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In situ antibody-loaded hydrogel for intravitreal delivery

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Abstract

Therapeutic protein medicines have transformed the treatment of blinding diseases (e.g. age-related macular degeneration, AMD) during the last 1-2 decades. Many blinding conditions such as AMD are chronic; and require multiple intravitreal injections over a long period to achieve a high and reproducible dose needed for clinical benefit. Prolonging the duration of action of ophthalmic drugs is critical to reduce the frequency of injections. Thermoresponsive hydrogels (e.g. *N*-isopropylacrylamide, NIPAAM) that collapse in physiological conditions can entrap and sustain the release of a therapeutic protein. However, most NIPAAM hydrogels are not biodegradable and often requires invasive surgery to remove the depot. Here, we report the preparation of a hydrogel derived from NIPAAM and acrylated hyaluronic acid (Ac-HA) as a biodegradable, macromolecular crosslinker. Ac-HA was prepared by the acrylation of hyaluronic acid (HA). Antibody (infliximab (INF), 5.0 mg/mL) or bevacizumab (BEVA), 12.5 mg/mL), NIPAAM (0.35 mmol) and Ac-HA (2.0-10.0 mg/mL, 40.0-200.0 nmol) were first mixed prior to redox polymerisation to ensure maximal protein mixing and to shorten the burst release. Hydrogels with lower amounts of Ac-HA (2.0-4.0 mg/mL, 40.0-80.0 nmol) showed favourable lower critical solution temperature (LCST) values and injectability (27-29G) than higher amounts of Ac-HA (>4.0 mg/mL, >80.0 nmol). These hydrogels were further characterised (swelling ratio (SR), water retention (WR) and rheology). All hydrogels degraded in presence of bovine testes hyaluronidase (0-50 U/mL, 37°C, 100 rpm). Release studies of BEVA-loaded hydrogels were investigated *in vitro* using the PK-Eye™ model, which estimates the human clearance times of proteins from the back of the eye. Phosphate buffered saline (PBS, pH 7.4, 37°C) was used rather than simulated vitreous to more effectively map trends between the formulations. A zero-order release profile was observed between days 5 to 50 with 43.3 ± 9.5% protein released at day 50. Determining protein binding and functionality from a formulation is crucial to determine the optimal formulation prior to more detailed studies that might be necessary. BEVA showed binding to human vascular growth endothelial factor ($VEGF_{165}$) throughout the study (two months) while still maintaining a therapeutic dose (123.5 ± 45.6 ng) in the posterior cavity of the PK-Eye™ model. These encouraging results suggest that extended release of proteins in the vitreous can be achieved using injectable hydrogels derived from NIPAAM and HA.

Introduction

Antibody-based medicines that bind to vascular endothelial growth factor (VEGF) are administered by intravitreal injection to treat wet age-related macular degeneration (AMD), which is one of the major causes of vision loss and blindness in the elderly population (>60 years old) (del Amo and Urtti, 2008). Drug administration via intravitreal injections is currently the best way clinically to ensure a sufficient and reproducible therapeutic dose is administered into the back of the eye/posterior segment (del Amo *et al.*, 2015). Current intravitreal treatment regimens however are onerous and are not without risk (Jager *et al.*, 2004) when considered in the long-term management of controlling the progression of chronic blinding conditions. There remains the risk of bleeding, retinal detachment and infection each time an intravitreal injection is performed (Jager *et al.*, 2004). Aflibercept (Eylea®, Regeneron Pharmaceuticals, Inc), a fragment crystallisable (Fc) fusion protein, has come to dominate the AMD market because intravitreal injections can be given once every two months. Aflibercept achieves a longer dosing interval compared to the other AMD products (ranibizumab, Lucentis®, Genentech/Novartis, a fragment antigen-binding, Fab) simply because it is given in a higher dose. The monoclonal antibody (mAb), bevacizumab (Avastin®, Roche) is also widely used as an unlicensed medicine to treat AMD. IVAN and CATT clinical trials compared the efficacy and safety of ranibizumab and bevacizumab to treat wet AMD and no difference in visual acuity outcome was reported between the two (Chakravarthy *et al.*, 2012; Martin *et al.*, 2012). Regeneron Pharmaceuticals, Inc was recently successful in a supplementary Biologics License Application (BLA) for aflibercept to have a three-month dosing interval (Regeneron, 2017). The barrier to entry for new medicines is now at least a three-monthly dosing schedule with the goal being to have even longer dosing intervals to prevent frequent administration.

Maintaining the tertiary structure of therapeutic proteins is important to preserve their biological activity. Implants or devices require that proteins be formulated in either a solid or a highly concentrated form. Inserts and devices can often cause a foreign body response when implanted in the body. The eye is susceptible to inflammation and endophthalmitis (Lance *et al.*, 2016) due to protein aggregation. It is very difficult to maintain protein tertiary structure in both these forms. There are no clinically registered implantable, long acting formulations for therapeutic proteins. While pumps exist for peptides (e.g. insulin), the clinical use of implants to extend the duration of action of

protein therapeutics is limited. A surgically implanted port delivery system for ranibizumab is currently in clinical trials (Genentech, 2018).

Hydrogel formulations are thought to be biocompatible (Hamidi, Azadi and Rafiei, 2008) and may result in the sustained release of proteins (Drapala *et al.*, 2011; Turturro *et al.*, 2011; Awwad *et al.*, 2018; Egbu *et al.*, 2018). The high water content within hydrogels can be important to help avoid protein aggregation (Jatav, Singh and Singh, 2011). It is crucial that the protein is mixed properly within the hydrogel network to ensure polymer entanglement of the protein to avoid burst release profiles (Awwad *et al.*, 2018). *In situ* collapsing injectable gels have been examined to sustain drug release from hydrogels (Hwang *et al.*, 2013; Rauck *et al.*, 2013; Prasannan *et al.*, 2014). Thermoresponsive hydrogels potentially offer the advantage of an injectable implant capable to extend the release of a protein (Klouda, 2015).

N-isopropylacylamide (NIPAAM) hydrogels has been reported for the delivery of proteins (Drapala *et al.*, 2011; Osswald *et al.*, 2017; Awwad *et al.*, 2018; Egbu *et al.*, 2018) and NIPAAM is thought to have minimal retinal toxicity (Turturro *et al.*, 2011). NIPAAM hydrogels are soluble in water below a lower critical solution temperature (LCST) of approximately 33°C depending on gel composition and crosslink density. Gel collapse occurs above this temperature. The swollen hydrogel has relatively large mesh sizes, which allow the drug to be released quickly at room temperature (RT). The drug molecules will be entrapped inside the hydrogel structure potentially creating a depot once injected *in vivo*. The pore size between polymer chains will be reduced above the LCST slowly allowing protein diffusion from the hydrogel (Drapala *et al.*, 2011). Hydrogels typically contain a lot of water and are often burdened by an initial burst release of the drug. The release of a drug in a burst can be clinically advantageous as a ‘loading dose’, but it is often a major concern because it may shorten the total duration of release and result in dose-dependent adverse effects. Therefore, the hydrogel is prepared in the presence of protein in solution to ensure good protein encapsulation and entanglement within the hydrogel network (Awwad *et al.*, 2018).

Most collapsible NIPAAM hydrogels are not biodegradable. It is necessary that an *in situ* NIPAAM depot is degradable to avoid accumulation. One strategy is to utilise hyaluronic acid (HA), a predominant glycosaminoglycan in the vitreous humour (Sebag and Balazs, 1989; Kogan *et al.*, 2007). HA is a biocompatible, biodegradable and viscoelastic biomaterial that consists of a repeating disaccharide of β-1,4-D-glucuronic acid-β-1,3-*N*-acetyl-D-glucosamine (Kim and Park, 2002; Kurisawa *et al.*, 2005; Leach

and Schmidt, 2005). The major clinical ophthalmic use of HA is as a vitreous substitute during procedures such as lens implantation, cataract progression surgery and vitrectomy (Kogan *et al.*, 2007). HA is an essential component of the extracellular matrix (ECM) and aids in cellular signaling, wound repair and matrix organisation (Burdick and Prestwich, 2011; Khunmanee, Jeong and Park, 2017). HA is degraded by enzymes such as hyaluronidase, b-D-glucuronidase and β-N-acetyl-hexosaminidase (Necas *et al.*, 2008). Hyaluronidase rapidly hydrolyses HA which increases its permeability (Silverstein, Greenbaum and Stern, 2012) and has been used in ophthalmology (Silverstein, Greenbaum and Stern, 2012; Filas *et al.*, 2014). HA can be chemically modified due to the hydroxyl groups present within its structure (Khunmanee, Jeong and Park, 2017).

Therefore, we report the preparation and use of acrylated hyaluronic acid (Ac-HA) as a degradable crosslinker that undergoes polymerisation in the presence of NIPAAM resulting in the preparation of temperature responsive antibody loaded hydrogels. Two different antibodies (infliximab (INF) and bevacizumab (BEVA)), important for ocular angiogenesis and inflammation, were evaluated.

Materials and Method

Materials and Instrumentation

Infliximab (INF, ~150 kDa, Remicade®, 10.0 mg/mL, Genentech, South San Francisco, California) was collected from pooled leftovers from Great Ormond Hospital (London, UK). Bevacizumab (BEVA, ~150 kDa, Avastin®, 25.0 mg/mL Genentech, South San Francisco, California) was purchased from Moorfields Eye Hospital (London, UK). NIPAAM (97%), *N*,*N*,*N′*,*N′*-tetramethylethylenediamine (TEMED, ~99%), ammonium per sulfate (APS), sodium azide, acryloyl chloride (Ac-Cl), triethylamine, hyaluronidase from bovine testes and human VEGF ($VEGF_{165}$), bovine serum albumin (BSA), anti-human IgG (Fc specific)-peroxidase antibody produced in goat, 3,3',5,5'-Tetramethylbenzidine substrate and hydrochloric acid (1.0N) were purchased from Sigma-Aldrich (Gillingham, Dorset, UK). HA (~50 kDa) was supplied from Glentham Life Sciences (Corsham, Wiltshire, UK). Visking dialysis membrane (molecular weight cut off, MWCO: 12–14 kDa) was obtained from Medicell International Ltd (London, UK). Deuterated oxide (D_2O, Cambridge Isotope Laboratories, Andover, UK) was used for nuclear magnetic resonance (NMR). Phosphate buffered saline (PBS) tablets were purchased from Fisher

Scientific (Loughborough, Leicestershire, UK) and the pH was adjusted to 7.4. Sodium hydroxide solution (1N) was purchased from VWR International Ltd (Leicester, UK).

A 16-channel Ismatec peristaltic pump (Michael Smith Engineers Ltd., Woking, Surrey, UK) was used to maintain fluid flow in the PK-Eye™ (Awwad *et al.*, 2015) models. Ultraviolet-visible (UV) spectroscopy measurements were performed using a Hitachi U-2800A spectrometer, Quartz cuvettes (Starna Scientific Ltd) a Wallac Victor2 1420 plate reader with a wavelength range of 200 to 800 nm. Scanning electron microscopy (SEM) was achieved using a QuantaTM 200F instrument (FEI Quanta200 FEGESEM, Eindhoven, The Netherlands). NMR spectra were obtained using a Bruker Avance 400 MHz spectrometer with Bruker NMR Suite 3.5 and TopSpin 1.3. Different scanning calorimetry (DSC) experiments were performed on DSC Q2000 (TA instruments, Waters, LLC) with TA Instruments Universal Analysis 2000 software. A VIRTIS-Advantage freeze-dryer was used for freeze-drying. Bohlin CVO rotational rheometer (Malvern Instruments Ltd, Malvern, UK) was used to study rheological properties of the hydrogels. An Innova® 40 incubator (New Brunswick Scientific, Edison, USA) was used for incubation of the samples.

Methods

Synthesis of Ac-HA crosslinker

Ac-HA crosslinker was synthesised through the reaction between the primary hydroxyl group of HA (for both *N*-acetylglucosamine and glucuronic acid units) and Ac-Cl (δ: 1.12 gcm^{-3}) following the procedure previously reported (Kumar and Gross, 2009) (**Scheme 1**). HA (3.0 g, ~50 kDa, 0.06 mmol) was dissolved in deionised water (250.0 mL) with a magnetic stirrer. Catalytic amount of hydroquinone was added to the reaction and the flask was protected from light by covering it with foil. The temperature was then reduced to 0-5°C in an ice bath and monitored with a thermometer. The pH was gradually increased from 6.5 to 10.0 with drop-wise addition of sodium hydroxide for 40 minutes. Ac-Cl (15.0 mL, 16.8 mol) was added drop-wise to the solution with a constant pH (8.0-10.0) and stirring for 1 hour. The reaction mixture was dialysed (MWCO: 12-14 kDa) against ultrapure water (5.0 L) for 48 hours with water change every 12 hours. The solution was then freeze-dried for 72 hours.

All samples for freeze-drying were transferred to glass vials with a maximum volume of 2.0 mL. The samples were frozen in dry ice and then transferred to the freeze-drier at -40°C with a condenser temperature of -60°C. The vacuum pressure was

maintained less than 200 mBar. The primary freeze-drying step was at -20°C for 48 hours. The temperature was increased to 20°C for 2 hours at the final stage before opening the freeze-drier. A white, fluffy product was obtained after freeze-drying and it was stored between 4-8°C. Acrylation was confirmed using proton NMR (^{1}H-NMR). Freeze-dried Ac-HA (5.0 mg) and HA alone (5.0 mg, negative control) were dissolved in D_2O (1.0 mL) and the chemical shifts were assigned (in parts per million, ppm) to indicate the presence and degree of acrylation.

Preparation of empty and protein loaded hydrogels

Empty hydrogels were prepared by dissolving NIPAAM (40.0 mg, 0.35 mmol) in deionised water (1.0 mL) in a glass vial. Ac-HA (2.0-10 mg/mL, 40.0-200.0 nmol) and APS (4.0 mg, 0.018 mmol) were added to the NIPAAM solution with a mixing time of 15 minutes at RT (~25°C). TEMED (ð: 0.775 g/mL, 20°C, 50 µL, 0.33 mmol) was added to the mixture and was vortexed for 10 seconds. The mixture was incubated at 4°C for 24 hours to ensure complete polymerisation. INF (0.5 mL from 10.0 mg/mL; final concentration of 5.0 mg/mL, volume: 1.0 mL) or BEVA (0.25 mL from 25.0 mg/mL, final concentration of 12.5 mg/mL, volume: 0.5 mL) was added to the NIPAAM (40.0 mg, 0.35 mmol), Ac-HA (2.0-4.0 mg/mL, 40.0-80.0 nmol) and APS (4.0 mg, 0.018 mmol) in deionised water (0.5 mL for INF-loaded hydrogels and 0.25 mL for BEVA-loaded hydrogels). The entire content was allowed to mix for 1 hour at RT before the addition of TEMED (50 µL, 0.33 mmol, 10 seconds vortexing). The mixture was incubated at 4°C for 24 hours. Upon polymerisation, the gel was washed using several vials of PBS (4×, 5.0 mL each, 20.0 mL total volume) with gentle shaking/swirling for 10 minutes for each wash cycle. Wash fractions were analysed using UV-spectroscopy (280 nm).

INF-loaded hydrogels were used for general characterisation (e.g. SR, WR and rheology), whereas BEVA-loaded hydrogels were used for *in vitro* release experiments using the PK-Eye™ models.

Hydrogel characterisation

Determination of lower critical solution temperature (LCST)

The LCST was measured using differential scanning calorimeter (DSC) at 2.0°C/min from 20 to 50°C. Calibration with Indium (Tm = 156.6, ΔHf =28.71 J/g) was performed according to the manufacturer instructions. Nitrogen was used as purge gas with a flow

rate of 40.0 mL/min for all the experiments. The onset temperature of the DSC endothermic peak was considered as the LCST.

Scanning Electron Microscopy (SEM)

Samples were cut and adhered onto aluminium SEM stubs using carbon-coated double-sided tape prior to SEM analysis. The samples were then sputter coated with gold prior to imaging to make them electrically conductive.

Swelling ratio (SR) and water retention (WR) measurements

Freeze-dried hydrogel samples (1.0 mL reaction mixture) were weighed (*Wd*) and incubated in deionised water (2.0 mL) at 25 and 37°C for 48 hours to ensure complete swelling. The swollen gels were gently wiped with filter paper after incubation and reweighed (*Ws*). The SR was calculated using **Equation 1**.

$$SR = \frac{Ws - Wd}{Wd} \qquad \textbf{Equation 1}$$

WR percentage (WR%) was measured using a gravimetric method at 50°C. Freeze-dried hydrogel samples (1.0 mL reaction mixture) were weighed (*Wd*) and fully hydrated in deionised water (10.0 mL) at 25°C for 48 hours. The fully hydrated gels were weighed (*Ws*) and quickly transferred to a pre-heated water bath at 37°C in an incubator. The samples were weighed (*Wt*) at pre-determined time intervals and returned back to the incubator before each measurement. WR% was calculated according to **Equation 2**.

$$WR\% = \frac{Wt - Wd}{Ws} \times 100 \qquad \textbf{Equation 2}$$

Rheological properties

A peltier plate with a diameter of 20.0 mm and 0° angle was used. The rheometer was calibrated to zero prior to sample analysis. Swollen gel samples (empty and protein loaded, 2.0 mL) were placed on the plate with a gap size of 0.4 mm. The chuck was unlocked prior to each experiment to allow rotation of the plate. The slipped gel around the plate was cleaned with a plastic spoon before every run to avoid erred reading of the rheometer due to slippage of samples during loading. Viscosity was measured at a range of shear rates from 0.1-200 sec^{-1} at 25 or 37°C.

In vitro degradation of hydrogels

Hydrogel degradation was measured with respect to weight loss after the addition of hyaluronidase. Empty hydrogels were incubated in deionised water (2.0 mL) at 25°C for 48 hours to ensure complete swelling. Various concentrations of hyaluronidase (0-50 U/mL) were prepared in deionised water. Empty hydrogels were incubated in hyaluronidase solutions (20.0 mL) at 37°C with constant shaking (100 rpm). The incubated hydrogels were removed and weighed at various time intervals. The surface of the hydrogel was gently dabbed with tissue before each weight measurement. The hydrogels were transferred back to the hyaluronidase solution with fresh hyaluronidase solution (with the same concentration) prepared every 24 hours.

In vitro release studies using the PK-Eye™ models

The PK-Eye™ (Awwad *et al.*, 2015) is a two-compartment *in vitro* model comprised of an anterior (~0.2 mL) and posterior (~4.2 mL) cavity separated by a washer and a Visking membrane (MWCO: 12-14 kDa). The inlet port was connected to a peristaltic pump with a constant flow of PBS (pH 7.4, 0.05% sodium azide) at 2.0 µL/min (37°C). One outlet port is present in the anterior cavity for continuous sampling. Both cavities were filled with PBS and the temperature was maintained at 37°C throughout the studies. Washed BEVA-loaded hydrogels were injected (1.1 mg, 100.0 µL) with a 1.0 mL syringe fitted with 27-29G needle via the injection port of the model. Samples were collected at regular time intervals via the outflow port present in the anterior cavity and stored in the freezer (-20°C) prior to protein quantification.

Protein quantification

Enzyme-linked immunosorbent assay (ELISA)

Human $VEGF_{165}$ diluted with a coating buffer (bicarbonate buffer, pH 9.5; 100.0 µL, 0.1 µg/mL) was added to 96-well flat-bottomed microplates. The plates were sealed and incubated overnight (2-8°C). The plates were then washed with washing buffer (PBS, 0.05% Tween 20) and aspirated. Assay diluent (10% BSA in PBS, 200.0 µL) was added to each well and the plates were incubated with shaking at RT for 1 hour to prevent non-specific binding. The washing step was repeated and diluted BEVA standard samples were then loaded into the microplates with constant shaking at RT for 2 hours. Anti-human IgG (Fc specific)-peroxidase (1:50000 dilution, 100.0 µL/well) was added after washing the plates. The plate was incubated for 1 hour with shaking and then washed

again. 3,3',5,5'-Tetramethylbenzidine substrate solution (100.0 µL/well) was added to the microplates and incubated in the dark for 2-5 minutes or until color was developed. Hydrochloric acid (1.0 N, 100.0 µL) was then added to stop the reaction before monitoring the endpoint absorbance at 450 nm. The absorbance reading of the blank (PBS solution) was subtracted from the reading of standards/unknown samples. Calibration curve was produced for each measurement and was fitted with a four-parameter dose response curve. The developed VEGF-ELISA allowed the detection of native BEVA in a concentration range of 0.006-25.0 µg/mL (R^2: 0.986).

Micro Bicinchoninic Acid (BCA) assay

The protocol followed was according to the manual provided by ThermoFisher Scientific. Briefly, working reagent solution was prepared by mixing 25 parts micro BCA reagent A (MA), 24 parts reagent B (MB) with 1 part of reagent C (MC) (25:24:1). Samples were pipetted (150.0 µL) to a 96-well plate followed by working reagent (150.0 µL). The plate was gently mixed on a plate shaker for 30 seconds prior to incubation at 37°C for 2 hours. The plate was then cooled at RT and the absorbance was measured at 562 nm with a plate reader. Negative control (PBS) was subtracted from the readings of the unknown sample. A calibration curve was prepared with BEVA (0.0-100.0 µg/mL, R^2: 0.993).

Data analysis

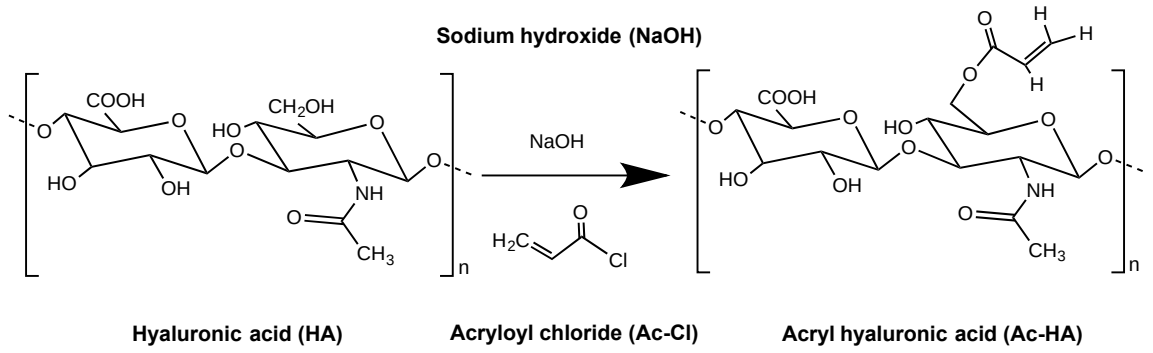
All results are presented as the mean and standard deviation (± STD), and data were plotted using Prism 7 and GraphPad Software. The level of significance was determined using one-way ANOVA, Mann Whitney U *t*-test and an unpaired *t*-test using Prism 7. Probability values less than 0.05 ($p<0.05$) were considered as indicative of statistically significant differences.

Results and Discussion

Preparation of crosslinked hydrogels

We synthesised a biodegradable crosslinker (Ac-HA) to provide a degradable crosslink element into a NIPAAM gel (**Scheme 1**). The acryl group was introduced into the primary hydroxyl groups of the HA backbone by nucleophilic displacement of the chlorine of Ac-Cl (**Scheme 1**). The hydroxyl group of HA was deprotonated to hydroxide ions by the addition of sodium hydroxide prior to the acrylation process. The hydroxide

ion nucleophilically attacked the Ac-Cl carbonyl group to displace the chloride ion and form Ac-HA. Sodium hydroxide was necessary to maintain the pH at 8.5-10.5 due to the possible lowering of pH from acrylic acid. The degree of acrylation was determined to be approximately 40-60% by ^{1}H-NMR by comparing the relative amount of the vinylic acrylate proteins to the N-acetyl protons on HA.



Scheme 1. Schematic representation of the synthesis of Ac-HA crosslinker. The primary hydroxyl group of hyaluronic acid (HA) was deprotonated by sodium hydroxide (NaOH) to form hydroxide ion that nucleophilically attacked the carbonyl group of acryloyl chloride (Ac-Cl) and displaced chlorine to form acrylated hyaluronic acid (Ac-HA).

NIPAAM is an acrylamide, which results in a water-soluble polymer. Many hydrogel systems do not require organic solvents during preparation (Stile, Burghardt and Healy, 1999; Vermonden, Censi and Hennink, 2012; Shi *et al.*, 2013), therefore, hydrogels were prepared with water. Empty hydrogels was prepared using NIPAAM monomer (0.35 mmol) in water (1.0 mL) and varying amounts of Ac-HA (2.0-10.0 mg/mL, 40.0-200.0 nmol) while maintaining a constant amount of radical initiator (APS, 0.018 mmol and TEMED, 0.33 mmol). Gentle vortexing did not affect the stability of the antibody, which was confirmed by size exclusion chromatography (SEC, **Figure S1**) and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE, data not shown). The prepared hydrogels after polymerisation (24 hours) were soluble and transparent at 25˚C. A white, turbid solid was seen at 37˚C. Hydrogels prepared with 2.0-4.0 mg/mL (40.0-80.0 nmol) Ac-HA were easily injectable via a 27G needle, whereas hydrogels prepared with more than 4.0 mg/mL (>80.0 nmol) Ac-HA were difficult to inject (**Table 1**).

It is not possible to mix a large molecule such as a therapeutic protein efficiently with a preformed hydrogel (Awwad *et al.*, 2018). A strategy to address this 'mixing problem' is to form the gel while in the presence of the protein in solution (Awwad *et al.*, 2018). Inadequate mixing time leads to the protein precipitating out of the solution.

Therefore, a mixing time of at least 1 hour was needed to efficiently mix the antibody (INF, 0.5 mL from 10.0 mg/mL or BEVA, 0.25 mL from 25.0 mg/mL), NIPAAM monomer (0.35 mmol), Ac-HA (2.0 and 4.0 mg/mL, 40.0 and 80.0 nmol), APS (0.018 mmol) and deionised water (0.5-0.75 mL) before the addition of TEMED (0.33 mmol).

LCST is the critical temperature at which a thermoresponsive gel collapses (Makino, Hiyoshi and Ohshima, 2001; Gandhi *et al.*, 2015). The LCST of freeze-dried hydrogels were measured with DSC. Empty hydrogels showed LCST values between 33.7 ± 0.1 to 36.9 ± 0.4°C for the Ac-HA hydrogels prepared with increasing amounts of Ac-HA (**Table 1**). Hydrogel with 2.0 mg/mL Ac-HA had the closest LCST to pNIPAAM (31-33°C, (Jain *et al.*, 2015)). The LCSTs were below physiological temperature (33.7 ± 0.1 and 34.5 ± 0.1°C) for hydrogels with 2.0 and 4.0 mg/mL (40.0 and 80.0 nmol) Ac-HA respectively; whereas the LCSTs were closer to physiological temperature (36.4 ± 0.2 and 36.9 ± 0.4°C) for hydrogels with 7.0 and 10.0 mg/mL (140.0 and 200.0 nmol) Ac-HA respectively. These findings support previous work reported in literature. Hydrophilic amide groups of pNIPAAM interact more with water below the LCST. Hydrophobic isopropyl groups of pNIPAAM dominate above the LCST making the gel insoluble (Sato Matsuo and Tanaka, 1988; Makino, Hiyoshi and Ohshima, 2001). HA is a hydrophilic saccharide-based macromolecule which would be expected to interfere with NIPAAM conformational interactions to reduce the thermo-transition above the LCST (Erbil, Kazancıoğlu and Uyanık, 2004; Gökçeören, Şenkal and Erbil, 2014; Samanta and Ray, 2014). The hydrophilic property of Ac-HA can form strong bonds with water and hence, this higher interaction of the gel with water would require higher energy (temperature) to overcome.

LCST of INF-loaded hydrogels were also investigated with DSC. INF was used instead of BEVA for general proof of concept/characterisation experiments prior to release studies and the results obtained for these experiments with INF is expected to be similar for BEVA. LCST values of 34.7 ± 0.3 and 35.5 ± 0.5°C were observed for INF-loaded hydrogels (5.0 mg/mL) with 2.0 and 4.0 mg/mL (40.0 and 80.0 nmol) Ac-HA respectively (**Table 1**). A slight increase in LCST in presence of protein is expected due to the hydrophilic nature of the protein (Wu *et al.*, 2005; Egbu *et al.*, 2018). The LCST remained below physiological temperature for the INF-loaded hydrogel. These results are consistent with previously reported work with BEVA-loaded NIPAAM hydrogels (Awwad *et al.*, 2018). It was decided to continue further with Ac-HA amounts of 2.0 and 4.0 mg/mL (40.0 and 80.0 nmol) as these hydrogels displayed favorable properties in

terms of injectability and LCST (**Table 1**). Microscopic investigation of the freeze-dried hydrogels with SEM is shown in **Figure 1**.

Table 1. Optimisation of hydrogels with varying amounts of crosslinker (Ac-HA)

Sample	Crosslinker amount		Injectability via 27G needle	Lower critical solution temperature (LCST, °C)
	mg/mL	nmol		
Empty	2.0	40.0	Easy	33.7 ± 0.1
	4.0	80.0	Easy	34.5 ± 0.1
	7.0	140.0	Difficult	36.4 ± 0.2
	10.0	200.0	Difficult	36.9 ± 0.4
INF-loaded	2.0	40.0	Easy	34.7 ± 0.3
	4.0	80.0	Easy	35.5 ± 0.5

Note: LCST values are displayed as the average (n=3) and its standard deviation (± STD).

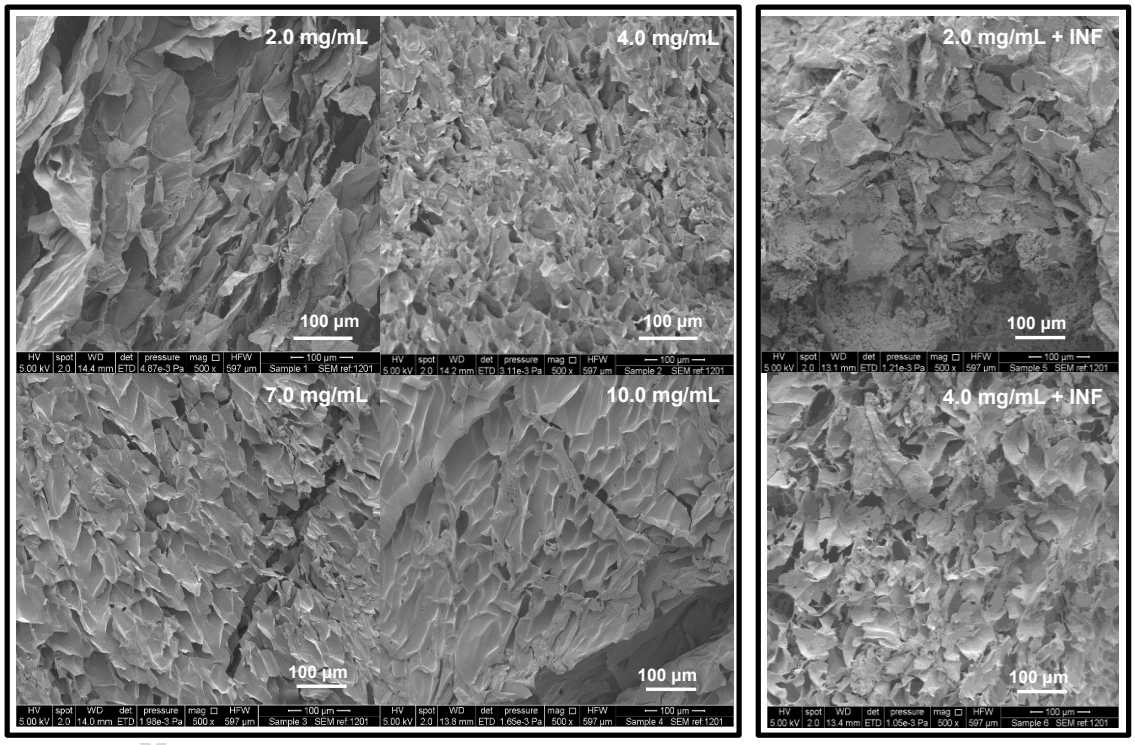


Figure 1. Scanning electron microscopy (SEM) of freeze-dried hydrogels (Ac-HA amounts of 2.0-10.0 mg/mL i.e. 40.0-200.0 nmol respectively) with and without infliximab (INF, 5.0 mg/mL) with a scale of 100 µm.

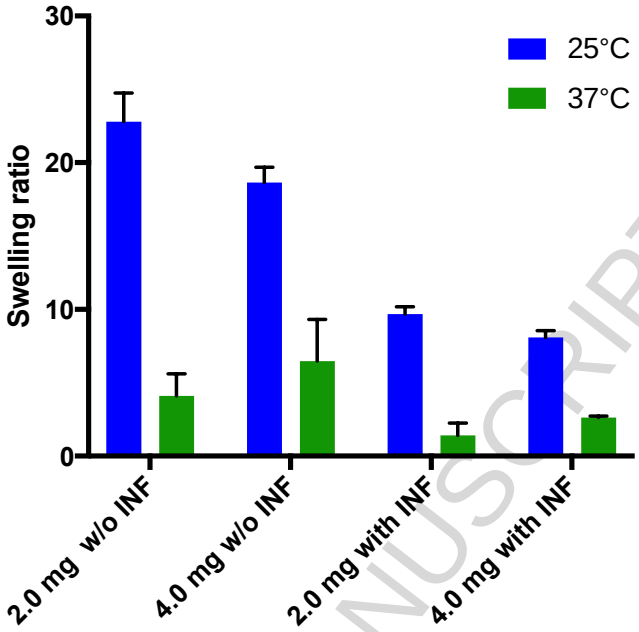
Hydrogel characterisation

Swelling ratio (SR) and water retention (WR) properties

The swelling ratio (SR) is an important parameter because the diffusion property of drug loaded into the gel is dependent on the amount of water within the gel. Freeze-dried

hydrogels were swollen in deionised water for 48 hours to ensure complete hydrogel swelling prior to analysis. SR values of 22.8 ± 1.7 and 18.6 ± 1.0 at 25°C and SR values of 4.1 ± 1.5 and 6.5 ± 2.8 at 37°C were observed for empty hydrogels with 2.0 and 4.0 mg/mL Ac-HA respectively (**Figure 2A**). The SRs at 37°C were much lower than the SRs at 25°C indicating the loss of water due to gel collapse. The decrease in SR with higher crosslink density is consistent with previously reported results (Awwad *et al.*, 2018). Similarly, the SR of INF-loaded hydrogels (5.0 mg/mL) was determined at both 25 and 37°C. INF-loaded hydrogels with Ac-HA amounts of 2.0 and 4.0 mg/mL displayed SR values of 9.7 ± 0.5 and 8.1 ± 0.5 at 25°C and SR values of 1.4 ± 0.8 and 2.6 ± 0.1 at 37°C respectively (**Figure 2A**). The balance between the hydrophilic and hydrophobic groups of the NIPAAM gels does not appear to be affected by the presence of protein ($p>0.05$) at 37°C which is consistent with other findings previously reported (Drapala *et al.*, 2011; Awwad *et al.*, 2018).

A)



B)

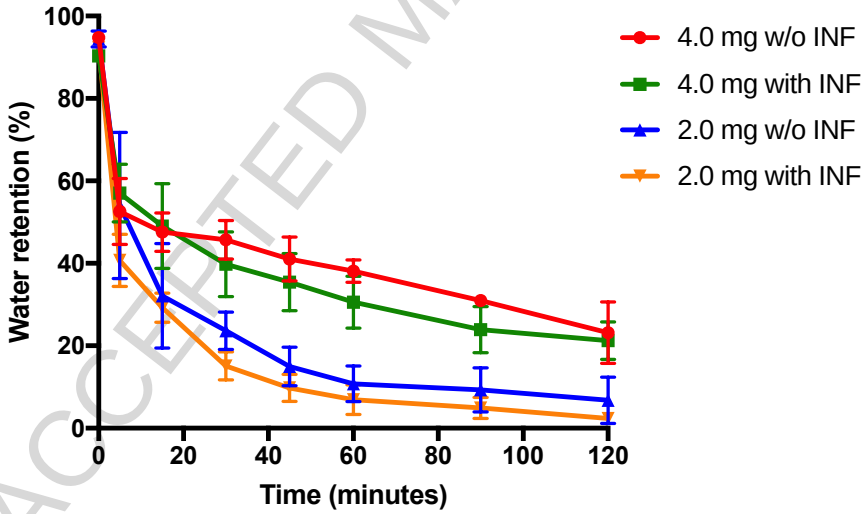


Figure 2. A) Swelling ratio (SR) at 25 and 37°C and **B)** water retention (WR) at 37°C of empty and infliximab- (INF) loaded hydrogels. The SR and WR of the hydrogels were not affected by the concentration of INF (5.0 mg/mL) used in these studies at temperatures above its LCST. All data is presented as its mean (n=3-4) and standard deviation (± STD).

The de-swelling kinetics is often referred to 'water retention' (WR) and it is the ability of the hydrogel to expel water above its LCST. It is defined as the weight of water retained inside a hydrogel versus the weight of the initially absorbed water. The difference between WR and SR is that WR characterises the total 'water capacity' of the

hydrogel and is the ratio of the weight of water contained within the swollen hydrogel to its dry weight (Yoon *et al.*, 2010). WR is used to examine how the hydrogel will behave above its LCST regarding the change in volume of the hydrogel and the time required to achieve that change. The effect of the relative amount of crosslinker on WR (%) was measured by observing the decrease in weight/volume for 120 minutes. Hydrogels with 2.0 mg/mL Ac-HA showed water loss of 54.0 ± 17.7, 23.6 ± 4.6 and 9.3 ± 5.4 % (empty hydrogels) and 40.7 ± 6.4, 15.1 ± 3.4 and 4.9 ± 2.6 % (INF-loaded hydrogels, 5.0 mg/mL) at respective times of 5, 30 and 90 minutes (**Figure 2B**). A slower water loss was observed for gels with 4.0 mg/mL crosslinker. Hydrogels with 4.0 mg/mL Ac-HA showed water loss of 52.5 ± 8.0, 45.7 ± 4.7 and 31.0 ± 1.4 % (empty hydrogels) and 57.1 ± 7.0, 39.8 ± 7.9 and 23.8 ± 5.6 % (INF-loaded hydrogels) at respective times of 5, 30 and 90 minutes (**Figure 2B**). The WR would be expected to significantly differ with higher amounts of Ac-HA or higher protein concentration. The hydrophilic nature of a protein molecule is expected to interact with water and can possibly reduce the sensitivity of the NIPAAM hydrophobic groups to temperature. Therefore, higher protein concentration can lead to lower thermoresponsive gels with higher amount of water being retained within the hydrogel.

Rheology studies

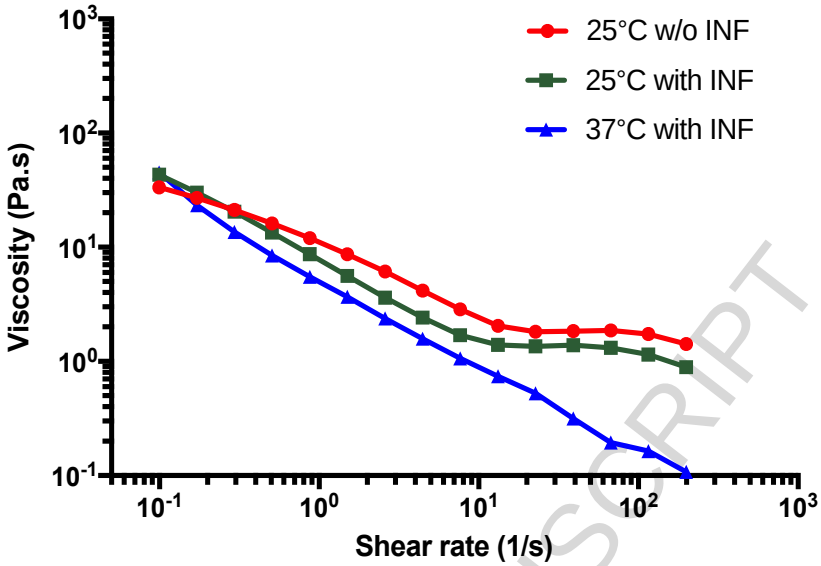
An understanding of the mechanical properties of the hydrogels could potentially elucidate information about gel resorption as well as release drug by the gel (Lian *et al.*, 2012). The effect of crosslinker concentration (2.0 and 4.0 mg/mL) on the viscosity of the hydrogel (empty and INF-loaded hydrogels, 5.0 mg/mL) were studied using a rotational rheometer with a shear rate ranging from 0.1 to 200 s^{-1} so that the trend of viscosity at different shear rates can be determined from the curves. An important factor for injectability from the rheology data is whether the hydrogel is shear-thinning (Chen *et al.*, 2017). Shear-thinning hydrogels decrease in viscosity with shear (Chen *et al.*, 2017).

Table 2. Viscosity values of empty and infliximab- (INF) loaded hydrogels (5.0 mg/mL) with varying amounts of Ac-HA (2.0 and 4.0 mg/mL, 40.0 and 80.0 nmol) at 25 and 37°C

Temperature (°C)	Viscosity (Pa.s)			
	2.0 mg/mL (40.0 nmol)		4.0 mg/mL (80.0 nmol)	
	Empty	INF-loaded	Empty	INF-loaded
25	33.3 ± 9.5	37.3 ± 17.1	81.5 ± 20.3	115.8 ± 48.6
37	-	45.2 ± 28.9	-	26.5 ± 8.9

Note: Rheology values are displayed as the average (n=6) and its standard deviation (± STD).

A) 2.0 mg/mL hydrogels



B) 4.0 mg/mL hydrogels

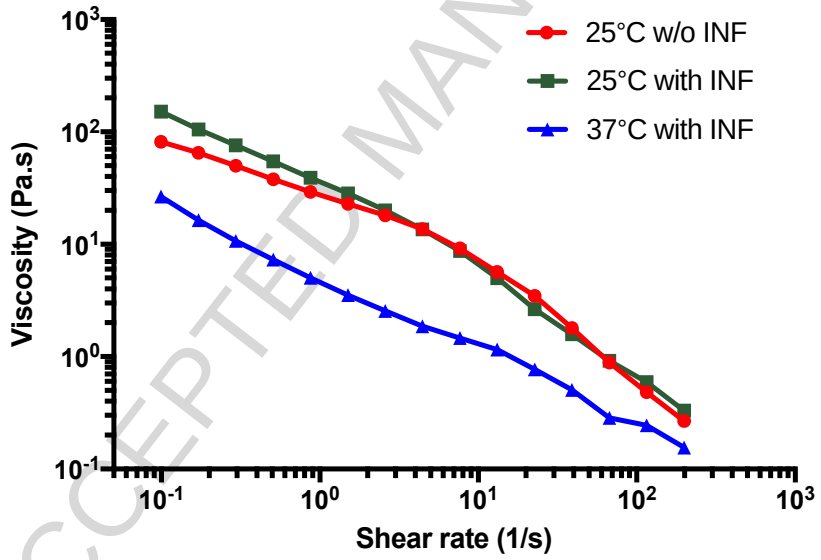


Figure 3. Viscosity measurements of A) 2.0 mg/mL (40.0 nmol) and B) 4.0 mg/mL (80.0 nmol) empty and infliximab- (INF) loaded hydrogels (5.0 mg/mL) at 25 and 37°C. A significant difference in viscosity ($p>0.05$) was seen with 4.0 mg/mL hydrogels at both temperatures. All data is presented as its mean (n=6) and standard deviation (± STD).

The continuous flow experiment (**Figure 3**) showed a decrease in viscosity with the application of shear at both 25 and 37°C indicating all of the hydrogels tested have shear-thinning capabilities. Viscosity values at 0.1 s^{-1} of 33.3 ± 9.5 Pa.s (empty, 25°C), 37.3 ± 17.1 Pa.s (INF-loaded, 25°C) and 45.2 ± 28.9 Pa.s (INF-loaded, 37°C) were observed for 2.0 mg/mL hydrogels, whereas values of 81.5 ± 20.3 Pa.s (empty, 25°C),

115.8 ± 48.6 Pa.s (INF-loaded, 25°C) and 26.5 ± 8.9 Pa.s (INF-loaded, 37°C) were observed for 4.0 mg/mL hydrogels (**Table 2**). No significant differences ($p>0.05$) were observed between the two concentrations (2.0 vs 4.0 mg/mL) at 25 and 37°C with and without INF loading. However, there was a significant difference ($p<0.05$) in viscosity values of the 4.0 mg/mL crosslinked protein hydrogel at 25 and 37°C (which was not seen with the 2.0 mg/mL hydrogel, $p>0.05$). This could be a result of lower equilibrium swelling that INF causes for hydrogels at both temperatures (**Figure 3A**).

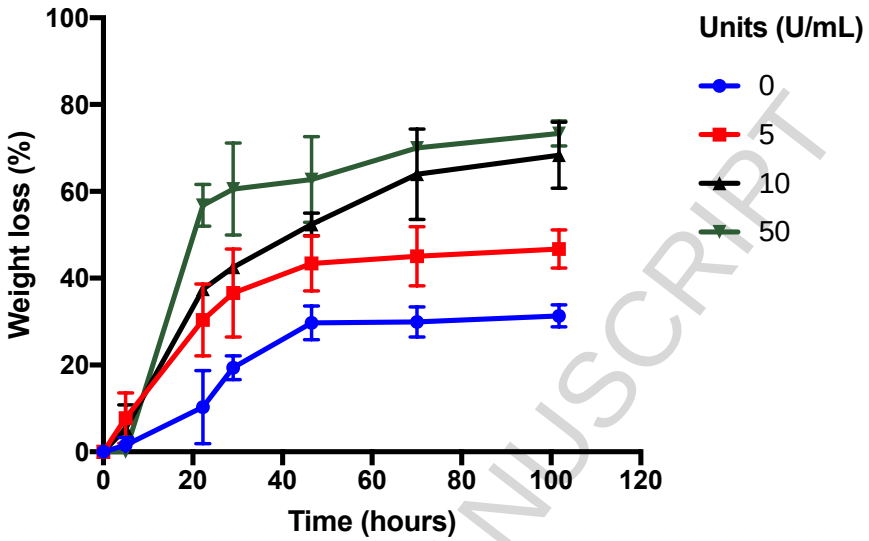
***In vitro* enzymatic degradation**

HA will likely be degraded by hyalocyte, hyaluronidase or other mechanisms in the vitreous (Jacobson, 1984; Haddad and André, 1998; Bishop, 2000; Ballios *et al.*, 2010; Yu *et al.*, 2015). Hyaluronidase has been reported to be present at 20 turbidity reducing units (TRU) per mL in human vitreous (Schwartz *et al.*, 1996; Wells, Furukawa and Sheardown, 2011). Hyaluronidase promotes HA turnover and is responsible for the maintenance of the vitreous structure (Guter and Breunig, 2017). Vitreal HA has an estimated half-life of 10-70 days depending on the species. The half-life is approximately 72 days in rabbits and no data is available for human (Ghosh *et al.*, 2017). HA in other parts of the body has a shorter half-life (not more than a week and in some cases less than a day) (Guter and Breunig, 2017). Hyaluronidase is usually used as a liquefaction agent prior to vitro-retinal surgery in treatment of retinal detachment (Guter and Breunig, 2017) and has also been used for the clearance of vitreous hemorrhage (Silverstein, Greenbaum and Stern, 2012). In some instances, hyaluronidase is administered during the delivery of microparticles to rapidly hydrolyse the ECM structure of the sclera to ensure feasible delivery (Gaudana *et al.*, 2010).

Preliminary screening on gel biodegradability (**Figure 4**) was conducted with the 2.0 and 4.0 mg/mL crosslinked gels as a function of time in presence of bovine testes hyaluronidase (0-50 U/mL, 37°C, 100 rpm). By day 4, 2.0 mg/mL crosslinked Ac-HA hydrogels displayed weight losses of 31.3 ± 2.5, 46.7 ± 4.5, 68.3 ± 7.6 and 73.3 ± 2.9 % with 0-50 U/mL hyaluronidase respectively; whereas 4.0 mg/mL crosslinked Ac-HA hydrogels displayed weight losses of 19.1 ± 1.6, 35.6 ± 0.7, 31.5 ± 4.3 and 41.5 ± 7.2 % respectively. Most of the hydrogels were difficult to weigh and handle due to the fragmentation of the hydrogels. The solution of all samples also appeared turbid, which is an indication of the degrading components of the hydrogels. A slower degradation rate

was seen with a higher Ac-HA within the hydrogel. A higher chance of enzyme perforation is possible with lower amounts of Ac-HA.

A) 2.0 mg/mL hydrogels



B) 4.0 mg/mL hydrogels

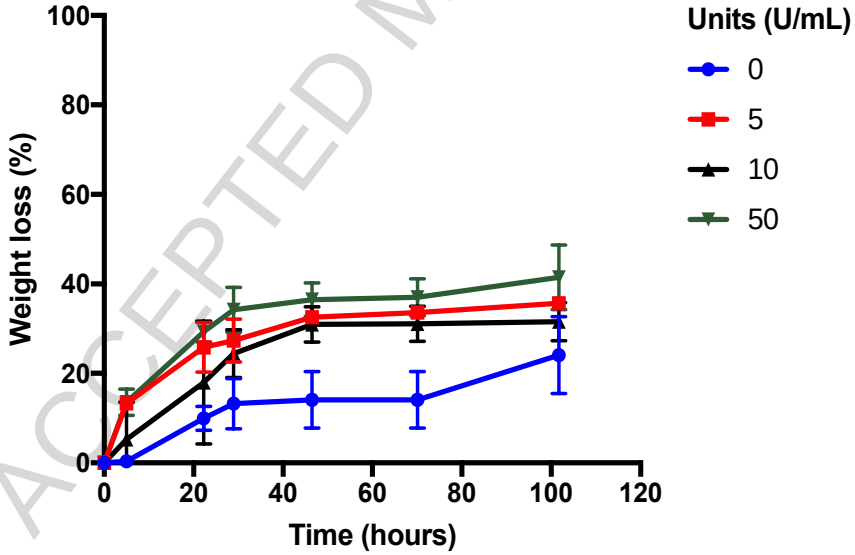


Figure 4. *In vitro* enzymatic (bovine testes hyaluronidase) degradation of empty hydrogels at 37°C, 100 rpm. Enzyme degradation was confirmed by hydrogel fragmentation and weight loss with time. All data is presented as its mean (n=3) and standard deviation (± STD).

In vitro release with the PK-Eye™

Assessment of protein-based medicines in animals for 2-3 months or more is almost impossible and prohibitively expensive. The current total reliance on animal models for intravitreal pharmacokinetic studies of proteins to develop intraocular medicines has

limitations. Animal models (i) vary in their predictive capacity due to anti-drug antibodies (ADAs), anatomic and aqueous outflow differences, (ii) are challenging to evaluate protein stability over time and (iii) are extremely expensive/time consuming to conduct. ADA formation is a particularly insidious problem. Human therapeutic proteins will generate ADAs in animal models, which result in rapid clearance of the candidate drug making allometric modelling impossible/inaccurate to develop longer-acting formulations. The formation of ADAs is an intractable problem for industry, which can be solved by an appropriate model such as the PK-Eye™ (Awwad *et al.*, 2015, 2017).

In vitro drug dissolution and release testing is described in many national pharmacopeia and is widely used in the pharmaceutical industry to provide drug release profiles for both quality control purposes and preclinical development. There are many uses for *in vitro* models in preclinical research including (i) optimisation of drug solubility and dissolution properties, (ii) evaluation of new formulation designs and compositions and (iii) development of *in-vitro* and *in-vivo* correlations (IVIVCs) and extrapolations (IVIVEs). The PK-Eye™ is a two-compartment, aqueous outflow model scaled to the eye. A visking membrane separates the two compartments. The larger (~4.2 mL) and smaller (~0.2 mL) compartment mimics the posterior and anterior cavity respectively. Aqueous flow within the PK-Eye™ mimics the anterior hyaloid flow pathway in the human eye. The PK-Eye™ has been shown to estimate the human clearance time of therapeutic proteins from the vitreous cavity when using simulated vitreous (Awwad *et al.*, 2015). The PK-Eye™ offers a practical means to conduct comparative studies to optimise novel formulations of protein therapeutics (Awwad *et al.*, 2015) and long acting implant (Awwad *et al.*, 2017).

Prolonging the duration of action of a drug can be achieved via multiple steps that occur from a dosage form. Deconvoluting these steps to map what may happen in humans is important for efficient preclinical development. For example, a long acting gel formulation of a therapeutic protein requires the protein to first diffuse from the gel followed by protein diffusion at a suitable therapeutic concentration from the vitreous cavity. In an animal model, such a stepwise scenario would be expected to generate ADAs when using a human protein. Deconvoluting these steps to optimise a formulation can be more efficiently studied in a relevant non-animal model. This strategy is common for the development many oral dosage forms, which are first evaluated and optimised using in *vitro* models prior to or in concert with conducting animal studies.

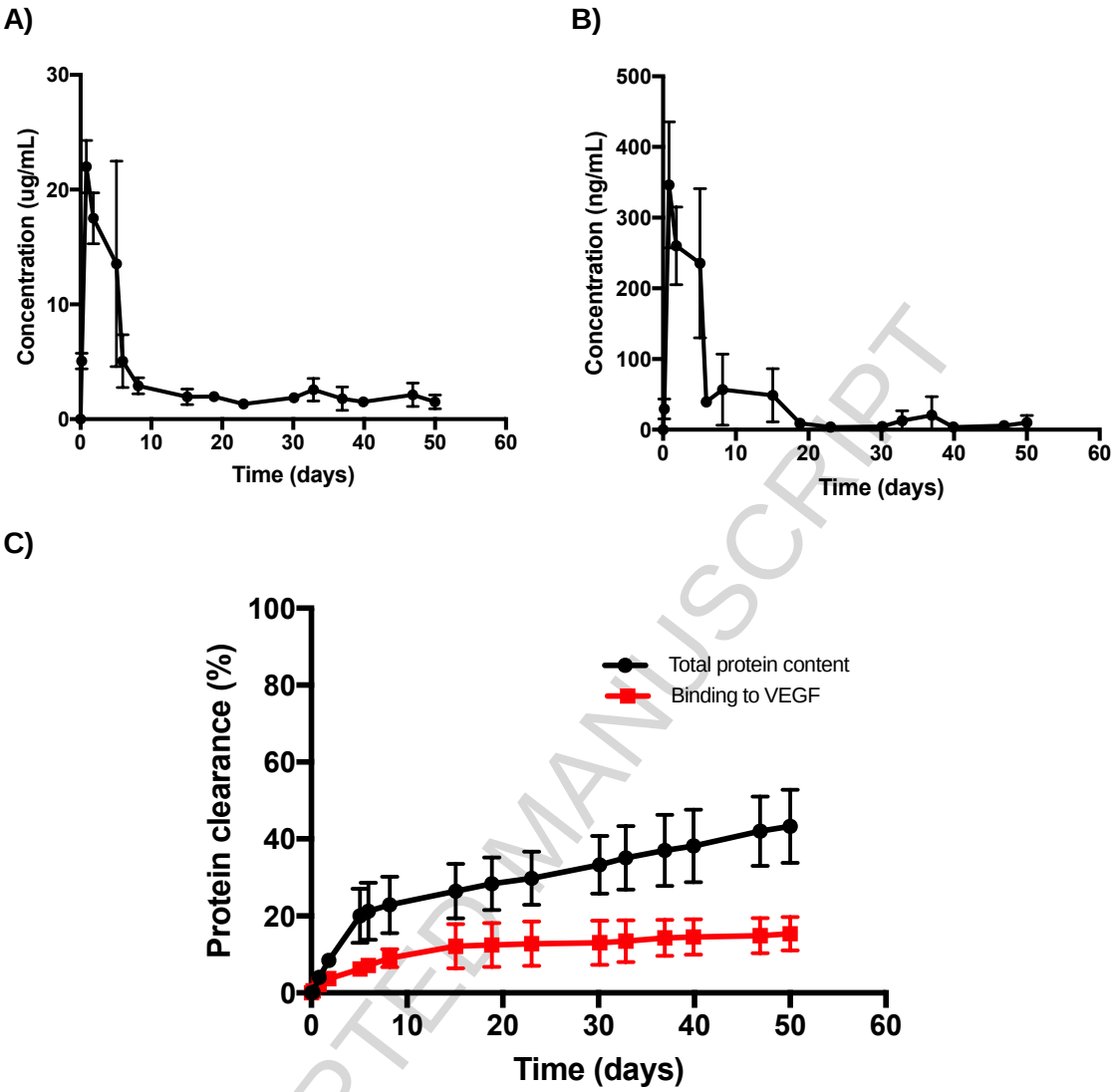


Figure 5. *In vitro* release kinetics of bevacizumab-(BEVA) loaded hydrogels (4.0 mg/mL, 80.0 nmol Ac-HA) in the PK-Eye™ containing PBS, pH 7.4 at 37°C. Concentration profiles after (A) microBCA and (B) ELISA analysis. (C) Cumulative release (%) of BEVA, total protein content vs binding (%) to human vascular endothelial growth factor (VEGF). A continuous protein release was seen for at least 50 days. All data is presented as its mean (n=3) and standard deviation (± STD).

In vitro release experiments were conducted with BEVA instead of INF as BEVA is used for the treatment of AMD. BEVA- (6.25 mg/0.25 mL from 25.0 mg/mL vial) loaded crosslinked (4.0 mg/mL Ac-HA) hydrogels (0.5 mL reaction) were prepared and washed with PBS, pH 7.4 (20.0 mL) to remove any leachables. A protein loss of approximately 16-17% was observed (approximately 5.2 mg/0.45 mL left). Studies were conducted in PBS instead of simulated vitreous (Awwad *et al.*, 2015) to obtain quicker clearance data. A slower release rate and diffusion rate is expected in simulated vitreous

than PBS (Awwad *et al.*, 2018). A dose of approximately 1.1 mg (100 µL) was injected in the PK-Eye™ at 37°C containing PBS, pH 7.4 (**Figure 5**). The microBCA assay was conducted to determine the total amount of protein. A C_{max} value of 22.0 ± 2.3 µg/mL was observed with a burst release of approximately 20.1 ± 6.9% in 5 days (**Figure 5A and C**). A zero-order release clearance profile from day 5 to day 50 was seen with 43.3 ± 9.5% protein release at day 50.

Previous work reported an *in vitro* half-life of 1.2 ± 0.1 days of BEVA (1.25 mg dose) in PBS with almost all BEVA being released in 10 days (Awwad *et al.*, 2015). In a similar study reported in our lab, 74.2 ± 3.5, 87.6 ± 6.4 and 95.8 ± 2.3% BEVA was released after a month with 4, 8 and 12 µL/mL (0.018 to 0.091 equiv. to NIPAAM) poly(ethylene glycol) diacrylate (PEGDA) respectively (BEVA-loaded hydrogels with 2.5 mg BEVA dose using PEGDA as a crosslinker (Awwad *et al.*, 2018)). A 50.4 ± 0.8 and 40.9 ± 0.5% INF release (from INF-loaded hydrogels with 1.0 mg INF loading) was observed from an interpenetrating polymer network (IPN) pNIPAAM-HA acid with 1 and 3% PEGDA crosslinking respectively (Egbu *et al.*, 2018). The results in this study were encouraging to sustain the release of BEVA for almost 2 months.

Given the current design of the PK-Eye™ model, there is now an opportunity to establish correlations with *in vivo* models using different injectable dosage forms. An important characteristic of the PK-Eye™ model is that the ease of sampling provided a practical method to evaluate protein function and stability at different time points. It is important to be able to easily evaluate protein stability and function at different time points for formulations with extended half-lives. Majority data present in literature on release studies do not determine protein functionality or binding. Most studies simply seek to get total protein release, however, protein function throughout the release period is critical.

An ELISA assay was conducted to determine the amount of functional protein present during the *in vitro* study. The lowest concentration of BEVA sampled from the PK-Eye™ against VEGF and detected by ELISA was 4.0 ± 0.8 ng/mL. The VEGF-ELISA assay did not detect the formulation excipients (e.g. NIPAAM) or non-specific binding, demonstrating that the absorbance reading corresponded to BEVA binding (data not shown). To compare the total protein content (a) and bound protein to VEGF (b), % binding was calculated ((b/a)*100%), (**Figure 5C**, red line). The ELISA assay showed binding for the duration of the experiment, however, there was a decrease in binding as compared to the total protein sampled. The loss of binding for the released antibody

indicates the antibody remaining in the gel may have partially degraded. Loss of protein function is a key outstanding challenge for developing long acting formulations.

The amount of BEVA required to block VEGF in the vitreous has been estimated to be approximately 83 ng (minute amount) (Hattori *et al.*, 2010). A dose of 1.25 mg of BEVA will decrease to 76.29 ng in approximately 60 days; therefore, an intravitreal dose of 1.25 mg of BEVA is necessary for a period of 2 months (Hattori *et al.*, 2010). By approximately week 7, the amount of BEVA bound to VEGF from the BEVA-loaded hydrogels that was sampled from the anterior outflow was 123.5 ± 45.6 ng.

The *in vitro* study conducted in the PK-Eye™ was important to help optimise the formulation before costly *in vivo* studies. The use of excipients (e.g. arginine, trehalose, glycine, histidine and other amino acids) can help maintain protein stability (Bhambhani *et al.*, 2012; Ratanji *et al.*, 2014; Awwad and Angkawinitwong, 2018). The appropriate excipient should be thoroughly screened and optimised to prevent any aggregation as one formulation excipient stabilising a particular antibody might not be suitable for another antibody (due to differences in their sequence) (Wang *et al.*, 2007; Falconer *et al.*, 2011; Ratanji *et al.*, 2014). In addition, sterilisation is an important criterion to consider in the preparation of antibody-loaded hydrogels. Sterilisation is not trivial and is critical since infection and endophthalmitis can rapidly cause painful vision lost and often blindness. Sterilisation also involves developing formulation and dosage form fabrication processes with low bioburden and the avoidance of endotoxin. HA is already currently used for intraocular applications in the clinic and an injectable Ac-HA-NIPAAM gel would be expected to be sterilised in an analogous fashion, which we believe is by a combination of aseptic and filtration processes.

Conclusions

One of the most important goals in ophthalmic drug development is to extend the therapeutic level of drugs, especially proteins and antibodies, in the posterior segment to treat blinding diseases. With an ageing population and with people living longer, there is a need to develop extended formulations for ocular use. Protein loaded thermoresponsive hydrogels were prepared with NIPAAM and investigated in hopes of extending the duration of action of a protein. A biodegradable crosslinker (Ac-HA) was synthesised to improve the biodegradability of the NIPAAM hydrogel. Having a biodegradable implant for drug delivery is beneficial for ocular tissues, given the small

size of the eye and significant risk in first placing and then potentially needing to remove a non-degradable or non-resorbable implant.

Ac-HA was synthesised by the acrylation of the primary hydroxyl groups of HA and hydrogels were then crosslinked with Ac-HA by redox polymerisation. Different hydrogels were prepared with varying amounts of Ac-HA (2.0 to 10.0 mg/mL, 40.0 to 200.0 nmol). Hydrogels with more than 4.0 mg/mL (80.0 nmol) of Ac-HA failed the injectability (via 27-29G needles) and LCST characterisation tests; hence they were discontinued for further studies. Lower Ac-HA amounts (2.0-4.0 mg/mL, 40.0-80.0 nmol) were used for the preparation of empty and protein-loaded hydrogels. Polymerising NIPAAM with Ac-HA (APS and TEMED) in the presence of antibody (INF and BEVA) did not result in any obvious detrimental effects to the antibody. Although the antibody did not appear to be conjugated to the NIPAAM, more work would be needed to ensure no radical induced reactions occurred at a susceptible amino acid residue such as tyrosine in the antibody. Fortunately tyrosines are hydrophobic and generally solvent inaccessible. Both set of hydrogels showed favourable LCST, WR, SR and enzyme degradation test.

In vitro release was conducted using PK-Eye™ models, which displayed BEVA release for at least 50 days with zero order kinetics post 5 days. The amount of BEVA detected was within the therapeutic dose range of BEVA. A key use of the PK-Eye™ model would be to evaluate new drug delivery strategies and devices that are being developed to prolong the vitreous half-life and the duration of action of a protein therapeutic. Using the PK-Eye™ to estimate the release profiles of long lasting dosage forms during preclinical research may accelerate development. The PK-Eye™ was designed to be simple and practical to use to allow for iterative processes to occur so that formulations can be optimised efficiently and while minimising the use of animal models for suboptimal preclinical candidates. Continued study of HA degradation properties is required to determine structure-property correlations, e.g. cross-link density vs degradation profile. Since protein function and stability can be easily monitored with the PK-Eye™ during the clearance period, a more elaborate stability study can be conducted to have a better undertstanding of the formulation and its possible behaviour *in vivo*.

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Declaration of interest

The authors state no conflict of interest.

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Graphical abstract