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A self-healing hydrogel eye drop for the sustained delivery of decorin to prevent corneal scarring



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ABSTRACT

Scarring/Opacity on the surface of the eye and vascularisation following infectious diseases, inflammation and corneal trauma are often a leading cause of blindness. The ‘gold standard’ treatment to prevent corneal scarring is the application of amniotic membrane (AM) to the ocular surface in the acute stage of injury. Although clinically effective, the use of the AM is associated with biological variability and unpredictable responses. Potential health risks including disease transmission, significant ethical issues surrounding the tissue donation process and stringent regulations/storage conditions, preclude widespread use. Consequently, there is a demand for the development of a new, synthetic alternative, that is stable at room temperature, capable of protecting the wound and has the capacity to deliver anti-scarring and anti-inflammatory mediators. Here we have developed a micro-structured fluid gel eye drop, to deliver a potent anti-scarring molecule, decorin. We have compared the release of decorin from the formulated dressing to a typical gel film, demonstrating enhanced release for the fluid gel eye-drops. Therefore, we have investigated the effect of the fluid gel system in 2D human corneal fibroblast culture models, as well as shown the retention of the gellan fluid gel in an *in vivo* rat model. At the same time the efficacy of the fluid gel eye drop was studied in an organ culture model, whereby the fluid gel containing decorin, significantly ($P < 0.05$) increased re-epithelialisation within 4 days of treatment.

1. Introduction

The ocular surface is a highly organized and delicate structure, comprising the cornea and conjunctival tissues. Its avascular nature and the intricately aligned layers of collagen fibrils are responsible for the optical transparency of the cornea [1]. Various diseases, nutritional deficiency or traumatic injury can lead to: corneal ulceration, stromal loss, tissue remodelling, scarring and limbal corneal epithelial stem failure, resulting in recurrent ocular surface breakdown. This increases the risk of secondary infection associated with loss of biomechanical integrity such as: corneal distortion, perforation and irreversible blindness [2]. Generally, when the cornea becomes damaged, the tissue is typically able to repair itself, resulting in normal function. However, in cases where it becomes exposed to pathogenic microorganisms, this does not occur. Breaches in the corneal epithelium, often observed as a consequence of prolonged contact lens wear, enable bacterial invasion

into the corneal stroma leading to amplified innate immune responses; characterized by heightened inflammatory cell recruitment, in an attempt to eradicate infection. Concurrent up-regulation of various growth factors such as transforming growth factor (TGF- β 1), and platelet-derived growth factors (PDGF) stimulate the tissue repair process including corneal stromal keratocyte activation, myodifferentiation, and laying down of extracellular matrix (ECM) [3,4]. This combination of growth factors triggers fibrotic pathways leading to further disruption of the highly ordered collagen fibrils, and ultimately drives scar formation. The presence of scar tissue, primarily composed of irregularly deposited collagenous matrix, with or without corneal neovascularization, results in loss of corneal clarity and irreversible loss of sight. Thus, once the acute phase of injury has subsided, surgical removal of visually disabling scar tissue is required, with corneal transplantation remaining the mainstay.

The current ‘gold standard’ treatment in preventing the formation of

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corneal scars and minimizing tissue damage, is the application of de-nuded amniotic membrane (AM) to the ocular surface; throughout the acute phase of injury [5]. The bandaging effect of the amnion dampens cascading of fibrogenic signalling during the various stages of healing [6–8], as well as providing anti-fibrotic/anti-inflammatory factors to the wound site, to assist healing [9,10]. Although clinically effective, the use of AM is typically limited due to reproducibility/repeatability depending on the inter- and intra-amnion variability. Furthermore, the technique requires additional surgery, as reapplication after 2–3 weeks is needed. The widespread use of AM has been hindered, as it is highly regulated and requires very specialised storage conditions. This is also coupled with significant ethical issues surrounding the tissue donation process, and potential health risks such as disease transmission. As such the drive towards alternative treatment strategies including the development of amniotic membrane extract drops [11,12], has been sought. Lyophilized amniotic membrane extract is powdered and reconstituted with saline (0.9% NaCl). The treatment is applied directly to the ocular wound, delivering healing factors to the site of trauma. Here the duration of therapy is guided by the individual healing response. However, to date, no technology has matched or replaced the therapeutic capability of AM products, with regards to surface healing responses.

Historically the most common forms of ocular therapy are eye drops systems, for their ease of application and direct delivery to the active site. However, typically such systems lead to pulsed release mechanisms, as a consequence to rapid elimination through lid overflow and drainage through the nasolacrimal duct [13]. As a result of poor retention, much effort has been placed into engineering novel methods of extending residence times upon the ocular surface. Gellan gum has been at the forefront of this drive, its inherent mucoadhesive properties aid adsorption to the cornea, binding the water, and prolonging the residence of the therapeutic [14]. As such gellan has been used in many eye drop formulations, commonly as a viscosity modifier in very low concentrations (<0.1%). Unfortunately, the timescales required for sufficient bioavailability/efficacy is still not met using topical eye drops. This has led to a considerable body of work focusing on *in situ* gelation of gellan based ophthalmic solutions, with the intention of controlled release/delivery [15]. In such cases solutions are applied to the eye where gelation is mediated through pH [16], temperature [17], or salt ions found within the tear fluid [18]. However, many gels formed in this manner have alterations to the polymer backbone or secondary co-polymers, providing the necessary chemistries for mechanical stability or delivery [19–21]. However, the multiple components of which many are not currently FDA approved prevents many of the systems from being translated through to the clinic. Therefore, there remains a clear need for systems that previously undergone regulatory approval, where by manipulation of the microstructure in a chemically inert way gives rise to functional material properties.

Fluid or shear gels are a prime example of a system where control over the processing has led to microstructures which enhance the bulk properties [22]. Fluid gels, unlike solutions with added viscosity modifiers are micro-particulate suspensions produced by applying a constant mechanical separation process (shear field) during the sol-gel process [23]. This results in a system where the chemical composition of the components remains unchanged, but the physical properties can be controlled; thus providing a direct route to translation [24]. Viscoelastic behaviours arise through weak structuring between particles, as a function of the microstructure formed during processing [23,25,26]. This gives solid-like behaviour at rest (like *in situ* gels) but can flow on manipulation to slowly be eliminated over time. Furthermore, unlike previously reported micro gel systems [27,28], the gelled micro-particles are formed through a nucleation and growth mechanism, while the applied shear limits the degree of long ranged ordering [23]. Therefore, rate and extent of the ordering during the shearing process ultimately dictates the degree of inter-particle structuring and ability to form reversible particulate to continuous networks

[29].

This study seeks to control the microstructure of gellan gum hydrogels, in order to produce a novel topical ocular therapy that can dynamically invert through solid-liquid-solid transitions during the natural blinking mechanism of the eye. As such an ocular bandage can be achieved in the form of a topical eye drop, with the ability to deliver actives whilst retaining a high degree of retention upon the surface. Therefore, a comparison of the release properties has been made between two micro-structured gels: quiescently formed gels (equivalent to *in situ* gels reported in literature) and the fluid gels, subsequently moving on to describing its ability to aid wound healing in both *in vitro* and *ex vivo* models. The work uses a potent anti-scarring molecule (decorin), present in AM, as the active ingredient. Decorin, is a leucine rich glycoprotein that attenuates fibrotic scarring by sequestering TGF β 1&2, directly interacting with collagen fibrils and limiting fibrillogenesis [30,31]. Previously, decorin has been shown to reduce scarring in animal models for: intraocular pathologies including glaucoma and proliferative vitreoretinopathy [32], together with brain injury, spinal cord injury [33] and inflammatory hydrocephalus [34]. Although highly effective in scar reduction, decorin is delivered in systems that cannot be sustained upon the surface of the cornea long enough to demonstrate a therapeutic effect. Therefore, this work demonstrates the enhanced bioactivity and efficacy of decorin from fluid gels as a potential topical treatment in ocular diseases.

2. Materials and methods

2.1. Materials

Low acyl gellan gum (Kelco Gel™) was kindly donated by Azelis, UK, sodium chloride was purchased from Sigma-Aldrich (UK), and Galacorin (decorin) in PBS – 4.76 mg/ml was purchased from Catalant, USA.

2.2. Preparation of gellan delivery devices

2.2.1. Gellan films

Gellan films were produced by adding low acyl gellan gum (gellan) at a concentration of 2% (w/v) to MilliQ water at 90 °C while stirring. The resultant solution was cast on plastic polystyrene dishes (10 cm × 10 cm) and left to cool for 10 min. The cast gels were further dried using an oven (VacuTherm, UK) at 50 °C for 17 h. Films containing decorin were prepared through the addition of the protein in PBS to the gellan sol at 60 °C to a final concentration of 0.24 mg/ml before casting the films in the plastic polystyrene moulds.

2.2.2. Gellan fluid gels

Fluid gels were prepared by adding low acyl gellan gum (gellan) at a concentration of 1% (w/v) to MilliQ water at 90 °C whilst stirring. Once fully dissolved the hydrocolloid solution was cooled to 60 °C, and subsequently cooled further after being loaded into an AR-G2 Rheometer (TA Instruments Ltd, UK) at 1 °C/min whilst being sheared at a shear rate of 450/s using a cup and vane geometry (cup: 35 mm diameter, vane: 28 mm diameter). Upon reaching a temperature of 40 °C, decorin (Galacorin) was added to a final concentration of 0.24 mg/ml. Further crosslinker (NaCl) was then added at concentrations of 0.05, 0.1 and 0.2 M; resulting in final concentrations of 0.9% w/v gellan, 2.5, 5 and 10 mM NaCl. Shear viscosity was monitored during fluid gel production. The onset of gelation was identified at the temperature when a 0.08% change in viscosity was observed.

2.3. Dynamic oscillatory rheology

The structure and particle interactions of the fluid gels were elucidated by dynamic oscillatory measurements on a AR-G2 Rheometer (TA Instruments Ltd, UK) using a sandblasted parallel plate geometry (1 mm

gap, 40 mm diameter). Dynamic storage modulus (G') was determined at constant frequency of 1 Hz at a range of 0.1–100% strain.

2.4. Decorin release

Decorin release from the hydrogel systems was conducted in phosphate buffered saline (PBS) and evaluated using a decorin ELISA (R&D, UK), following instructions from the manufacturer. In brief, dried gellan films were prepared to fit in a 6 well plate, containing a total 0.24 mg of decorin. Films and fluid gel (1 ml containing 0.24 mg/ml of decorin) were added to the well of a 6 well plate. Following, PBS (4 ml) was pipetted into the well so that the hydrogels were completely submerged. In the case of the fluid gel, precaution was taken not to disrupt the gelled phase, preventing break-up of the gel and the need for dialysis tubing (Fig. S1). The samples were incubated at 37 °C and aliquots (0.5 ml) removed at each time point (5, 10, 15, 20, 25, 30, 45, 60, 75, 90, 105, 120, 150, 180, 210, 240 min), replenishing the volume in each case with fresh PBS. Active concentration was subsequently analysed using ELISA with all experiments undertaken in triplicate.

2.5. Fluid gel transparency measurements

Measurements of optical transparency were performed using a CE 7500 double beam UV/visible spectrophotometer. Fluid gels were added into 1 mm cuvettes; transmittance (%) at 600 nm was recorded. The instrument was zeroed using sterile MilliQ water as a control. Three tests were performed and results averaged. Standard error bars are plotted as the standard deviation.

2.6. In vitro studies

2.6.1. Corneal fibroblast culturing

Corneal fibroblasts (CFs) were cultured in Dulbecco's Modified Eagles Medium supplemented with 10% FBS (Sigma Aldrich, UK), 2.5% Penicillin Streptomycin (Sigma Aldrich, UK) and 2.5% L-Glutamine (Sigma Aldrich, UK). Corneal fibroblasts were trypsinised and seeded in 6 well plates at a seeding density of 2×10^5 cells/ml and left to attach to for 16 h, 10 ng/ml TGF- β 1 was added to the cultures to differentiate primary fibroblasts to myofibroblasts for 7 days. Fluid gels with and without decorin were added to the cultures. Cell viability and metabolic activity were assessed using Calcein AM and MTT respectively. Collagen deposition was determined using Sirius red staining and the hydroxyproline assay.

2.6.2. Live/dead (Calcein AM/PI) assay

Corneal fibroblasts were cultured as in section 2.5.1. Viability was assessed using Calcein AM (50 μ g/ml) and propidium iodide (PI, 100 μ g/ml). The dyes were added to the cultures and left for 5 min in an incubator. The stained cultures were observed using a fluorescence microscope (Carl Zeiss Ltd, Hertfordshire, UK) and images of live and dead cells stained green and red respectively were captured using a digital camera (Powershot G5, Canon UK Ltd, Surrey, UK).

2.6.3. Metabolic activity (MTT) assay

Corneal fibroblasts were cultured as in section 2.5.1. Thiazolyl blue tetra-zolium bromide (MTT) (5 mg/ml) was added to the cell cultures and incubated for 4 h at 37 °C to assess cell metabolic activity. MTT is reduced to purple formazan crystals in living cells by mitochondrial reductase. The formazan crystals were dissolved using HCl-isopropanol solution and read within the hour at 620 nm on a plate reader (Glomax Multi Detection System Plate reader – Promega).

2.6.4. Sirius red assay

Corneal fibroblast cultures were washed with phosphate buffered saline (PBS) and fixed with Bouin's fluid for 1 h. Fixed cultures were washed with distilled water and left to air dry for 15 min. Sirius red

solution (1 mg/ml) prepared in saturated picric acid, was added to the cultures and left on a plate shaker for 1 h at room temperature. Excess dye was aspirated and the stained cultures were washed with hydrochloric acid (0.01 N). Images were taken using an inverted light microscope. For semi-quantitative analysis, the stain was lifted using NaOH (0.1 N). The dissolved dye was transferred to a 96 well plate and read using a plate reader (Glomax Multi Detection System Plate reader - Promega) at an optical density (OD) 550 nm against NaOH (0.1 N) blank.

2.7. Ex-vivo studies

2.7.1. In situ eye drop structuring

Structuring upon the ocular surface was tested on freshly enucleated pig eyes. 4 pig eyes were sourced from a local abattoir (Coventry, UK) and used on the same day that they were collected. Eyes were refrigerated between collection and use. The eyes were washed with Dulbecco's Modified Eagles Medium (DMEM) and placed in a plate with the eyes facing upwards. 150 μ l of the fluid gel was placed on the surface of the eyes; PBS was used as a control. The eyes were photographed (Nikon, D7100, UK) to observe spreading over time.

2.7.2. Organ culture model for wound healing

The efficiency of the fluid gel with and without decorin in an ex-vivo organ culture model of corneal wound healing. Eight-to ten-week old, male, 175–200 g, Sprague Dawley rats (Charles River, Kent, UK), Rats were sacrificed by exposure to increasing concentrations of CO₂ at the Biomedical Services Unit at the University of Birmingham (Birmingham, UK) in accordance with the Home Office guidelines set out in the 1986 Animal Act (UK). Wounding was performed within 1 h of sacrifice. The globe was then irrigated with PBS. A full thickness 5 mm corneal abrasion was produced using a 15° micro-scalpel. The globes were enucleated, fixed in 6 well plates and treated with 40 μ l of fluid gel with and without decorin ($n = 4$). The globes were topped up with DMEM 2.5% Penicillin Streptomycin and 2.5% L-Glutamine. Specimens were maintained at 37 °C and 5% CO₂ for 48 h. For H&E staining the eyes were removed and fixed in 4% formaldehyde. The corneas were dissected, washed in PBS for 30 min and cryo-protected by sequential immersion in increasing concentrations of sucrose solutions in PBS (10%, 20% and 30% v/w) at 4 °C. The corneas were then embedded in Tissue-Tek® O.C.T.™ compound (Sakura Finetek UK Ltd, Thatcham, UK) and frozen in embedding moulds with the dimensions of 22 mm \times 22 mm square \times 20 mm deep. The corneas were placed in a convex position, while pushed against one of the block walls Solidified embedding blocks were mounted on a chuck in a Bright cryostat (Bright Instruments, Luton, UK), radially sectioned (15 μ m thick; 12.5° angle of blade), adhered onto Xtra™ adhesive slides (Surgipath, Peterborough, UK), allowed to dry, and stored at –20 °C before staining. Sections are differentiated in 1% v/v acid-alcohol solution (1% HCl in 70% ethanol) for 5 s which removes the excess staining, rinsed in running tap water for 1 min, blued in 0.1% w/v sodium bicarbonate (NaHCO₃; VWR International Ltd) for 2 min, and then rinsed in running tap water and immersed \times 2 in 95% ethanol for 1.5 min. This was followed by exposure to the cytoplasmic counterstain in alcoholic Eosin Y solution (0.5% w/v in acidified 90% ethanol) for 1 min. Sections were then dehydrated through an ascending series of alcohol solutions: 70%, 90% and 2 changes of 100% ethanol and cleared in 3 changes of HistoClear, and finally mounted in Vectamount™ permanent medium (Vector Laboratories). The histological images were captured using Axioplan 2 microscope equipped with an Axiocam HRc lens running Axiovision software Version 4.8.2.0 (Zeiss).

2.8. In vivo studies

2.8.1. Gellan fluid gel retention on rat corneas

Rats were anaesthetised by intraperitoneal injection of a mixture of

ketamine hydrochloride (2.4 ml of 100 mg/ml), medetomidine hydrochloride (1.0 ml of 1.0 mg/ml), and injectable water (9.0 ml) at a dose of 3.0 ml/kg body weight. Six rats ($n = 12$ eyes) received 4 different eye-drop formulations (3 eyes/treatment). Having received 40 μ l of the respective eye drop formulation, anterior segment OCT images were acquired immediately (0 h) and repeated every 30 min while anaesthetised for up to 2 h using a Spectralis HRA3 confocal scanning laser ophthalmoscope (Heidelberg Engineering, Heidelberg, Germany). Rats were killed at the end of the 2 h by cervical dislocation. Images were analysed using ImageJ software (National Institutes of Health, Maryland, USA) at 3 different regions of the corneal surface (45° (nasal), 90° (central), 135° (temporal)).

3. Results

3.1. Formation of gellan gum films and fluid gels

Gellan gum may easily be gelled into a rehydratable, optically transparent structure. Casting of a 2% w/v solution and cooling to < 37 °C forms a gel through ordering of the biopolymer from random coil to structured helices [35]. These cast gels may be further processed via a drying stage at 50 °C, resulting in transparent films of thickness 25–30 μ m that can be rehydrated immediately prior to use (Fig. 1B). Molecules such as decorin may be absorbed into the structure in a facile manner, and undergoing release when placed in an aqueous environment. The optically inert nature of the gellan gel means that it is perfect for use as an eye dressing, however, the irregular contouring of the ocular surface and gel dehydration make the material impractical for use on the cornea. By modifying the processing conditions, it is possible to make a substrate with similar optical properties, that can be simply delivered onto the surface of the cornea, in the form of an eye drop.

Existing topical drugs such as eye drops, although commonly used, are relatively inefficient as drug delivery systems. This is due to the high drop to pre-corneal ratio, where eye drops often range from 25 to 50 μ l and the pre-corneal volume in humans is ca. 7 μ l [36]. As such, bio-efficiency is often very low as firstly, the excess volume is quickly

cleared as previously discussed, followed by rapid clearance of the pre-corneal tear film (approximately 20%/min [36]). One way to increase the effectiveness of ocular delivery systems would, therefore, be to design materials which can remain on the corneal surface for extended timeframes. Simultaneously, these drops would need to deliver therapeutic levels of active drugs, locally or to the posterior segment. Such materials, in the form of lenses have been widely investigated, where the synthetic lens swells in the presence of water subsequently loading with targeted therapeutics [37–39]. Controlled delivery can then be achieved by tailoring diffusion of the molecules from the lens to the eye, once applied. Although promising the use of contact lenses for drug delivery has not taken hold, commonly due to their application to infected or otherwise damaged eyes, exacerbating the condition [40]. Therefore, resorbable structured gels or fluid gels, are gaining attention. Such structuring allows for materials such as gellan to be formed into a flowable material exhibiting similar optical properties to unmodified gels. The inserts to Fig. 1C visually illustrate the properties of a fluid gel, whereby on inversion in the absence of shear, *i.e.* mixing or shaking, the gel does not flow. However, under force, the system begins to flow. Such transitions were probed using dynamic, small deformation oscillatory techniques, measuring the material viscoelasticity (G' (storage modulus) and G'' (viscous modulus)). The plot in Fig. 1C demonstrates the strain dependent properties of a typical gellan fluid gel. At low strains, within the linear viscoelastic region (LVR), G' dominates G'' highlighting a solid-like system [41]. At larger strains a decrease in G' is observed as the weak interactions between the ribbon-like network (seen in the light microscopy image Fig. 1A) become cleaved, allowing the system to untangle and flow. This transition from solid to liquid like behaviour, effectively increases the retention time of the gel upon the eyes' surface, forming a soft structure that becomes slowly removed by blinking.

As previously described, tailoring of the fluid gel properties can be controlled during the processing stage. Here the fluid gels were produced on a rheometer, whereby the formulations can be characterized in real time, during processing (Fig. 1D). Fig. 1D demonstrates the change in viscosity as the system is forced through the gelation process

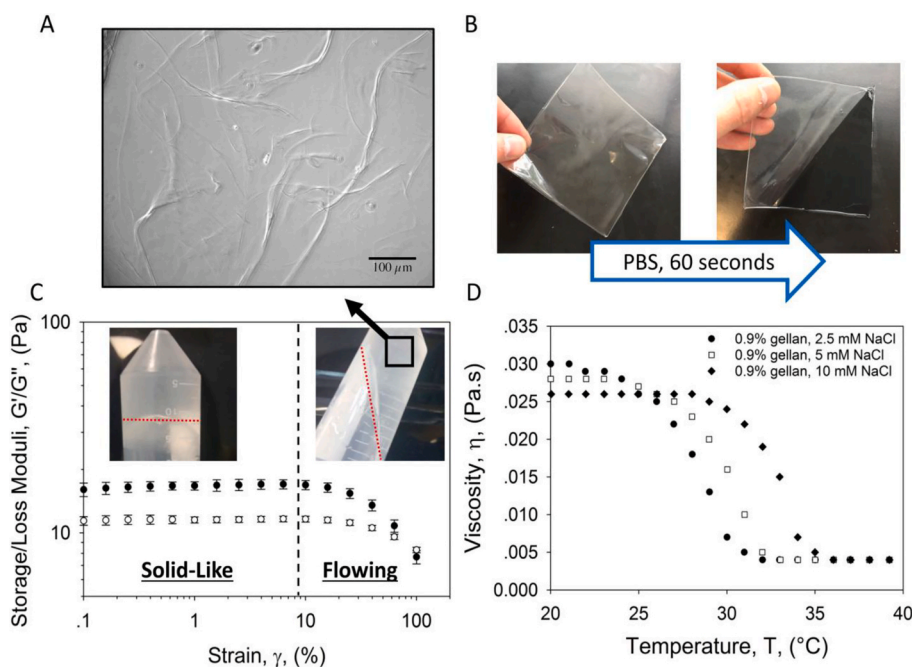


Fig. 1. Micrograph illustrating the fluid gel structure using light microscopy (A). Images illustrating a Gellan Gum film, pre- and post-rehydration in PBS (B). Strain sweep obtained at 1 Hz for a 0.9% w/v gellan fluid gel crosslinked using 5 mM NaCl. Inserts show photos demonstrating the flow of a fluid gel before and after shear is applied to the sample. Shear can be applied by either mixing or shaking of the tube, which disrupts the weak interactions between gelled ribbons (C). Graph demonstrating the formation of fluid gels with various concentrations of NaCl in real time; as temperature decreases the viscosity of the system increases as the gellan fluid gel is formed (D).

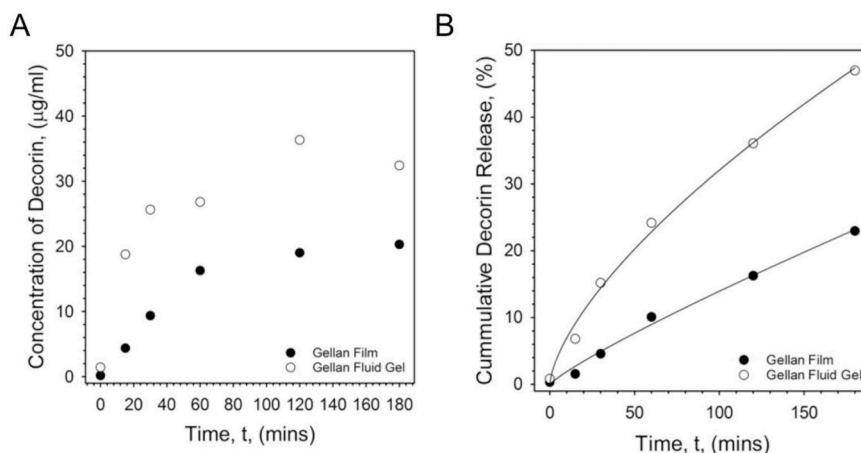


Fig. 2. Decorin release from the two different gellan formulations, films and fluid gels. Fluid gels exhibited increased release of decorin 35 µg/ml when compared to the films (20 µg/ml) across the same timeframe, 3 h (A). Cumulative % release of decorin, demonstrating changes in release for both the fluid gel and gellan film. Equations for lines of best fit are as follows: $y = 0.26x^{0.88}$ ($R^2 = 0.99$) and $y = 1.5x^{0.69}$ ($R^2 = 0.99$), for the film and fluid gel respectively (B).

under shear for 0.9% w/v gellan systems crosslinked using: 2.5 mM, 5 mM and 10 mM NaCl. As the temperature is lowered a rapid increase in viscosity is observed resulting from the gelation of the gellan hydrocolloid, until a maximum viscosity is reached (T_{max}). Beyond this point no further structuring is observed, as illustrated by the plateau in viscosity. The flow profiles indicate a salt dependency for both the onset of gelation and final viscosity. The onset of gelation increased as a function of the salt concentration, shifting to higher temperatures. However, the inverse correlation was observed for the final gel viscosities (it is important to note that viscosity values obtained at 450 s^{-1} , 20°C are within the shear thinning region, as such are much higher at lower shear rates (data not presented)). Enhanced handling properties, coupled with enhanced stability at previously reported corneal surface temperatures [42], suggest that the fluid gel formulation containing 10 mM NaCl was the best to investigate further in this study.

3.2. Decorin release from gellan films and fluid gels

The release of decorin from both gellan films and fluid gels was evaluated over 3 h using a dynamic system, whereby the samples were placed in culture media at 37°C and agitated on a shaker at 250 rpm. At sampling points, the eluting solution was removed and replaced with fresh media to simulate absorption within the eye. The difference in release profiles between the two systems can be clearly seen in Fig. 2A, where decorin concentration gradients nearing equilibrium are reached much more rapidly within the fluid gel system (ca. 0.5 h) compared to the film (ca. 1 hr). It is argued that the extent and rate of decorin released is controlled by morphological changes arising from drying, resulting in glass-to-rubber transitions associated with rehydration; here a greater extent of swelling/structuring occurs as films become rehydrated (Fig. S2), leading to an effective reduction in the elution media, preventing as much protein leaving the film [43]. Accompanying kinetics were therefore observed to be a function of the gelled microstructure, with constant (close to zero-order, release index = 0.86) elution of decorin from the gellan films and release for the fluid gel much closer to Fickian with a release index of 0.66 (Fig. 2b) [44]. As such, cumulative profiles showed 25% and 45% release over the 3 h for the film and fluid gel respectively. Therefore, enhanced release profiles for the fluid gel systems when compared to the films prompted further studies *in vitro*, *in and ex vivo*.

3.3. Fluid gel-decorin activity *in vitro*, using primary human corneal fibroblasts

Gellan fluid gel toxicity was tested in 2D using human corneal

fibroblast (hCF) cultures, characterized via MTT assay. Cultures with and without fluid gels didn't provoke toxic effects to the cells over the 12 days of study (Fig. 3A). Furthermore, no significant differences between fluid gels with and without decorin (Fig. 3B) were observed in cultures stained with Calcein AM and propidium iodide (PI), used as an indicator for live and dead cells respectively. Collagen deposition was assessed using a semi-quantitative staining, Sirius Red, which binds the total collagen expressed by the hCF cultures post TGF- β 1 treatment. Reduced collagen deposition was seen in the hCF cultures treated with decorin containing gellan fluid gel after 7 and 12 days of culturing. This was when compared to hCF cultures treated with just fluid gel (Fig. 3C). The stain was further dissolved and measured using UV spectroscopy at 550 nm, a significant ($p < 0.05$) reduction in absorbance was observed at day 12, as a function of the decorin preventing the corneal cells from depositing collagen.

3.4. Fluid gel transparency and retention on the ocular surface

Transparency of the gellan fluid gels was determined using UV spectroscopy over 35 days, illustrated by Fig. 4A, showing optical stability at 90% transmittance throughout the study. Following optical clarity studies, flow and re-structuring of gellan fluid gel, that is, the time between the removal of the shear force (during application) and re-thickening, was monitored using freshly enucleated porcine eyes. The gel was applied topically to the cornea, inducing liquid-like behaviour. Immediate thickening of the gel was observed on contact with the ocular surface, demonstrating a thickening time which was lower than experimentally obtainable in this manner ($< 1 \text{ s}$) (Fig. 4B).

The thickening of the gel was further tested *in vivo*, whereby 50 µl of the gel was dropped on the surface of anaesthetised rat eyes and clearance time evaluated (Fig. 5A). A thin layer of gellan fluid gel remained on the surface of the rat cornea after 90 min (Fig. 5C). When compared to PBS, the retention time was greatly enhanced, as the PBS cleared within the first 60 min (Fig. 5B). The mean thickness of the gel was 1000 µm at t_0 and reduced to 50 µm after t_{120} . However, the PBS drop had a mean thickness of 800 µm at t_0 and could no longer be observed at the 60min evaluation point post-application. Interestingly, when comparing the spreading of the fluid gel using thickness measurements centrally (90° - See Fig. 5C), temporally (135°) and nasally (45°) across the eye's surface, there was no significant difference. This indicates an even coverage as the gel flowed and spread upon the ocular surface for 2 h (Fig. 5D and E).

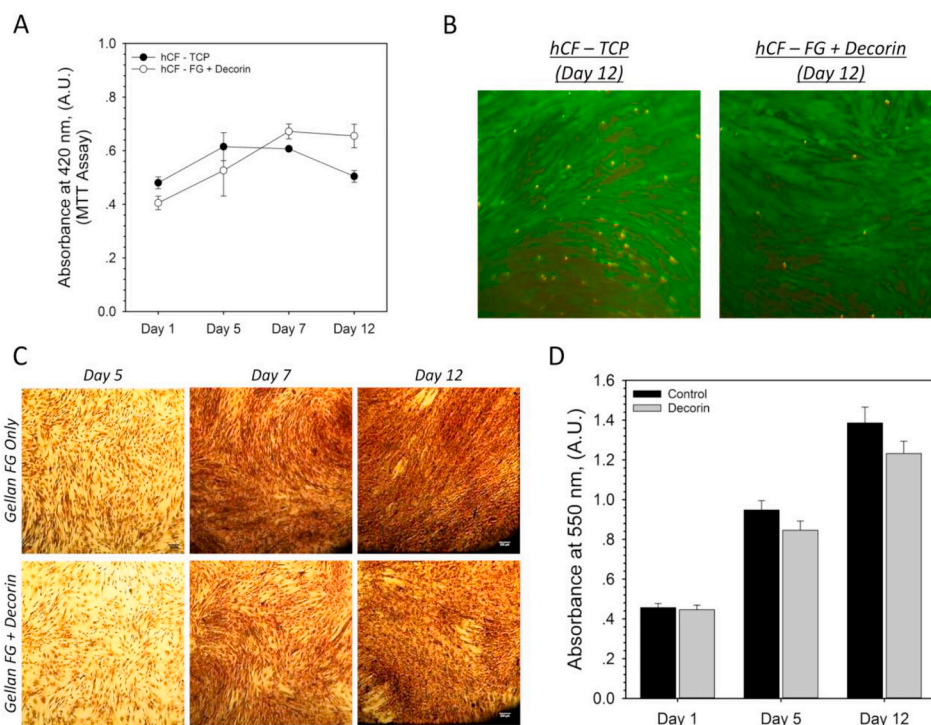


Fig. 3. The effect of toxicity in primary human corneal fibroblasts (hCF), cultured on tissue culture plastic only (TCP) and TCP with the addition of decorin loaded fluid gel. MTT analysis showed no significant difference in the TCP or gellan fluid gel samples over 12 days ($n = 3$, error bars show one standard deviation) (A). Fluorescent images of Calcein AM (green) and PI (red) staining, indicative of live and dead cells respectively (B). Micrographs demonstrating Sirius Red staining, shows total collagen deposition in hCF cultures treated using gellan fluid gel with/without decorin (C). Semi-quantitative analysis of collagen production in hCF cultures ($n = 5$, error bars show one standard deviation) (D). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3.5. Fluid gel-decorin activity assessed *ex-vivo* (organ culture model)

An *ex-vivo* corneal wound healing model, demonstrating corneal re-epithelization, was used to evaluate the bio-activity of the decorin system. Histology, using H&E staining, illustrated the complete removal of the epithelium across a 5 mm diameter wound (Fig. 6A). Application of the decorin in the absence of the gel carrier demonstrated that, even at significantly high concentrations (1 mg/ml (Fig. 6B) and 3 mg/ml (Fig. 6C)), decorin had no effect in re-epithelialisation of the cornea, potentially as a function of the extent of injury. However, data shown for abrasion and treatment using gellan fluid gel containing decorin resulted in stimulated re-epithelialisation within 2 days of treatment (Fig. 6D).

The *ex vivo* rat model of corneal injury shows delamination and full

thickness epithelial debridement across a ~ 5 mm diameter wound. The corneal epithelium is stained blue and the underlying corneal stromal layers are stained in pink. (A). H&E staining of a completely debrided cornea treated with 1.0 mg/ml of decorin (B) and 3.0 mg/ml Decorin only, insert shows the high-magnification view of the respective stroma (The stroma is stained in pink, however, no epithelial cells (blue stained cells) were present on the surface of the cornea, confirming the absence of re-epithelialisation) (C). Images illustrating re-epithelialisation in epithelia abraded rat eyes, when treated with gellan fluid gels with and without decorin, fluorescein staining was quantified using ImageJ (D). An increase in re-epithelialisation occurred in samples treated with gellan Fluid gel + decorin, shown by a reduction in fluorescein staining to 18% after 4 days when treated the gel containing decorin when compared to gellan fluid gels without decorin (E).

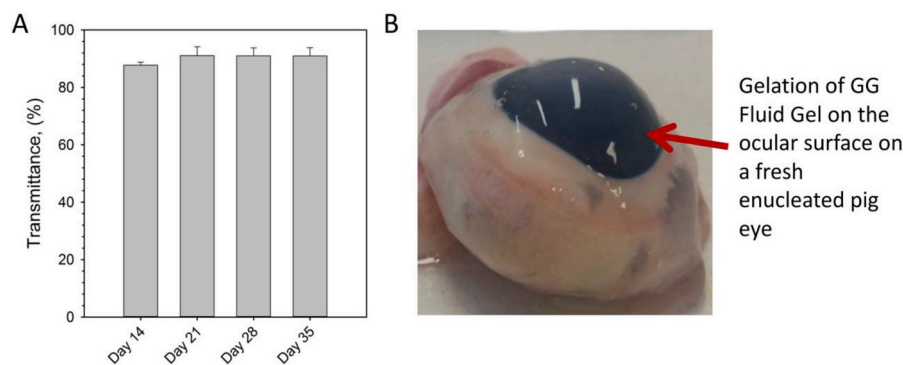


Fig. 4. Gellan fluid gels demonstrated 90% transparency when tested over 35 days using UV spectroscopy (A). Setting time of the gels were tested on freshly enucleated porcine eyes, whereby the gel was dropped on the ocular surface and thickening time was determined. Re-structuring of the gel occurred almost instantaneously (< 1 s) after application (B).

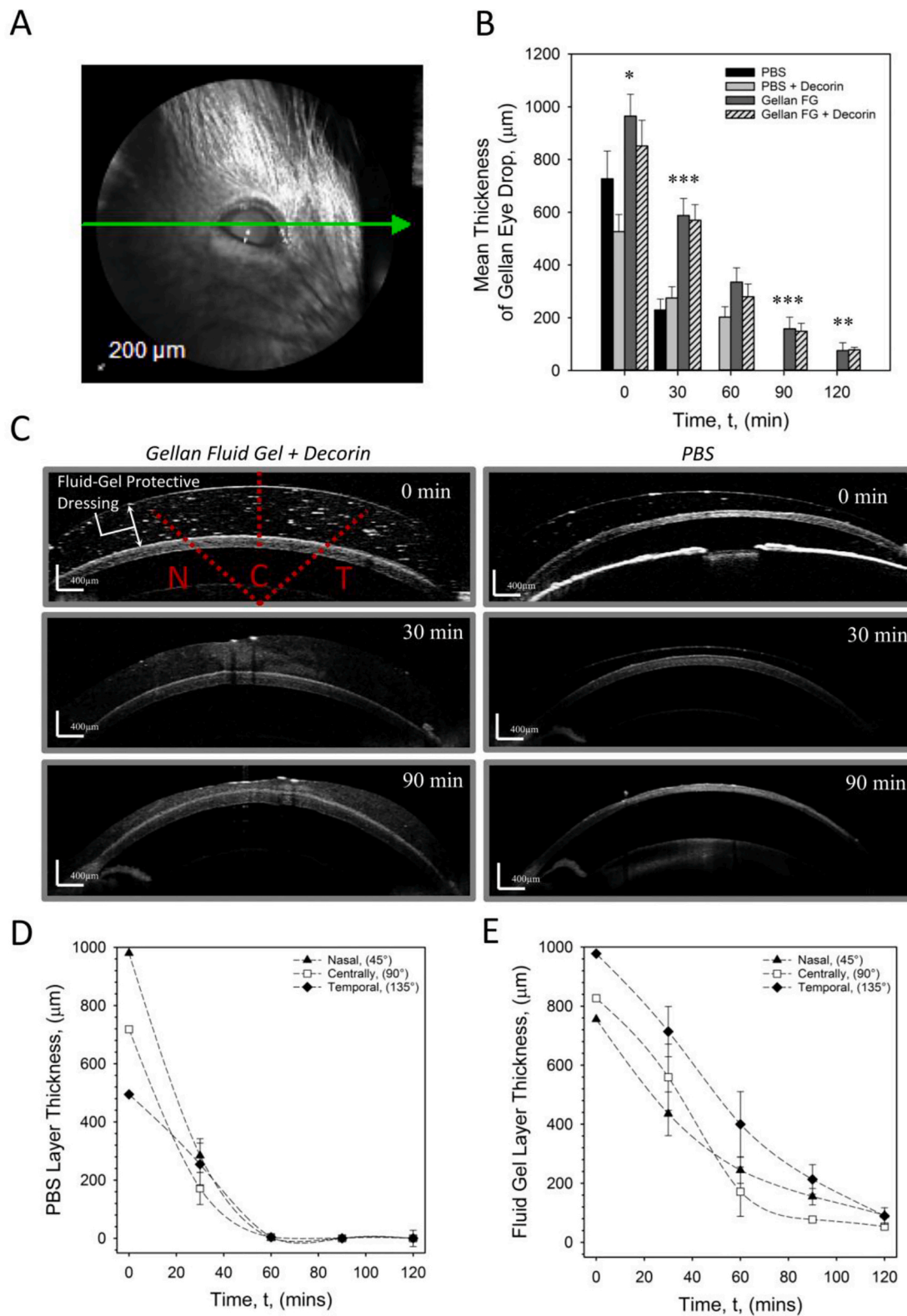


Fig. 5. To evaluate the *in vivo* clearance of the gellan fluid gel from the pre-corneal space, 40 μ l of the fluid gel was dropped on to the surface of anaesthetised rat eyes and imaged using OCT (optical coherence tomography) (A). Mean drop thicknesses for both PBS and fluid gels as a function of time were evaluated, demonstrating that only the fluid gel systems persist throughout the whole experiment (* = $p < 0.006$, ** = $p < 0.004$, and *** = $p < 0.001$ vs PBS + Decorin eye drops) (B). Typical representation of OCT images used to quantify eye drop layer thickness (C). Quantification using ImageJ of the mean thickness of OCT images (C) of eye drops at 30min intervals up to 120 min at different regions of corneal surface (45° (nasal, 'N'), 90° (central, 'C'), 135° (temporal, 'T') (inserted lines to guide the eye in "C") (D and E).

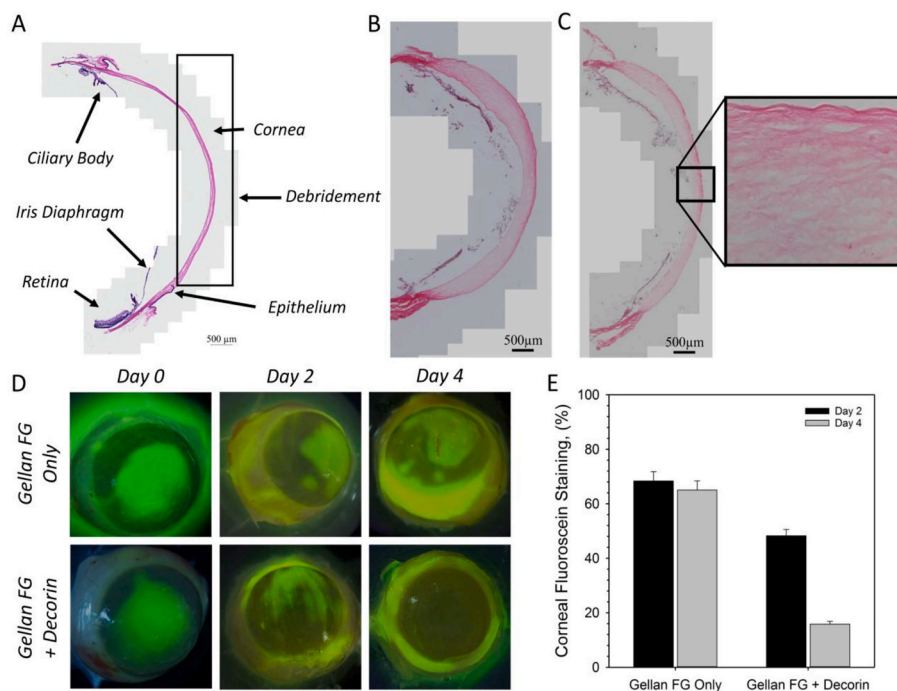


Fig. 6. Histological image of typically H&E stained rats' cornea, illustrating complete epithelial debridement.

Corneal re-epithelialisation was determined by measuring the surface area of fluorescent staining on the ocular surface, with respect to Day 0. Fluorescent staining on the day of injury was measured at 100% for both treatment groups. An approximate 40% reduction in fluorescence was seen at Day 2 of organ culture, in both treatment groups with and without decorin. A further significant ($p < 0.05$) reduction in staining at Day 4 was observed, whereby the cultures containing decorin were reduced to 15%, in comparison to the treatment group without decorin, still showing 48% fluorescence remaining (Fig. 6F).

4. Discussion

Topical administration of ophthalmic drugs remains a popular choice of therapy for the treatment of corneal diseases, due to their ease of use and patient compliance. However, ocular bioavailability is poor due to rapid clearance rates, leading to increased levels of reapplication e.g. often hourly, day and night. Often the primary strategy to improve ocular bioavailability after topical administrations, is to promote the retention of therapeutics or biologicals upon the corneal surface. Currently, this is achieved by injections to the peri-ocular depot [45], increasing contact time through viscosity modification or vehicles [46,47], or applying delivery devices such as therapeutic bandages (e.g. contact lenses) [39]. However, very few physical dressings have been clinically approved, as prolonged wear can often further damage or result in further complications, for example microbial keratitis [40]. Application of corneal sheet dressings, for example amniotic membrane, often requires surgery due to required suturing. Although such dressings have been shown to work [7], they are less favourable therapies due to reasons such as, surgical intervention, risk of transplant related diseases and availability.

Recently, the development of micro-structured hydrogel systems, called fluid gels, have been widely studied as drug-delivery systems for various biomedical applications [48]. Fluid gels can be developed from various natural biopolymers including both polysaccharides and proteins [29]. This study investigated a commonly used thickening agent, gellan gum, as the gelling polymer to make fluid gels that house decorin, for delivery to the ocular surface. The preparation of fluid gels requires a cooling process, whereby a fixed shear rate is applied to the

hydrocolloid sol, resulting in for the formation on micro-gelled “ribbon-like” matrices, which are largely dispersed in an un-gelled medium [23,49]. This hydrogel system exhibits reversible weak gel to liquid-like transitions upon external stimuli such as syringing, allowing application to the ocular surface. This forms a temporary occlusive bandage that can dynamically change to the eye's blinking environment, retarding elimination from the ocular surface and enhance retention.

Based on the mechanical findings, the gellan fluid gels exhibit excellent potential as a wound dressing. The dressing is easy to apply, reversibly shear thinning to provide an occlusive transparent layer. Initial *in vivo* studies highlight the fluid gels' retention to the ocular surface, demonstrating visible fluid gel layers for up to 2 h on a rodent's eye. Gels exhibited uniform layers, having spread across the corneal surface, heterogeneously thinning (thinner in the centre to the peripheries) across the 2 h as it became cleared from the tear film. While this is expected in a rodent model, due to the size of the drop and the curvature of the eyeball, it doesn't present any significant problems for the sustained release of the decorin, still remaining occlusive over the cornea.

The unique suspension hysteresis makes these suspended hydrogels solutions highly attractive for use in ocular drug-delivery applications. Release profiles for decorin loaded fluid gels were compared to the gellan films, a comparative mimetic to *in situ* gelled therapies. Differences in release between the two systems were observed, dependent on the microstructure. Shear applied during processing and resulting particulate suspension enhanced the rate of release, as expected due to the subsequent increase in surface area. Additionally, a transition from zero order to kinetics more closely controlled by Fickian diffusion were observed when analysed using a simple power function described by Ritger et al. (1987) [44]. Results agreed with similar studies probing the release from *in situ* gelation of gellan and eye drops, where typical zero-order plots were obtained for the gellan gel, reducing in the release index for the drop system [50]. It is argued that the relatively large release index for the gellan fluid gel (0.66), in comparison to those previously shown for pure Fickian release (0.43–0.5, for slabs spheres and cylinders [44]), are a function of the ribbon like morphology and ability to entwine forming a weak network between particles. This ultimately resulted in a much more rapid and controlled release for the fluid gels in comparison to the gellan films, leading to an

eye drop system with much higher therapeutic dosing within the timescale associated ocular retention. Thus, the controlled behaviour of release allows the eye drop to give sustained dosing, using repeat applications at clinically relevant administrations (3–4 times daily), and improving bio-efficiency.

Bio-efficiency and activity were addressed both *in vitro* and *ex vivo* probing cytotoxicity and the system's ability to heal the cornea. Both the gellan fluid gel and the decorin showed good compliance with cells, as expected; having already been approved for both medicinal and food uses (gellan), and successfully proven in multiple animal models (decorin), for the reduction in scarring [32,51]. Semi-quantitative analysis of collagen production using Picro Sirius Red staining highlighted the efficacy of the decorin *in vitro*, significantly reducing the degree of collagen deposition, corresponding with the current understanding in literature [54,55]. Such control over the collagen production is facilitated through strong decorin-collagen interactions. Naturally decorin 'decorates' the fibrils, both playing key roles in both spacing and the regulation of growth factors, through sequestration [53]. In turn, controlled lay down of collagen is achieved which ultimately directs fibre alignment and prevents the formation scarred tissue [52].

Further understanding of the decorin loaded fluid gel efficacy was achieved using a previously, well-established, *ex vivo* organ culture model for ocular healing, whereby a corneal keratectomy was applied to a rodent eye and visualised using staining [56–59]. The model is based on a clinically employed technique of apply a fluorescein stain to the ocular surface which directly exposes corneal debridement, with staining proportional to the damaged epithelial surface. Decorin only controls were applied using PBS as a carrier to mimic conventional eye drops. Haematoxylin and eosin histology showed no signs of healing with an absence of re-epithelialisation at both decorin concentrations studied, 1 and 3 mg/ml. It is proposed that in these cases, although no blinking mechanism is present, the combination of drying and low viscosity of the PBS is insufficient to keep the decorin upon the curved surface of the cornea. Application of the fluid gel vehicle in the presence of the active, however, increased epithelial closure across the cornea, shown via fluorescein staining, as a result of the decorin's matrikine properties [60], providing modification to the cellular environment. When compared to the fluid gel only control, no change in wound closure was observed with no reduction in stained areas observed. Such observations reinforced the requirement of a delivery vehicle, augmenting the retention needed for sufficient bioavailability and a therapeutic response. Furthermore, the gellan device appeared to be inert, only enhancing wound healing by retaining the therapeutic.

In summary, this study has highlighted the use of gellan fluid gel as a potential vehicle for the delivery and retention of therapeutics upon the ocular surface. The novelty within this vehicle is derived through the processing of the polymer as it goes through its sol-gel transition, resulting in a system that can dynamically transition between solid-liquid-solid to provide sustained delivery with enhanced longevity in comparison to typically used *in situ* gelation. Furthermore, the system has demonstrated its bio-efficacy in *in vitro*, *vivo* and *ex vivo* models. In doing so, enhanced retention has proved essential to gaining a therapeutic response providing an ideal system for ease of application and therapeutic activity.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.biomaterials.2019.04.013>.

5. Data availability

The raw and processed data required to reproduce these findings cannot be shared at this time due to both legal reason and ongoing work.

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