found to be inversely proportional to shear rate. These findings are supported by previous work in this area, which found the same relationship explained by the viscoelasticity exhibited by the system (El-hefian and Yahaya, 2010). The benefit of this viscoelastic behaviour is that it facilitates the movement of the eyelid during movement and allows the gel to retain its shape, whilst enabling surface spreading with the shearing force applied during blinking (Li and Chauhan, 2006).

4.1.6 Effect of solution on gel elastic modulus

After one week in solution (PBS), CGP hydrogel (500 kDa) displayed a significantly lower elastic modulus (G') than control gel. Weight measurements of the hydrated CGP gel showed that water uptake is 7% of initial gel weight. This value is relatively low, which is to be expected due to the presence of GP which forms cross links with chitosan and limits the degree of swelling that can occur (Dusek et al., 2014).

4.2 Addition of therapeutic agents

4.2.1 Effect of peptide concentration on gel elastic modulus

Although there was no significant difference between gels containing the polyarginine antimicrobial peptide at 1.9% (v/v) and 3.8 % (v/v), the significant difference ($p < 0.05$) between gel with peptide at 3.8 % (v/v) and control suggests that gel elastic modulus increases with peptide concentration. Previous studies showed that tensile elastic modulus of chitosan-PVA gels (Sung et al., 2010) and the storage and loss moduli of chitosan-collagen

hydrogels (Reis et al., 2012) were unaffected by the addition of proteins. However, Rask et al., (2010) found that elongation was significantly greater in chitosan formulations containing peptide than those without. As discussed in section 1.4.2, the polyarginine antimicrobial peptide used in this study is highly positively charged and therefore provides an abundance of sites for the electrostatically charged CGP network to bind to. In section 1.3.5 the effect of NH_2 groups on chitosan on CGP network formation was discussed and it was reported that a greater number (resulting from a higher DD) produced a more uniform and connected gel. It is possible therefore that the addition of polyarginine AMP, as a positively charged molecule, results in the formation of strong ionic bonds between chains which increases the integrity of the system.

4.2.2 Peptide release rate with molecular weight

As expected, peptide was successfully released from all CGP gels, however the rate of release was not significantly different between the molecular weights of chitosan tested which is contrasting to previous findings in this area (Desai, Liu and Park, 2006). In previous studies, chitosan hydrogels containing high molecular weight chitosan exhibit lower release rates than other molecular weights (Honory, Maleki and Karami, 2009). Khodaverdi et al. (2012) found that increasing both GP and peptide concentrations resulted in slower release rates. These findings indicate that the rate of peptide release involves a number of parameters and that in this project the effect of chitosan molecular weight may have been modulated by the concentrations of GP and peptide used. A release profile of insulin from CGP gels showed a biphasic response, with an initial low burst followed by steady release over the following days (Khodaverdi et al., 2012). Similarly, the results of this project show an increasing short-term

peptide release (0-7 hours) which indicates a steady peptide release immediately following application that would be advantageous to the wound healing properties of the eye dressing.

4.2.3 Imaging of peptide dispersion through gel

Results of peptide addition to the CGP gel show a non-homogeneous dispersion.

4.2.4 Microbiology of hydrogel

Results of antimicrobial testing on the CGP gel show that it has a significant effect against *H. influenza,* Methicillin Resistant *S. aureus* and Coagulase Negative *Staphylococcus* cells and CFU solution tests showed significant differences between Coagulase Negative *Staphylococcus* and compared to control. In the case of bacteria being less present on the surface of the antimicrobial gel than on control (Fig. 12), it is likely that the peptide has worked effectively in killing target bacterial cells. Results of CFU solution may have a number of explanations and will depend on the release of peptide out of the cell and movement of bacteria into the gel. For example, a low bacterial count in solution could mean either the peptide has migrated into solution and successfully targeted the bacteria, or that the bacteria have migrated into the gel and out of solution.

4.2.5 Effect of decorin concentration on gel elastic modulus

The results show that decorin concentration did not have a significant effect on gel elastic modulus. Although currently there has been little investigation into the addition of decorin to hydrogels, previous decorin studies suggest that its functionality is modulated by an ability to

self-associate (Bittner et al., 1996). This self-association may lead to low interaction with the CGP network, causing decorin to have a negligible effect on gel properties and rather act in a similar way to the solvent. Alternatively, by comparison to the increase in gel elastic modulus with peptide concentration, it is likely to be the charge density of each molecule which dictates the extent of its effect on the CGP network and as a molecule with low charge density, that decorin is unable to form strong bonds with the existing network structure. A combination of low charge density and self-assembly properties may be the reason for decorin having no effect on gel elastic modulus.

4.2.6 Release profile of decorin

Investigation was carried out by way of NanoDrop analysis on the release medium to measure short-term decorin absorbance. Results of this test, which measures all protein present in the sample, showed an increase in absorbance over 7 hours compared to control and indicates a steady release of decorin over this time period. As discussed by Li and Chauhan (2006), a longer residence time of the ophthalmic drug is beneficial for ensuring full drug delivery. Ideally, the CGP system would provide sufficient robustness to enable decorin to be fully released at a rate that it can be absorbed readily by the target tissue. To further increase the efficiency of the system and speed up the wound-healing process, decorin would be released steadily over a short time period, for example a couple of hours. This would decrease the treatment time and enable the patient to return to full health quickly.

5 CONCLUSIONS

A successful thermosensitive CGP gel system that is liquid at room temperature and which gels at 34 °C has been produced. Furthermore, antimicrobial and anti-scarring agents have been successfully incorporated into the system, at no expense of the mechanical properties of the gel and which are able to be released steadily from the gel into solution. Therapeutic properties and low cytotoxicity of the gel have been proved and provide promising evidence for the use of this system in a wound-healing application.

6 FURTHER WORK

6.1 Cytotoxicity studies

To develop the CGP gel for use in ocular wound healing, further cytotoxicity studies should be done to investigate possible toxic effects on a wider range of ocular cells. In application, the gel would interact with both endothelial cells and keratocytes on the cornea. Investigating possible cytotoxic effects on the latter cells would be particularly important due to their role in general repair and maintenance of the cornea. The studies should follow a similar process to the study described in this report.

6.2 Mechanical testing

Further mechanical tests should be carried out to investigate the effect of repeated stresses, caused by eye movement, on the performance and structure of the gel. Tensile testing would be useful to determine the tensile strain of the gel and understand its ability to return to its original shape following an applied tensile load, such as that of the eyelid. Perhaps most

useful would be to determine the tensile elastic modulus of the gel, as indicated by the tensile stress applied at the point of yielding. Tensile stress should be used to give the elastic modulus of the gel which indicates its hardness. Similarly, to be able to predict the movement of the gel in application, it would be necessary to model the movement of solution across the surface of the eye and measure the release rate of therapeutic agents from the gel in this dynamic environment.

6.3 Microbiology studies

Further testing should be conducted on a wider range of bacteria to develop understanding of the clinical potential for the gel. As well as bacteria related to corneal disease, bacteria commonly associated with other body conditions should be tested for toxicity to determine the effectiveness of the gel in other areas.

6.4 *In vivo* drug release study

Following satisfactory laboratory studies, the CGP-decorin system should be tested *in vivo*. In compliance with relevant standards in animal testing, the gel should be injected onto the surface of the mouse cornea following corneal damage (i.e. a cut). The robustness of the gel should be observed both with and without eyelid interference to determine the longevity of the system.

To determine the release profile of decorin *in vivo*, and also the effectiveness of the system at corneal-healing, the gel should be injected into a number of mice. At set time points, for example every hour, the gel would be removed from one of the mice and the extent of healing observed/measured by the amount of healthy tissue that has been regenerated.

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