Gellan gum and its methacrylated derivatives as in situ gelling mucoadhesive formulations of pilocarpine: In vitro and in vivo studies

Gellan gum and its methacrylated derivatives as *in situ* gelling mucoadhesive formulations of pilocarpine: *in vitro* and *in vivo* studies

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Abstract

Gellan gum was chemically modified by the reaction with methacrylic anhydride to produce derivatives with 6, 14 and 49 % methacrylation. The structure and substitutions degrees of these derivatives were confirmed by ^{1}H NMR- and FTIR-spectroscopy. These derivatives are more hydrophobic compared to pristine gellan and form turbid solutions in water. *In vitro* study performed with formulations of sodium fluorescein containing gellan gum and its methacrylated derivatives indicated that methacrylation enhances their retention on bovine conjunctival mucosa. *In vivo* experiments with the formulations of pilocarpine hydrochloride containing gellan gum and methacrylated derivatives have demonstrated that all polymers enhance the drug effect significantly, but best performance is observed for the polysaccharide with 6% methacrylation.

Keywords: gellan gum, methacrylation, *in situ* gelling, mucoadhesion, ocular drug delivery, pilocarpine, glaucoma, wash out$_{50}$.

1. Introduction

Glaucoma is a group of ophthalmic conditions accompanied with an increased intraocular pressure, which may eventually result in a damage of an optic nerve and potentially leads to blindness. There are two types of this ocular condition called open-angle glaucoma and angle-closure glaucoma. Unfortunately, glaucoma cannot be fully cured but if medication is administered regularly, it can control the intraocular pressure and prevent the damage of the optic nerve. There are several types of therapeutic agents that are used to treat glaucoma, which include prostaglandin analogues, beta-blockers, carbonic anhydrase inhibitors, sympathomimetics and miotics. All these medications are administered as eye drops (Moiseev et al., 2019).

Pilocarpine is a miotic that opens up an inefficient channel in the trabecular meshwork. Typically, pilocarpine is used for treatment of angle-closure glaucoma and adult patients with this condition are recommended to apply pilocarpine eye drops up to 4 times a day to control the intraocular pressure (British National Formulary, 2018). This requirement for frequent application of eye drops makes the therapy very inconvenient and less patient compliant. Advanced drug delivery strategies are needed to reduce the need for such a frequency for ocular administration of pilocarpine.

When conventional eye drops are used, drug retention in the ocular environment is generally very poor (Wilson, 2004). This is related to continuous production of tear fluid, blinking reflex, nasolacrimal drainage and poor permeability of ocular membranes. Therefore, the bioavailability of drugs administered via conventional eye drops is less than 5% (Hillery et al., 2001). Ocular bioavailability of eye drops could be substantially improved when mucoadhesive polymers are used as a part of the formulation. These materials have the ability to adhere to mucosal tissues on the eye and ensure better retention of the formulation on ocular surfaces leading to more efficient drug absorption (Hornof et al, 2003; Ludwig, 2005; Laffleur et al, 2015; Tighsazzadeh et al, 2019).

All water-soluble polymers exhibit some mucoadhesive properties (Khutoryanskiy, 2011, 2014). Polyelectrolytes (cationic and anionic) usually are more adhesive than non-ionic polymers. Adhesiveness of formulations and their retention on ocular tissues also depends on other factors such

as polymer molecular weight, chain flexibility, presence of cross-links, rheological properties of eye drops, etc. (Ludwig, 2005). Some polymers could also be used to formulate *in situ* gelling systems that are liquids during storage but form viscous gels upon administration on the eye, which leads to substantial improvements in their retention on ocular surfaces (Thrimawithana et al., 2012; Kirchhof et al., 2015; Al Khateb et al., 2016; Wu et al, 2019).

Gellan is a linear anionic hetero-polysaccharide that consists of tetra-saccharide repeating units including 1,3-ß-D-glucose, 1,4-ß-D-glucuronic acid, 1,4-ß-D-glucose and 1,4-α-L-rhamnose (Bajaj et al., 2007; Morris et al., 2012). Gel-forming properties of gellan, as well as its biocompatibility, allow using this polysaccharide not only in the food and cosmetic industry, but also for biomedical purposes, including drug delivery (Omoto et al., 1999; Rupenthal et al., 2011a; Ferris et al., 2013; Osmałek et al., 2014; Kudaibergenov et al, 2019). *In situ* gelling properties of gellan based formulations have been considered for application in ocular drug delivery in several publications (Rozier et al., 1997; Carlfors et al., 1998; Paulsson et al., 1999; Balasubramaniam et al., 2003; Rupenthal et al., 2011a, 2011b; Fernández-Ferreiro et al., 2015). Some attempts were also reported on chemical modification of gellan aiming to enhance its mucoadhesive properties. Yadav et al. (2014) synthesised gellan-thioglycolic acid conjugate and established that thiolation of gellan gum decreased its sensitivity to Ca^{2+}-induced gelation. However, formulations based on gellan thioglycolic acid conjugate containing metronidazole showed 1.82-fold greater mucoadhesive strength compared to parent polymer. Jalil et al (2019) conjugated gellan gum with 2-(2-amino ethyldisulfanyl) nicotinic acid and used it for formulating mucoadhesive films for vaginal administration.

Recently, Kolawole et al. (2018) reported the possibility of enhancing mucoadhesive properties of chitosan by its methacrylation. Methacrylated chitosan exhibited greater adhesion to and retention on porcine bladder mucosa. Methacrylated gellan has previously been used for preparation of chemically cross-linked hydrogels (Coutinho et al., 2010); however, it has not been explored with regards to the effect of methacrylation on mucoadhesive properties.

This paper reports the synthesis of methacrylated gellan and evaluates the possibility of its retention on freshly excised bovine conjunctival tissue using fluorescent microscopy *in vitro*. It also evaluates pilocarpine hydrochloride containing *in situ* gelling formulations with gellan and methacrylated gellan *in vivo* in rabbits.

2. Materials and methods

2.1. Materials

Gellan gum Phytagel™ (GG, MW~1000 kDa), methacrylic anhydride (MA), fluorescein sodium salt (NaFl) and pilocarpine hydrochloride were purchased from Sigma-Aldrich (Gillingham, UK). All other chemicals were of analytical grade and used without further purification.

2.2. Synthesis of methacrylated gellan gum

Methacrylated gellan gum (MeGG) was synthesised by reacting gellan gum (GG) with methacrylic anhydride (MA) at various molar ratios to produce derivatives with low (LMeGG), medium (MMeGG) and high (HMeGG) degrees of substitution using a protocol reported by Coutinho et al. (2010) with slight modifications. Briefly, 0.5 g (0.672 mmol) GG was dissolved in 100 mL of deionised water in a round-bottom flask at 90 °C for 30 min under constant stirring until a transparent homogeneous solution formed. Then, the temperature of the mixture was decreased to 50 °C and the desired amounts of MA were added dropwise. **Table 1** presents the data on the feed ratios used in this synthesis. The reaction proceeded at 50 °C and shaken at 100 rpm for 6 h. pH was maintained at 8.0 throughout the reaction by adding 5.0 M sodium hydroxide. The final product was re-dispersed in distilled water, purified by dialysis against distilled water (5 L; 8 changes) during 48 h using a dialysis membrane tube (12–14 kDa molecular weight cut-off; Medicell Membranes Ltd, UK), lyophilised and stored in a fridge for further use.

Table 1

Feed ratios for the synthesis of methacrylated gellan gum (MeGG).

Parameters	LMeGG	MMeGG	HMeGG
Concentration of gellan gum (GG)	0.5 g	0.5 g	0.5 g
Amount of methacrylic anhydride	1.035 g (1 mL)	2.59 g (2.5 mL)	4.14 g (4 mL)
Moles of MA per unit mole GG	5.0	12.5	20.0

2.3. Nuclear magnetic resonance spectroscopy (^{1}H NMR)

Solutions of gellan gum and its methacrylated derivatives (0.25% w/v) were prepared in D_2O. Solution of methacrylic anhydride (1% v/v) was prepared in CD_3Cl. ^{1}H NMR spectra of samples were recorded using a Bruker DPX 400 MHz NMR-spectrometer (Bruker, UK) at 50 °C.

The methyl group (–CH_3) on the rhamnose ring from GG repeating unit was used as a reference (δ 1.27 ppm) and the degree of substitution (DS%) was quantified using the following equation:

$$DS\% = \frac{\frac{1}{2}I_{double\ bond_{(methacrylate)}} \Big/ \frac{1}{3}I_{CH_{3(rhamnose)}}}{n_{OH_{repeating\ unit}}} \times 100\% \quad (1)$$

where $I_{double\ bond_{(methacrylate)}}$ is the integration of the double bond proton peak of the methacrylate groups and $I_{CH_{3(rhamnose)}}$ is the integration of the reference peak with the number of protons in each peak, respectively; $n_{OH_{repeating\ unit}}$ is the number of reactive –OH sites in GG structure.

2.4. Fourier transform infra-red (FTIR) spectroscopy

FTIR spectra of unmodified and modified gellan gums were recorded on Nicolet iS5 FTIR spectrometer (Thermo Scientific, UK) using an iD5 attenuated total reflectance (ATR) accessory

equipped with a diamond crystal. Samples were scanned from 4000 to 500 cm^{-1}; the absorbance mode was used and the spectral resolution was 4 cm^{-1}.

2.5. Dynamic light scattering (DLS)

Aggregation of unmodified and modified gellan gum was examined using dynamic light scattering (DLS) with a Zetasizer Nano-NS (Malvern Instruments, UK) at 25 °C. Samples were prepared by dispersing lyophilised polymers in deionised water to form 0.1; 0.5 and 1 mg/mL solutions and left stirring overnight. The pH of formed dispersions was adjusted to 2; 4; 6 and 8 by addition of HCl and NaOH solutions.

2.6. *Ex vivo* bovine mucoadhesion studies

2.6.1. Preparation of eye drop solutions

In order to demonstrate the applicability of modified and unmodified *in situ* gelling gellan gum (GG) formulations for ocular drug delivery, fluorescein sodium salt (NaFl) was employed as a model compound to load into GG and MeGG solutions. Briefly, 30 mg (0.6% w/v) of GG and its methacrylated derivatives were dissolved in 5 mL aqueous solutions of NaFl (1 mg/mL in deionised water) at a constant stirring and room temperature until homogenous solutions formed.

Simulated tear fluid (STF) used to wash a mucosal surface was prepared as reported previously (Lin and Sung, 2000). STF was composed of NaCl (6.7 g), $NaHCO_3$ (2.0 g), and $CaCl_2 \cdot 2H_2O$ (0.08 g) dissolved in 1000 mL of deionised water (pH 7.4) and the solution was kept at 37 °C throughout the experiments.

2.6.2. Retention on bovine conjunctival mucosa

The mucosal retention of modified and unmodified gellan gum (GG) on *ex vivo* bovine conjunctival tissues was evaluated using the methodology developed in-house with minor modifications (Tonglairoum et al., 2016). Whole bovine eyeballs with conjunctivae were acquired from P.C. Turner Abattoirs (Farnborough, UK) immediately after animal slaughter, packed and transported to the laboratory in a cold polystyrene container. The tissues were subsequently defrosted

upon delivery and bovine eyelids were carefully dissected within 2 h using a sharp blade, avoiding contact with the mucosal surface. Each eyelid mucosa (palpebral conjunctiva) was rinsed with 1 mL STF solution, mounted on a glass slide with mucosal side facing upward, placed in Petri dishes, wrapped with cling film to prevent dehydration and stored in a fridge. All tissues were used within 24 h of retrieval.

Experiments were conducted with a conjunctival tissue already mounted on a glass slide placed on a substrate at an angle of 45° and maintained at 37 °C in an incubator. Aliquots (200 µL) from NaFl-loaded modified and unmodified gellan gum formulations and free NaFl stock solutions were aspirated and pipetted onto a 2 × 2 cm^2 piece of conjunctival mucosa and irrigated with STF solution at a flow rate of 200 µL/min using a syringe pump over 60 min of total washing time. Fluorescence microscopy images of whole tissue were taken at predetermined time points after each wash using a Leica MZ10F stereo-microscope (Leica Microsystems, UK) with Leica DFC3000G digital camera at 1.6× magnification and 12 ms exposure time, fitted with a GFP filter (blue, $\lambda_{emission}$ = 520 nm). The microscopy images were then analysed with ImageJ software by measuring the fluorescence pixel intensity after each wash with STF. The pixel intensity of the blank samples (i.e. the background microscopy images recorded for each conjunctival mucosa without a fluorescent test material) was deducted from each measurement and data were normalised and converted into fluorescent intensity values using the following equation:

$$Fluorescence\ intensity = \frac{I - I_b}{I_0 - I_b} \times 100\% \quad (2)$$

where I_b is the background fluorescence intensity of a given tissue sample (a blank sample); I_0 is the initial fluorescence intensity of that sample (a tissue sample with mucoadhesive test material on it prior to the start of first wash out); and I is the fluorescence intensity of that tissue sample with a mucoadhesive fluorescent material after each wash out cycle.

In addition, wash out$_{50}$ (WO_{50}) values of fluorescent mucoadhesives were quantified *via* extrapolation of the average wash-off profiles to 50 % using polynomial fitting (5th order) and Wolfram Alpha (a computational knowledge engine). These WO_{50} values are used to evaluate and compare formulations retention efficacy on mucosal surfaces, which depict the volume of simulated tear fluid necessary to wash out 50 % of a mucoadhesive formulation from a substrate (Mun et al., 2016).

All measurements were carried out in triplicate and the mean values ± standard deviations were quantified and evaluated statistically.

2.7. *In vivo* experiments

Solutions of polymers were prepared by dissolving 0.03 g of each polymer in 5 mL deionised water. Then 0.05 g of pilocarpine hydrochloride was added to each sample to make 1 % solutions and these were left stirring overnight before use. *In vivo* experiments with these solutions were conducted in chinchilla rabbits of either sex (3700–3800 g, n = 4) according to the methodology adapted from (Lin et al., 2004). These experiments were approved by Kazan State Medical University ethics committee (approval No.5 from 28th May 2012) and were conducted following the ARVO Statement for the Use of Animals in Ophthalmic and Visual Research. Prior to experiments, rabbits were housed in standard cages and allowed free access to food and water. During the experiments, rabbits were restrained by gently wrapping them in a cotton tissue, where their eyes and eye-lid movements were not restricted. Eye drops (150 μL) were instilled into rabbit's left eye and their right one served as a control (150 μL of water were instilled). Digital images were taken at different time points with a web-camera and these were processed with ImageJ software to calculate the difference between the right (D_{right}, mm) and left (D_{left}, mm) pupil diameters:

$$\Delta = D_{right} - D_{left} \quad (3)$$

Each experiment was conducted for 210 min; then areas under the decrease in pupil diameter versus time profile in 210 mins ($AUC_{15\text{-}210}$) were calculated using the trapezoidal rule.

2.8. Statistical analysis

All measurements were performed in triplicates and data expressed as mean ± standard deviation (unless specified otherwise). Data were compared for significance using two-tailed Student's *t*-test and a one-way analysis of variance (ANOVA) with GraphPad Prism statistical analysis software (version 7.0; GraphPad Software Inc.), where $p < 0.05$ was set as the statistical significance criterion.

3. Results and discussion

3.1. Synthesis of methacrylated gellan gum (MeGG) derivatives

Methacrylated gellan was synthesised by reaction with methacrylic anhydride (**Fig. 1A**). Following purification by dialysis, methacrylated derivatives were studied using ^{1}H NMR spectroscopy (**Fig. 1B**). All four spectra displayed the characteristic peak that corresponds to the methyl ($–CH_3$) group from rhamnose ring (δ 1.27 ppm), which was used as a reference (Lu et al, 2019).

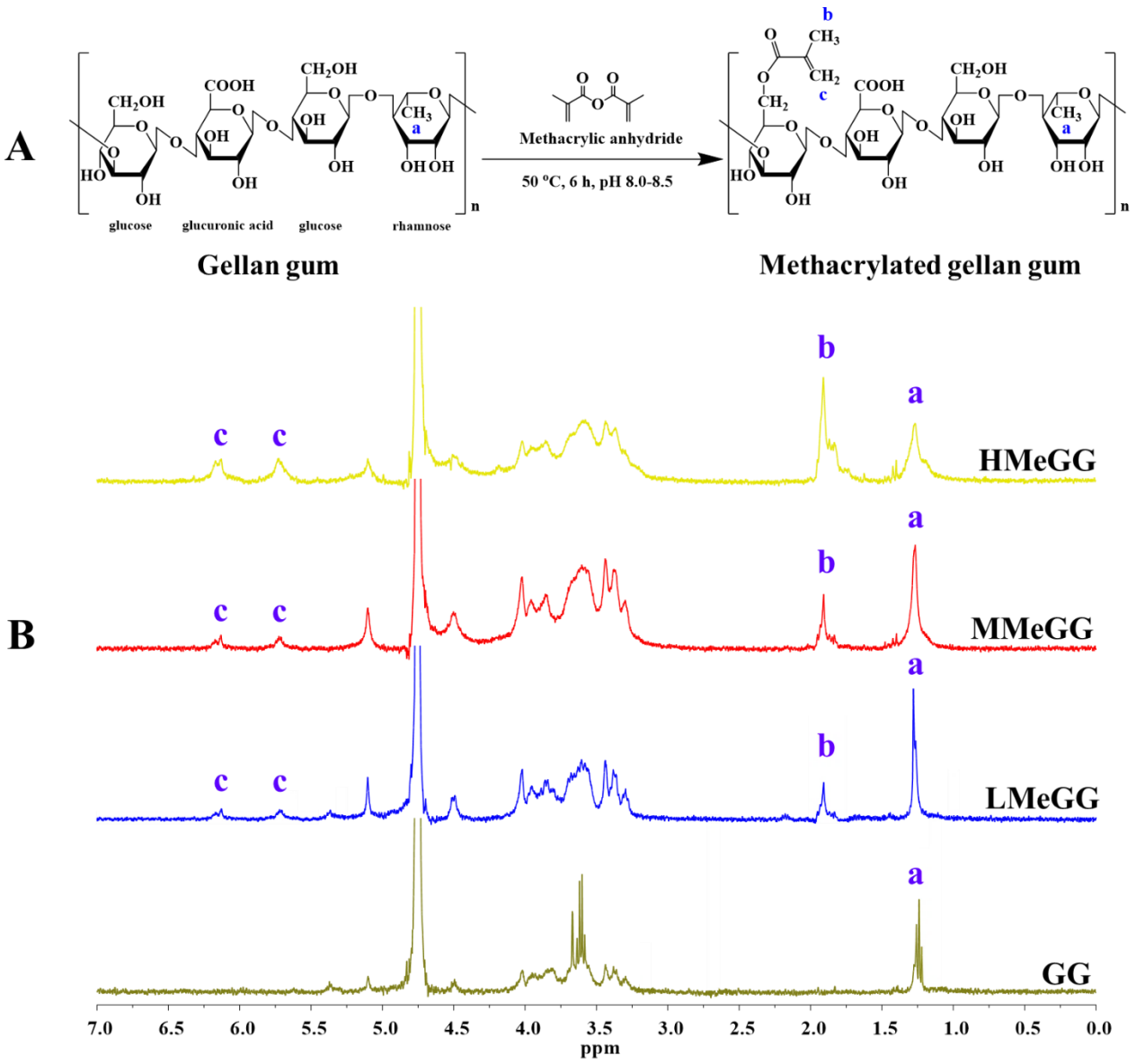


Figure 1. Synthesis and characterisation of methacrylated gellan gum (MeGG). (A) Schematic illustration of the methacrylation reaction. Please note that schematic structure show only one possibility of methacrylic reaction anhydride with –CH_2-OH groups of gellan gum. In reality it could react with any OH-group present in gellan gum; (B) ^{1}H NMR spectra of gellan gum (GG) with low (LMeGG), medium (MMeGG) and high (HMeGG) degrees of methacrylation recorded in D_2O at 50 °C. The characteristic methyl peak (a) from rhamnose structural unit and methyl group (b) of the methacrylic anhydride (MA) were detected at 1.27 and 1.91 ppm, respectively, and methylidene (CH_2=) peaks (c) of MA were identified at 5.72 and 6.13 ppm. Some broadening of methyl peak at 1.27 ppm could be related to partial aggregation of more hydrophobic methacrylated macromolecules.

Methacrylation was confirmed by the appearance of distinctive methacryloyl (**CH_2=**C(CH_3)-) group peaks (δ 5.72 and 6.13 ppm) and a peak corresponding to the –CH_3 group of the methacrylate moieties on the modified GG segment (δ 1.91 ppm). This is in good agreement with ^{1}H NMR data reported in the literature (Coutinho et al., 2010; Kolawole et al., 2018). The degree of substitution was quantified by determining the ratio of integrated methylidene group (CH_2=C) peaks on the methacrylate conjugate over the –CH_3 group on the rhamnose ring. The LMeGG, MMeGG and HMeGG displayed DS at 6, 14 and 49 %, respectively. The yields of methacrylated derivatives were: LMeGG (31 %), MMeGG (22 %) and HMeGG (11 %). This decrease in the yield shows a similar trend to the previously reported methacrylated chitosan (Kolawole et al., 2018).

The methacrylation of GG was further confirmed by FTIR spectroscopy (**Fig. 2**). The FTIR spectra of modified and unmodified GG display broad –OH stretching peaks appeared above 3000 cm^{-1} and skeletal vibration involving the C–O stretching at 1030 cm^{-1}, which are typical for all polysaccharides. The peaks at 1220, 1300-1470 cm^{-1} are due to C–C stretching and CH bending, respectively. The characteristic double bond peak signal observed in the spectra of methacrylated derivatives at 1630 cm^{-1} represents C=C stretching in methacrylate moiety of GG, while the absorption band at 1715 cm^{-1} attributed to the carbonyl (C=O) stretching confirming the chemical modification of GG and growth of peak intensity with increasing degree of methacrylation. The peaks at around 2300 cm^{-1} present in the spectra of all samples are typical for atmospheric carbon dioxide.

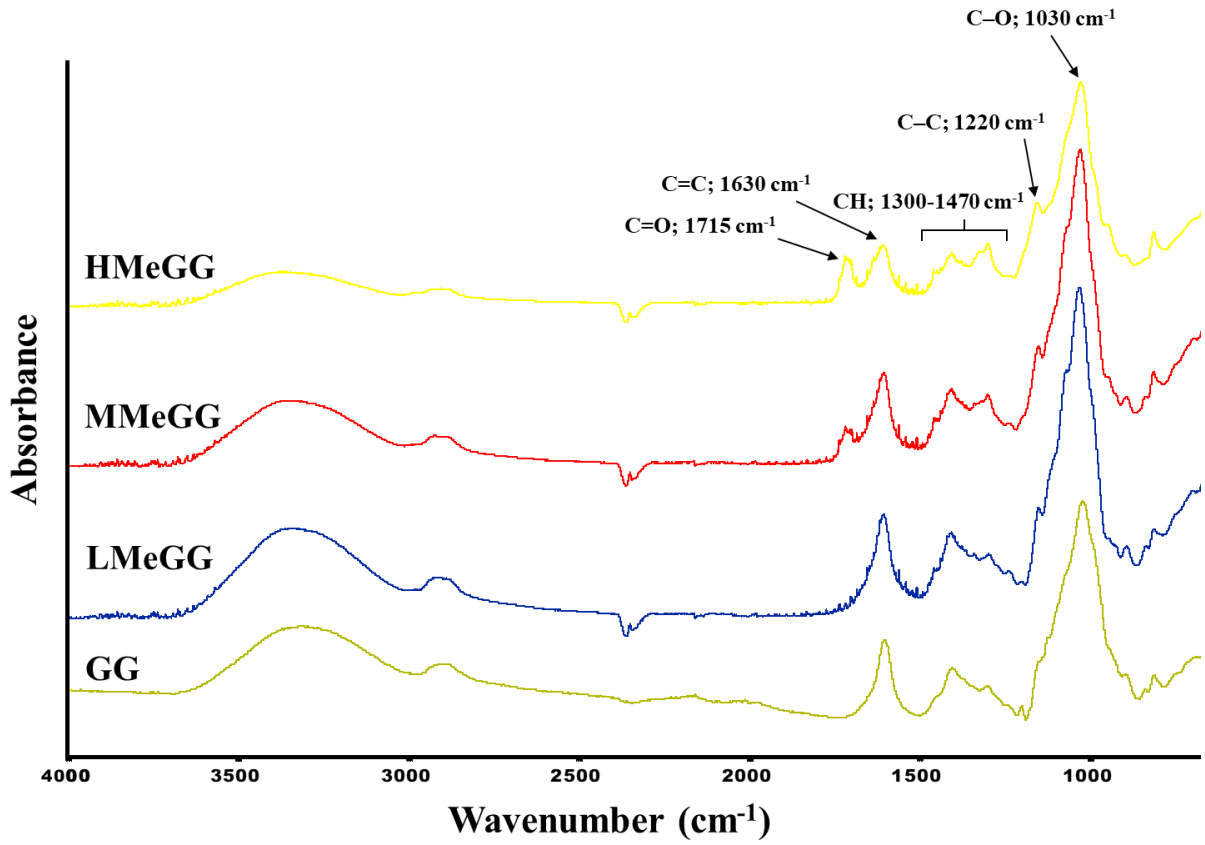


Figure 2. FTIR spectra of gellan gum (GG) and its methacrylated derivatives (LMeGG, MMeGG and HMeGG)

Solubility of methacrylated gellan gum in water

Unlike parent GG, methacrylated gellan gum derivatives were not fully soluble in water and formed slightly turbid solutions. This is likely related to a slightly hydrophobic nature of methacryloyl moieties and is in agreement with the observations reported for methacrylated chitosan (Kolawole et al., 2018). The solutions of parent and modified gellan gum were evaluated using dynamic light scattering (DLS) at three different concentrations (0.1, 0.5 and 1 mg/mL) and different pHs (2, 4, 6, 8), which indicated the presence of highly polydisperse aggregates even in solutions of parent gellan gum (Fig. S1 and S2). The highly polydisperse nature of these aggregates and the presence of particles whose sizes are > 1000 nm did limit the applicability of DLS for accurate characterisation of these colloidal dispersions. The presence of large particles in unmodified gellan gum is likely related to the

ability of this polysaccharide to form ordered helixes of double strands at low temperatures (Yuguchi et al., 1993). The tendency to aggregate is increased with methacrylation due to partially hydrophobic nature of methacryloyl moieties. More substantial aggregation was observed upon increase in polymer concentrations in all cases and also under very acidic pH (pH 2.0). The aggregation in strongly acidic solutions is likely related to suppression of carboxylic groups ionisation.

3.2. Mucoadhesion studies

The mucosal retention of unmodified and methacrylated gellan gum formulations containing fluorescein sodium (NaFl, 1 mg/mL) and free NaFl solution on freshly isolated bovine conjunctival tissue was evaluated using a wash-off *in vitro* technique with fluorescent detection. This method has been extensively used by our group to investigate the mucoadhesive properties of various materials on mucosal surfaces (Irmukhametova et al., 2011; Al Khateb et al., 2016; Tonglairoum et al., 2016; Kolawole et al., 2018; Porfiryeva et al, 2019). **Fig. 3** shows exemplar fluorescence microphotographs of the retention of gellan gum and its methacrylated derivatives (LMeGG, MMeGG, HMeGG) and NaFl (used as a control) on *ex vivo* bovine conjunctival mucosa taken after each washing with STF solutions (pH 7.4; flow rate 200 µL/min) over 60 min.

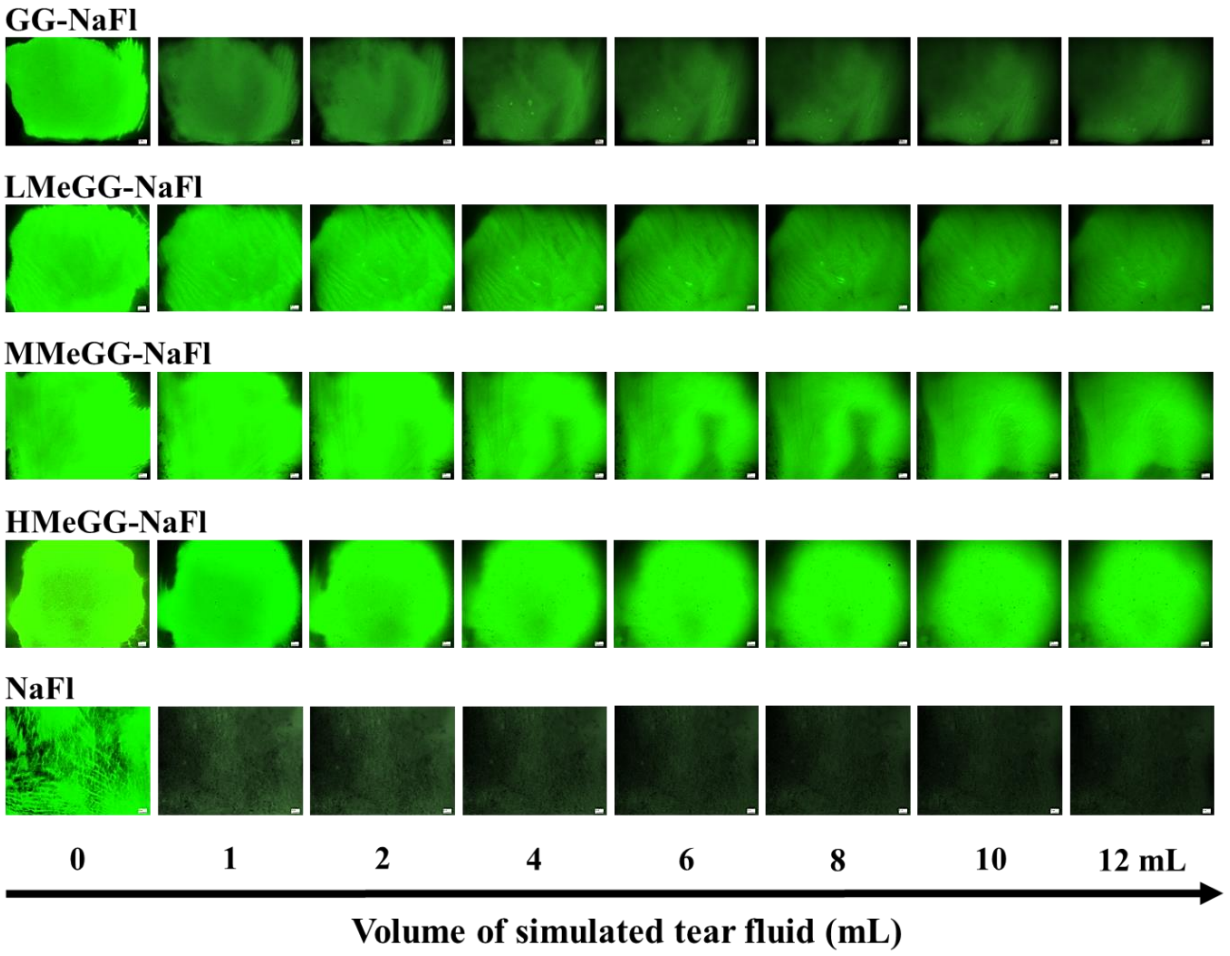


Figure 3. Exemplary fluorescent microphotographs showing mucosal retention of unmodified and methacrylated gellan gum (GG, LMeGG, MMeGG and HMeGG) formulations with fluorescein sodium (NaFl), and free NaFl (served as a control) on freshly excised bovine conjunctival tissue as washed with simulated tear fluid (pH 7.4; 200 μL/min) over 60 min. Scale bars are 200 μm.

The fluorescent images were then analysed using ImageJ software and fluorescence intensity values were normalised to 100 % (**Fig. 4**). During mucoadhesion experiments conducted at 37 °C, GG and its methacrylated derivatives formed *in situ* gels and the percentage of retention on mucosal tissues was estimated. It was revealed that methacrylation enhanced the mucoadhesive properties of GG on freshly excised bovine conjunctiva. HMeGG displayed significantly greater retention compared to its unmodified GG ($p < 0.001$), LMeGG ($p < 0.05$) and NaFl solution ($p < 0.0001$).

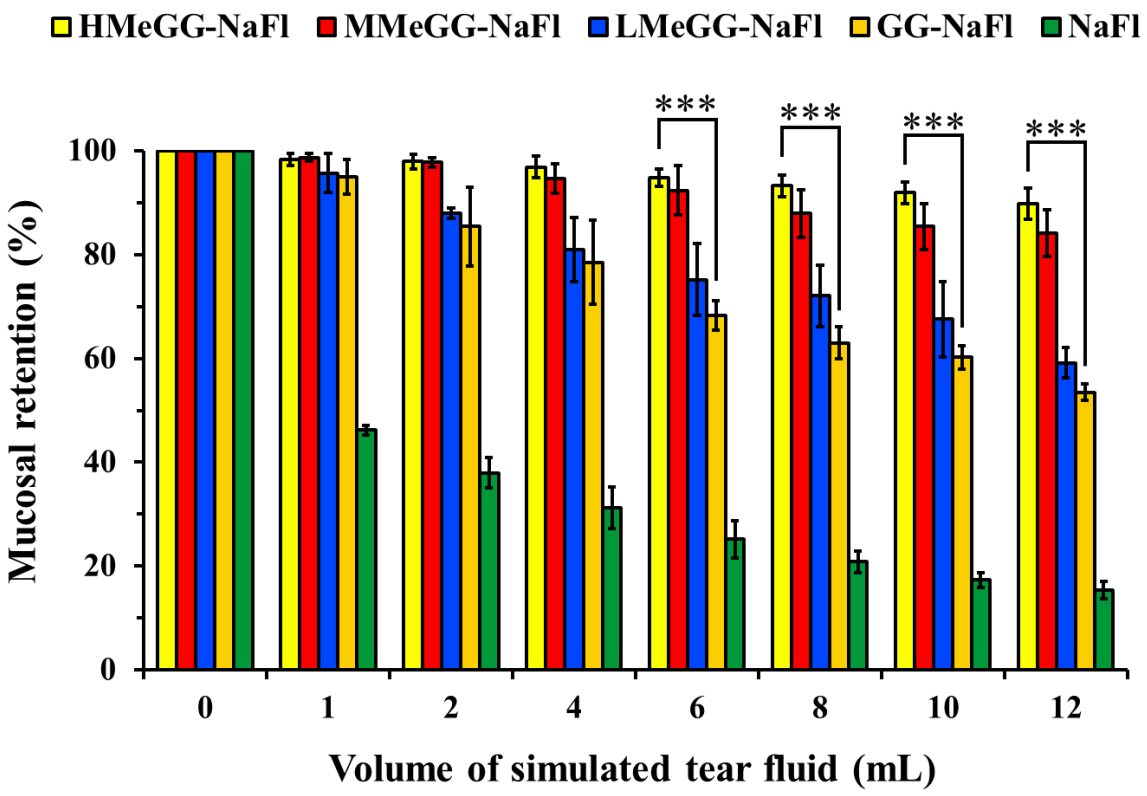


Figure 4. Mucosal retention of fluorescein sodium (NaFl) from GG, HMeGG, MMeGG and LMeGG, and NaFl (used as a control) on freshly dissected bovine conjunctival tissue as irrigated with simulated tear fluid (pH 7.4; 200 µL/min) over 60 min. All values are the means ± standard deviations of triplicate experiments. "***" depicts statistical significant differences between samples ($p < 0.001$).

Moreover, GG and LMeGG formulations exhibited almost the same retention ability ($p > 0.05$). They were washed out quicker than HMeGG but showed greater retention than NaFl solution. The retention of HMeGG and MMeGG on conjunctival mucosa was found not to be significantly different from each other ($p > 0.05$) expressing a similar retention trend and increased fluorescence intensity until the end of washing cycles. Additionally, NaFl solution showed significantly lower retention capability, approximately 85 % of it was washed out from the mucosal tissue. The remaining NaFl could be associated to its ability to stain mucosal surface as it is usually used in clinical practice for the diagnosis of ocular disorders (Korb et al., 2008).

In this study, the retention of GG, LMeGG, MMeGG and HMeGG on *ex vivo* bovine conjunctivae was also determined using a quantitative WO_{50} method developed by Mun et al. (2016).

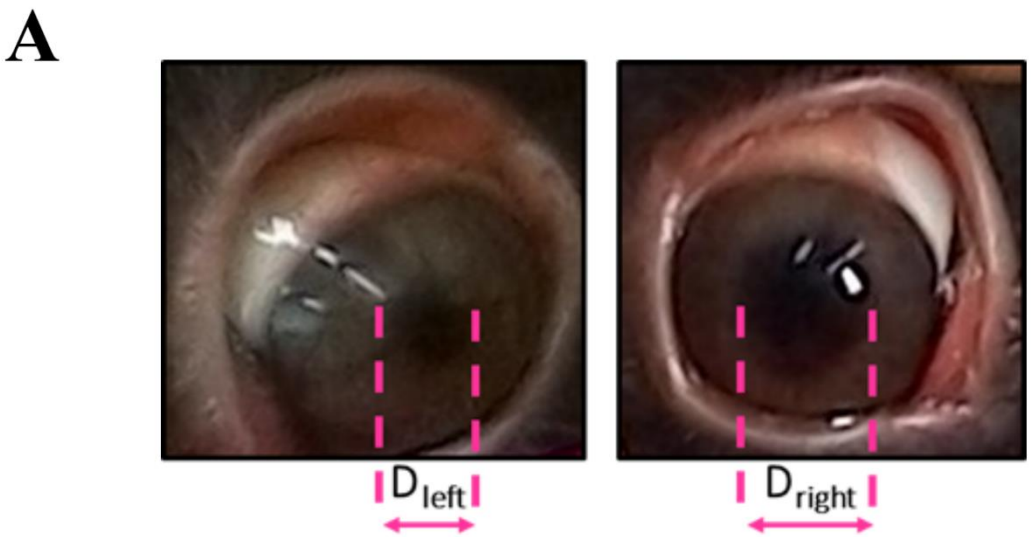
WO_{50} describes the volume of bio-relevant fluid required to wash out 50 % of the formulation from the mucosal surfaces. By analysing individual wash-off profiles for each NaFl-loaded GG, LMeGG, MMeGG and HMeGG excipients as well as free NaFl, the WO_{50} values were determined: 18 mL (R^2 = 0.9934), 23 mL (R^2 = 0.9979), 65 mL (R^2 = 0.9977), 75 mL (R^2 = 0.9988) and 3 mL (R^2 = 0.9958), respectively. According to these data, HMeGG has the highest WO_{50} value and this demonstrates its superior retention behaviour compared to other samples. This is likely attributed to the fact that methacrylation (similarly to acrylation) enhances the adhesion of GG on conjunctival tissues by forming covalent linkages between C–C double bond of GG methacrylate moieties and thiol groups present in conjunctival mucosa (Davidovich-Pinhas and Bianco-Peled, 2011; Brannigan and Khutoryanskiy, 2017; Kolawole et al., 2018; Porfiryeva et al, 2019). Therefore, these results confirm the retention properties of methacrylated gellan gum, which could also be used as a potential mucoadhesive formulation in the therapy of ocular disorders.

3.3. *In vivo* studies

In vivo studies were performed in rabbits using formulations of pilocarpine hydrochloride (pilocarpine · HCl) prepared with unmodified and modified gellan gum. Pilocarpine · HCl eye drops are mainly used in the treatment of glaucoma and this drug causes pupil constriction. This allows a non-invasive *in vivo* study where the efficiency of different pilocarpine formulations could be compared. Previously, Lin et al. (2004) have reported an *in vivo* study of pilocarpine formulated using sodium alginate, Pluronic F127 and their mixtures and established that the mixture of two polymers significantly improves the drug efficiency and bioavailability.

An administration of pilocarpine · HCl containing eye drops in rabbits does indeed cause their pupil constriction (**Fig. 5A**), which could be non-invasively measured using image analysis. **Fig. 5B** shows the difference in pupil diameter Δ recorded as a function of time following administration of different pilocarpine hydrochloride formulations. Despite the apparent ease of these measurements, there are some limitations related to the reaction of eye pupils to environmental light. Any changes in lighting of the environment could result in quick pupil reaction, which explains relatively high

values of error bars recorded in these measurements. Nevertheless, the analysis of these data indicates that there is a statistically significant difference between pure pilocarpine · HCl drops and the formulation containing MMeGG ($p < 0.05$), with the later exhibiting a more substantial pupil response at 180 min of experiment. The formulation containing MMeGG also showed greater response compared to unmodified gellan gum ($p < 0.01$).



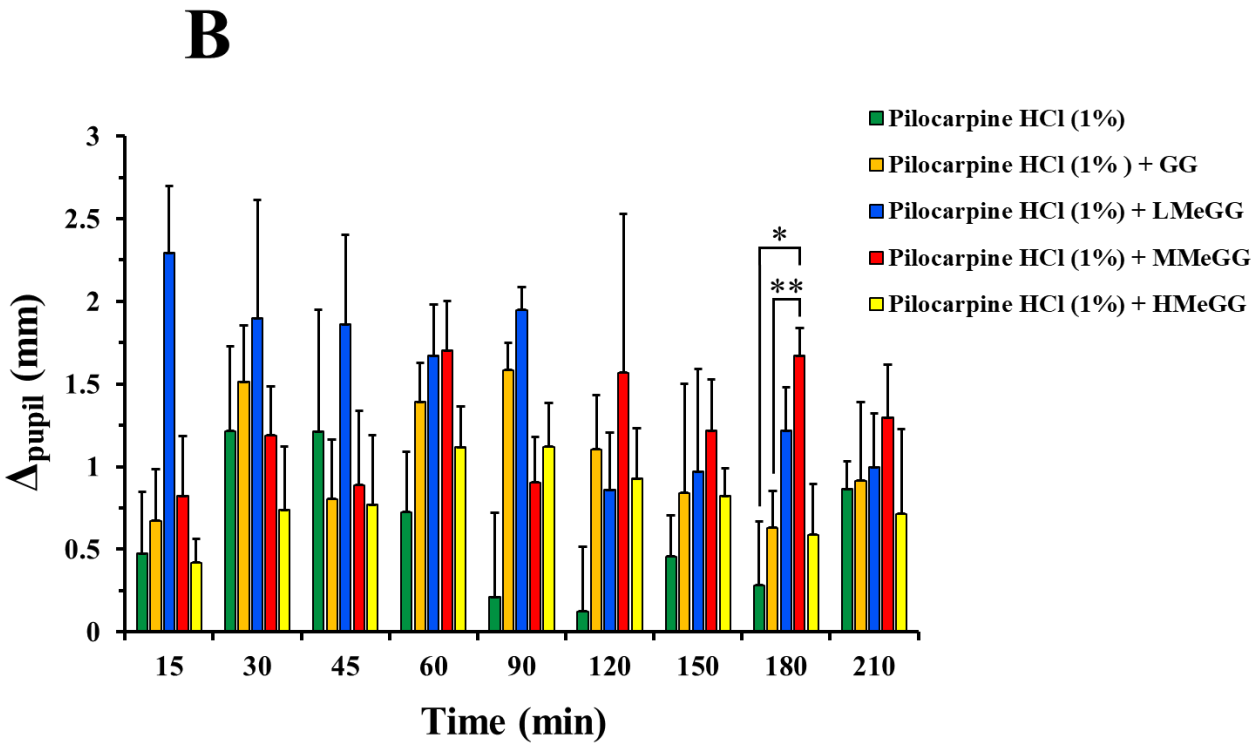


Figure 5. Exemplary images of rabbit eye with (left eye) and without (right eye) administration of pilocarpine · HCl formulations (A); Δ_{pupil} values recorded in rabbits from 15 to 210 min of experiment following administration of different pilocarpine · HCl formulations (B). "*" and "**" depict statistically significant differences between samples ($p < 0.05$) and ($p < 0.01$), respectively. All values are the mean ± standard error of the mean (n=4).

In order to see the overall performance of all five formulations in the time course of experiments the values of area under the Δ_{pupil} versus time profiles in 15–210 min were calculated (**Fig. 6**). These values showed the difference between these formulations clearer. The formulation containing unmodified GG did show significantly greater efficiency compared to pure pilocarpine · HCl ($p < 0.05$). The formulations containing LMeGG and MMeGG showed even better performance than GG, which is likely related to their enhanced mucoadhesive properties ($p < 0.0001$ and $p < 0.01$, respectively). However, no significant improvements were found when formulation with HMeGG was used compared to pure pilocarpine · HCl ($p > 0.05$). Also the formulation containing LMeGG did exhibit significantly greater performance compared to HMeGG ($p < 0.05$). The poor performance of HMeGG could be related to its more hydrophobic nature due to the highest levels of methacrylated groups. The difference between *in vitro* retention data and *in vivo* results observed in this work could also be related to the different active ingredients used in these formulations: sodium fluorescein versus pilocarpine · HCl. This could additionally be related to many other factors such some differences in the nature of mucosal surfaces between *ex vivo* bovine tissues and *in vivo* rabbit tissues (e.g. different thiol content), different tear production *in vivo* versus *in vitro* flow rate used, etc. Also *in vivo* the polymer interaction with the mucosa could affect drug absorption due to possible inhibition effects.

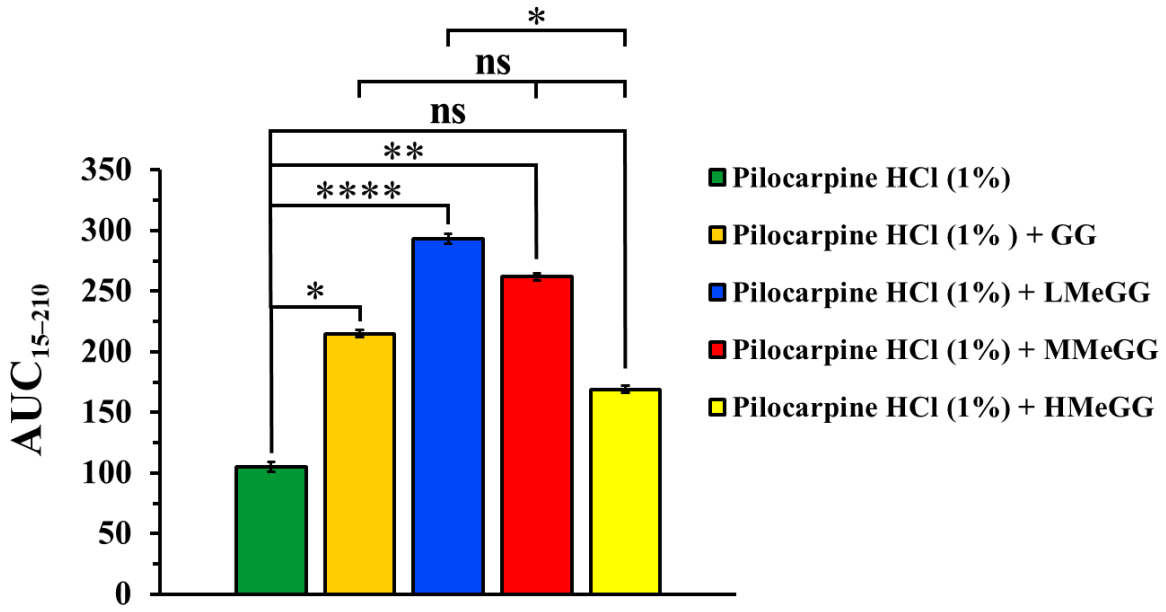


Figure 6. Area under the Δ_{pupil} versus time profiles in 210 min ($AUC_{15\text{-}210}$) for various formulations. "*", "**" and "****" depict $p < 0.05$, $p < 0.01$ and $p < 0.0001$, respectively, *ns* – no significance. All values are the means ± standard error of the mean (n = 4).

4. Conclusions

This study reports the synthesis of methacrylated gellan gum derivatives and their evaluation as potential mucoadhesive excipients for ocular drug delivery. Gellan gum was modified by reaction with methacrylic anhydride in order to improve its mucoadhesive properties. The methacrylation was confirmed using ^{1}H NMR and FTIR spectroscopic techniques and the degree of substitution was calculated. It was established that methacrylation makes this polysaccharide more hydrophobic. *In vitro* experiments performed using fluorescence technique indicated significant improvement in the retention of formulations with methacrylation of gellan gum on ocular mucosa. *In vivo* experiments conducted with pilocarpine hydrochloride formulations containing gellan gum and methacrylated derivatives indicated greater performance of the polysaccharide with low degree of modification.

Acknowledgements

Chemical Analysis Facility (University of Reading) is thanked for providing access to NMR-spectroscopy instrument. L.E.A., R.A.M and G.A.M acknowledge the Ministry of Education and Science of the Republic of Kazakhstan for the research grants (No. BR05234619 and No. BR05236446). D.B.K. acknowledges the Researcher Links Post-Doctoral Mobility Grant (No. 216046068, British Council Newton – Al-Farabi Partnership Programme) and Al-Farabi Kazakh National University for financial support of his postdoctoral fellowships. V.V.K and S.E.K. acknowledge the European Union's Horizon 2020 research and innovation programme under the Marie Skłodowska-Curie grant agreement Nanopol 823883. V.V.K. and R.I.M. acknowledge the Ministry of Education and Science of the Republic of Tatarstan (Russia) for "Algarysh" grant. P.C.

Appendix A. Supplementary material

The Supplementary Information is available free of charge.

Author contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no conflict of interest.

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