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A temperature-induced and shear-reversible assembly of latanoprost-loaded amphiphilic chitosan colloids: Characterization and in vivo glaucoma treatment

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ABSTRACT

Hydrogels composed of assembled colloids is a material class that is currently receiving much interest and shows great promise for use in biomedical applications. This emerging material class presents unique properties derived from the combination of nanosized domains in the form of colloidal particles with a continuous gel network and an interspersed liquid phase. Here we developed an amphiphilic chitosan-based, thermogelling, shear-reversible colloidal gel system for improved glaucoma treatment and addressed how preparation procedures and loading with the anti-glaucoma drug latanoprost and commonly used preservative benzalkonium chloride influenced the mechanical properties of and drug release from the colloidal gels. The results highlight that incorporated substances and preparation procedures have effects both on mechanical properties and drug release, but that the release of drug loaded in the colloidal carriers is mainly limited by transport out of the carriers, rather than by diffusion within the gel. The developed colloidal chitosan based gels hold outstanding biomedical potential, as confirmed by the ease of preparation and administration, low cytotoxicity in MTT assay, excellent biocompatibility and lowering of intraocular pressure for 40 days in a rabbit glaucoma model. The findings clearly justify further investigations towards clinical use in the treatment of glaucoma. Furthermore, the use of this shear-reversible colloidal gel could easily be extended to localized treatment of a number of critical conditions, from chronic disorders to cancer, potentially resulting in a number of new therapeutics with improved clinical performance.

1. Introduction

Here a highly biocompatible, shear-reversible, injectable drug delivery system based on assembly of amphiphilic chitosan colloids was developed for improved glaucoma treatment. During the development important observations were recorded regarding how drug loading and preparation procedures influenced biomedically relevant properties.

Glaucoma is a major cause of irreversible vision loss and blindness worldwide [1]. It is characterized by permanent damage to the optic nerve, resulting in visual field loss. The damage to the optic nerve is commonly associated with high intraocular pressure (IOP), caused by abnormal drainage of fluid produced in the eye (aqueous humor). Current treatment alternatives are medications and surgeries [2–4], both aimed at lowering the IOP. The surgeries can substantially alleviate the symptoms of glaucoma but involve several latent risks, and many patients still require long-term medical treatment after the surgery [5–8]. Therefore, surgery is not the primary treatment in cases when IOP can be controlled by medications. However, the required medications are lifelong

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and failure to comply will cause progression of the glaucoma, with worsened vision and possibly blindness as a consequence. Among the medications, the hydrophobic prostaglandin analogue latanoprost is the first-line treatment for glaucoma and ocular hypertension, and was approved by FDA in 2003 [9]. Eye drop formulations of latanoprost usually contain the quaternary ammonium compound benzalkonium chloride (BAK) as an antimicrobial preservative [10–12]. Even though such eye drops are clinically approved and effective, side effects such as ocular discomfort and temporary burning sensation are common [12]. Those side effects and elderly patients failing to follow punctual administration are the most likely reasons for poor patient compliance.

To overcome the side effects and reduce the need for frequent medication, a reliable dosing technology with sustained release of latanoprost over an extended time (weeks to months) would be highly beneficial. From a clinical perspective, an injectable drug depot with a sustained release of latanoprost from the subconjunctival region has been considered an attractive choice. In the very limited literature available, the group of Professor Venkatraman has published two very promising studies where they used an injectable liposome system for sustained latanoprost delivery [13,14]. Not taking anything away from the excellent results in those studies, it is recognized that liposomal drug delivery systems generally have some drawbacks, such as: multiple-step preparation involving hazardous volatile organic solvents (in the large-scale pharmaceutical industry even ethanol can be a concern), changed properties upon storage and risk of fast-burst release from non-encapsulated drugs. In addition, liposomes may enter the circulatory system with systemic and off-target effects as a consequence. To overcome and minimize such issues, a newly developed injectable carboxymethylhexanoyl chitosan (CHC)-based colloidal gel system was evaluated as a latanoprost-carrying depot formulation.

In water, the CHC self-assembles into nanocapsules of about 200 nm in diameter, and the amphiphilic nature of the CHC allows spontaneous and efficient encapsulation of both hydrophilic and hydrophobic drugs, as well as proteins [15–19]. This laboratory has previously demonstrated that, when mixed with β-glycerophosphate (β-GP), the CHC nanocapsules form injectable thermogelling solutions which, upon increased temperature, aggregate into a continuous colloidal network. The gels are composed of a polymer-rich CHC nanocapsule network phase and an aqueous inter-nanocapsule phase, both being continuous throughout the colloidal gels. Furthermore, the gels are highly biocompatible and offer excellent control of drug delivery through the encapsulation of drugs in the nanocapsules [20,21].

Unlike conventional hydrogels, where the continuous network phase is constituted from crosslinked individual polymer chains or phase-separated regions [22–24], colloidal hydrogels, such as the one in this investigation, are formed as a result of colloidal assembly/aggregation of the constituting nanocapsules or nanoparticles [20,25,26]. For such colloidal gels, the packing structure may vary with gelling conditions and kinetics. The packing structure may, in turn, determine or influence rheological, mechanical and drug-release properties of the gels. To the authors' knowledge, there is limited literature available on how gelation conditions and kinetics influence drug release and mechanical properties of drug-loaded colloidal gels. Therefore, in this article, while developing a colloidal CHC-based depot gel carrying latanoprost for glaucoma treatment, rheological properties and drug-release kinetics were investigated for different formulations and preparation procedures. Selected formulations were brought forward for cytotoxicity tests using an MTT assay and in vivo evaluation of biocompatibility and therapeutic efficacy in a rabbit model.

2. Materials and methods

2.1. Materials

Acetonitrile was of HPLC grade and was bought from J.T. Baker. Latanoprost, HPLC-grade dimethyl sulfoxide (DMSO), triamcinolone acetonide, hematoxylin, fetal bovine serum (FBS), trypsin–EDTA, trypan blue, eosin, MTT reagent, glycerol, β-GP and BAK were bought from Sigma–Aldrich. SIRC cells derived from the Bioresource Collection and Research Center (BCRC), Food Industry Research and Development Institute, Taiwan. Gibco minimum essential medium (MEM) and Gibco antibiotic antimycotic solution were bought from Life Technologies. Phosphate-buffered saline (PBS) solution was bought from UniRegion Bio-Tech (Taiwan). Deionized water was of Milli-Q grade. Carboxymethyl-hexanoyl chitosan (CHC), Mw ≈ 160,000 Da and viscosity ≈ 120 cP (2% solution), was purchased from Advanced Delivery Technology Inc. (http://www.adt-dds.com), Hsinchu, Taiwan, under the name AC-SAC (nanocarrier). Its chemical identity was confirmed to be similar to previously reported CHC using nuclear magnetic resonance imaging (Supplementary material). The molecular structures of CHC, BAK and latanoprost are shown in Fig. 1S.

2.2. Preparation of colloidal CHC hydrogels containing latanoprost/BAK

Colloidal CHC gels containing latanoprost/BAK for glaucoma treatment were prepared as follows: CHC polymer (3 g) was dissolved in deionized water (100 ml) and then cooled in an ice bath. Glycerol (0.5 ml) was added in a 3% CHC solution (8 ml) to prepare 8.5 ml glycerol/CHC solution. Where applicable, latanoprost (500 μg or 5 mg) in DMSO (0.5 ml) was mixed with the glycerol/CHC solution. Subsequently, β-GP solution (33.3% β-GP in 1 ml water), containing 0, 1 or 2 mg of BAK was added to the glycerol/CHC solution under stirring on ice to prepare CHC pre-gel solution containing latanoprost/BAK (CHC gel-(b)). For the investigation into how drug distribution affects the release properties, a different encapsulation method was also used. Briefly, dry CHC (0.24 g) was dissolved in latanoprost-containing solution (9 ml), prepared by mixing latanoprost (500 μg) in DMSO (0.5 ml) with glycerol (0.5 ml) and deionized water (8 ml), and was stirred for 1 day. BAK and β-GP were added to the latanoprost/CHC solution to prepare the CHC pre-gel solution containing 500 μg ml^{-1} latanoprost (CHC gel-(a)). The pre-gelling solutions were generally gelled at 37 °C, to form solid-like CHC colloidal gels. However, to investigate the effect of gelation time on the release properties, CHC gel-(b) was also gelled at 4 °C. To determine the drug encapsulation efficiency (EE), free latanoprost in supernatant and latanoprost encapsulated in the CHC nanocapsules were separated using a centrifuge (Hermle Labortechnik GmbH, Germany) at 12,000 rpm and 20 °C for 15 min. The per cent EE was calculated as:

$$EE = \frac{(A_{total} - A_{remaining})}{A_{total}} \times 100 \quad (1)$$

where A_{total} and $A_{remaining}$ are the absorbance, determined using a HPLC system (Agilent Technologies, U.S.), at 210 nm of the total latanoprost content and the latanoprost remaining in the supernatant after centrifugation, respectively.

2.3. Rheological characterization

The dynamic viscoelastic properties of formed CHC gels with different compositions were determined through rheological analysis using an ARES strain-controlled rheometer (Rheological Scientific, NJ, U.S.) with a parallel-plate fixture (diameter = 41 mm,

gap = 2 mm). The test methods employed were oscillatory strain sweep, step strain analysis and frequency sweep, monitoring storage modulus (G'), loss modulus (G'') or viscosity. The strain sweeps were performed at fixed frequency (ω = 10 rad s^{-1}) and temperature (37 °C), with the oscillatory strain being increased from 1 to 200%. Step strain analysis was performed with the same settings, but with the strain directly alternating between 10 and 250%. The frequency sweeps were set up by holding the temperature at 37 °C and applying strain with constant amplitude (γ = 10%) while increasing the frequency from 0.1 to 100 rad s^{-1}. The plots of G', G'' or viscosity vs. strain or frequency from the two sweep tests were obtained directly from the software controlling the rheometer.

2.4. In vitro release from CHC colloidal gels

From each formulation pre-gelling solution (0.5 ml) with latanoprost was placed in three 2 ml Eppendorf tubes, which were incubated at 37 °C for 1 day to form a solid gel. Subsequently, the release experiment was carried out in 1 ml of PBS (pH 7.4, containing 10% DMSO to accelerate the release) at room temperature. At predetermined times, 1 ml of the solution was sampled and the same volume of fresh medium was added. The amount of released latanoprost was determined using a HPLC system (1200 series, Agilent Technologies, U.S.) operating in the reversed-phase mode. Analysis was performed on an Eclipse XDB-C18 (Agilent Technologies, U.S.) packed column (150 mm length × 4.6 mm inner diameter, 5 μm particle size). The mobile phase was a mixture of acetonitrile and deionized water (55:45), the flow rate was 0.8 ml min^{-1} and the UV detector was used at 210 nm. The cumulative amount of released latanoprost was calculated. The percentage of released drug was calculated using the equation below:

$$\text{drug released invitro } (\%) = M_t/M_{\text{total}} \times 100 \quad (2)$$

where M_t is the amount of drug released at time t and M_{total} is the total amount of drug in the sample.

2.5. Cell culture cytotoxicity

SIRC cells (Statens Seruminstitut rabbit cornea, derived from BCRC; BCRC number: 60093) were cultured in MEM containing 10% FBS and antibiotic antimycotic solution (1%). The cells were cultured in the complete medium at 37 °C, in a 5% CO_2 humidified atmosphere. For all experiments, cells were harvested from subconfluent cultures using trypsin and were resuspended in fresh complete medium before plating. The CHC colloidal gels for cell culture cytotoxicity were prepared from 1 cm × 1 cm thin films, which were put under UV light overnight for sterilization. The in vitro cytotoxicity of CHC colloidal gel thin films with/without latanoprost and BAK was evaluated with an in vitro proliferation method using the MTT assay, cultures without added CHC, latanoprost or BAK being used as the control. Briefly, 1×10^4 cells were seeded into 24-well plates to allow the cells to attach, then CHC colloidal gels of different composition were added. After incubation at 37 °C, 5% CO_2 in air, for 24 and 48 h, 200 μl of MTT solution (MTT reagent:medium = 1:9) was added and incubation was continued for another 4 h. Subsequently, the MTT solution was removed and DMSO (200 μl) was added to each well to dissolve the purple formazan salt crystals. The absorbance was measured in a MicroELISA reader (Programmable MPT reader DV 990BV4, GDV, Italy) at 595 nm. Cell viability was determined by calculation according to the following equation:

$$\text{cell viability}(\%) = (A_{\text{sample}}/A_{\text{control}}) \times 100\% \quad (3)$$

where A_{sample} is the absorbance of the sample and A_{control} is the absorbance of control.

2.6. Animal studies

Male New Zealand white rabbits (12 weeks old with a weight of about 2 kg) were used in animal studies. The animals were treated in accordance with the standards of Association for Research in Vision and Ophthalmology. Approval for the study was given by the Institutional Animal Care and Use Committee of National Yang-Ming University, Taiwan. The animals were kept under the following conditions: light (7am–7 pm) and dark (7 pm–7am); temperature (22–25 °C); humidity (55–60%); feeding (100 g standard feed) twice per day (8AM, 6PM); no limit to water and activity.

2.7. Glaucoma animal model and subconjunctival injection

Six rabbits were randomly (i.e. no screening of weight or age) assigned to each experimental group. The size of the groups (n = 6) was decided based on past experience in evaluation of biomaterials and IOP. The investigator was not blinded during the study. All the eyes were examined completely by portable slit-lamp to exclude abnormalities before any procedure and each time IOP was measured. Glaucoma was induced in the rabbits' right eye by intravitreal injection of 0.1 ml of 4 mg ml^{-1} triamcinolone acetonide in PBS, the left eyes being used as normal controls. Two injections were performed 7 days apart (days 0 and 7) [27]. At day 21, 500 μl of latanoprost-loaded CHC gel (2.4% CHC, 500 μg ml^{-1} latanoprost, 0.02% BAK) and CHC gel without latanoprost was injected at subconjunctival sites in the right eye of the test and control group, respectively. IOP was measured with a Tonolab tonometer (Colonial Medical Supply, Franconia, NH) at predetermined time points. Both eyes were measured five times and the mean was calculated.

2.8. In vivo biosafety assessment

The in vivo biosafety and biocompatibility were evaluated by histological survey of the effect of latanoprost–CHC gels, containing 0.02% BAK, at subconjunctival and subcutaneous injection sites in six rabbits. One month after injection, tissue including the injection site was removed, and slices were stained with hematoxylin and eosin. The samples were investigated for inflammatory reaction, hemorrhagic angiogenesis, necrosis and scarring using an IX51 microscope (Olympus, U.S.).

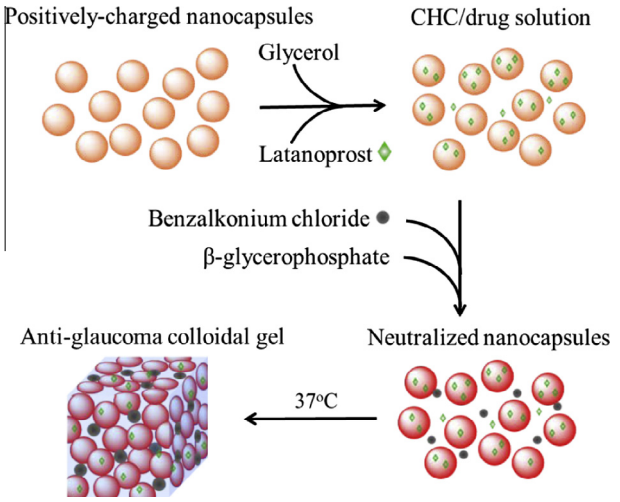
2.9. Statistical analysis

The mean and standard deviation were used for presentation of in vitro drug release. For cytotoxicity analysis and animal experiments, the median and interquartile range were used as the data could not be assumed to be normally distributed and the sample size was too small to test for normality with any statistical power. The significance of treatments was tested using the nonparametric Wilcoxon sum rank test, with the null hypothesis that there was no difference between compared populations.

3. Results

3.1. Formation of colloidal CHC gels containing latanoprost/BAK

Colloidal CHC-based thermogelling injectable hydrogels have been developed as a promising depot system for sustained drug delivery [20]. Here, thermogelling formulations of CHC nanocapsules were loaded simultaneously with hydrophobic latanoprost and/or the highly water-soluble stabilizer BAK. Scheme 1 shows the procedure for preparation of the drug-loaded gels. In the



Scheme 1. Schematic illustration of the latanoprost-carrying CHC colloidal gels for glaucoma treatment.

presence of β-GP, the dispersions of CHC nanocapsules, with or without BAK or latanoprost, exhibited thermogelation, as reported for a number of chitosan-based hydrogels [28–30]. For such thermogelling systems the gelation rate is dependent on the temperature, with higher temperatures generally corresponding to increased gelation rate [20,31,32]. For the present formulation the gelation took about 4 min at 37 °C and about 30 min at 4 °C, determined using the criterion of no flow upon tilting of the vial. The gel state was further confirmed by rheological analysis.

3.2. Rheological behaviour of the colloidal CHC gels

The colloidal CHC gels were subjected to rheological analysis, monitoring the storage modulus (G'), loss modulus (G'') and viscosity. Frequency sweep analysis revealed that the viscosity of the gels increased when loaded with latanoprost and BAK (Fig. 1a). Under 10% strain amplitude, a frequency of 10 rad s^{-1} and a temperature of 37 °C, the viscosity of gels without latanoprost showed an increase from 30 to 60 and 90 P with BAK concentrations of 0, 0.01 and 0.02%, respectively. For the gel containing both latanoprost and BAK, the viscosity was even higher, at 130 P. In addition, the G' and G″ values increased with BAK and latanoprost loading, and all investigated gels displayed solid-like gel behavior ($G' > G''$) over the frequency range investigated (0.1–100 rad s^{-1}), as seen in Fig. 1b. The above observations indicate that BAK and latanoprost strengthen and/or alter the structure of the formed colloidal network.

A highly relevant observation from the perspective of developing an injectable drug delivery system was that the gels exhibited a reversible shear-induced breakdown of the colloidal gel network. Above a critical strain, the gels transferred to quasi-liquid ($\tan\delta = G''/G' = 1$). The behaviour of gel without latanoprost is shown in Supplementary material (Fig. S2). For gel containing both BAK and latanoprost, the gel–liquid transition occurred at a strain of 70%, as seen in Fig. 1c. The recovery back to the gel state was very rapid after removing the high strain, as revealed by step-strain analysis (Fig. 1d). Under high shear strain ($\gamma = 250\%$; frequency = 10 rad s^{-1}) and resulting high shear stress, G' decreased from 1000 to 20 dyn cm^{-2}, resulting in a quasi-liquid state ($\tan\delta \approx 4$). However, when the strain was decreased to 10%, G' rapidly recovered to the initial value and the gel state ($\tan\delta \approx 0.14$)

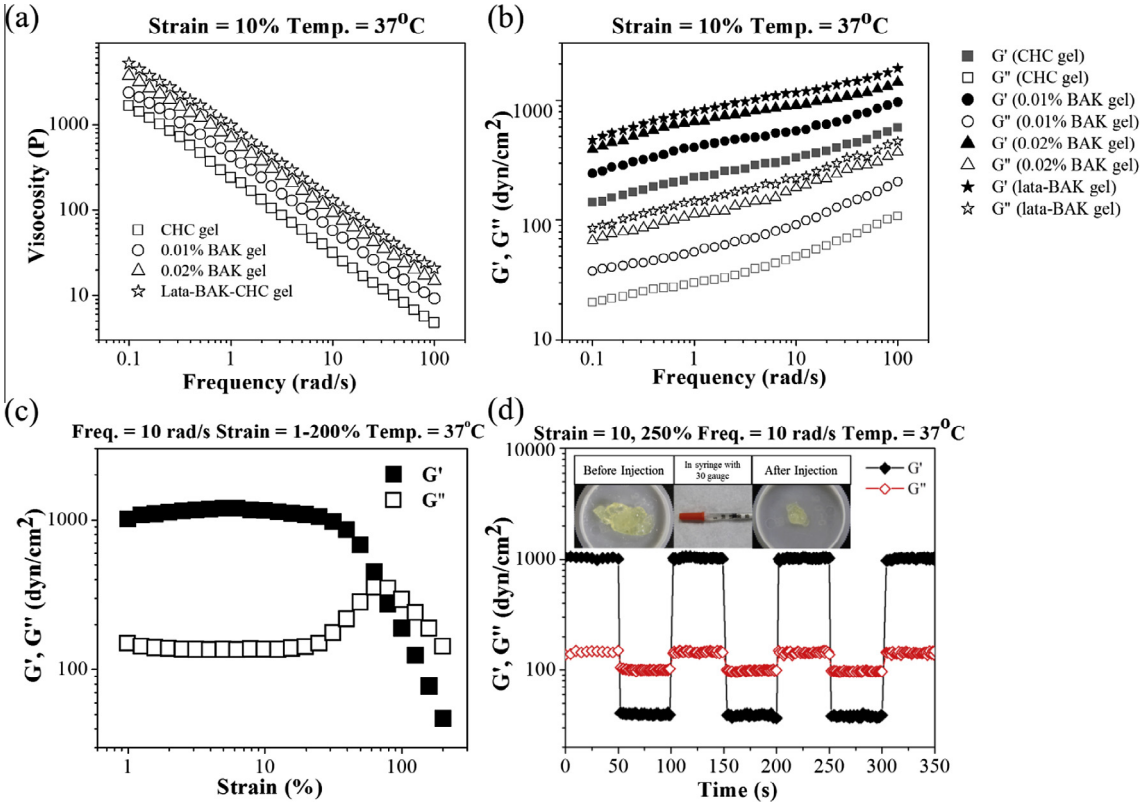


Fig. 1. Rheological characterization. (a) Viscosity and (b) storage (G') and loss modulus (G'') depending on frequency (ω = 0.1–100 rad s^{-1}) for gels containing 2.4% CHC with: No BAK or latanoprost (CHC gel); 0.01% and 0.02% BAK (BAK gel); 0.02% BAK and 500 μg ml^{-1} latanoprost (Lata-BAK–CHC). (c) Large strain sweep (γ = 1–200%) for CHC gel containing 0.02% BAK and 500 μg ml^{-1} latanoprost. (d) Continuous step-strain measurement (γ = 10 and 250%) for CHC gel containing 0.02% BAK and 500 μg ml^{-1} latanoprost. The inset shows the gel before injection, in a gauge 30 syringe and after being pushed through the syringe.

was restored. The extreme shear recovery of the colloidal gels is an excellent property for an injectable biomedical material. The extension of the shear reversibility to practical uses was confirmed by visually observing the gel before and after passing through a 30-gauge syringe (insert in Fig. 1d).

3.3. Effect of BAK concentration on drug release

In rheological tests, BAK was found to increase the hardness of the resulting colloidal gels. The influence of BAK on the release of latanoprost from the gels was subsequently investigated. As shown in Fig. 2a, the CHC gel without BAK released 45% of the loaded drug over a period of 4 days, while the BAK–CHC gel (0.02% BAK) released only 35% over the same time period. However, for a testing period as long as 30 days, the total amounts of released drug from both the CHC gel and the BAK–CHC levelled off at similar levels, i.e. about 60%. The fact that the drug release levelled off at 60% indicates that a fraction of drug is released within the time-frame of the experiments, while another fraction is released very slowly.

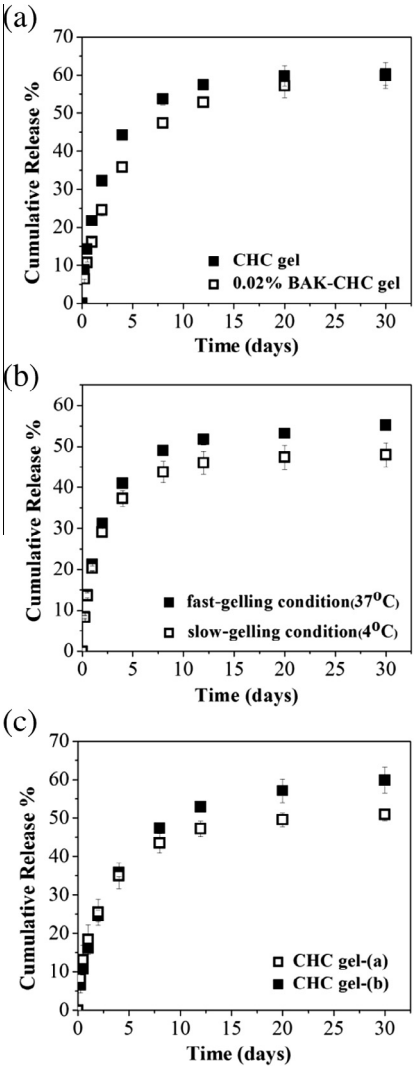


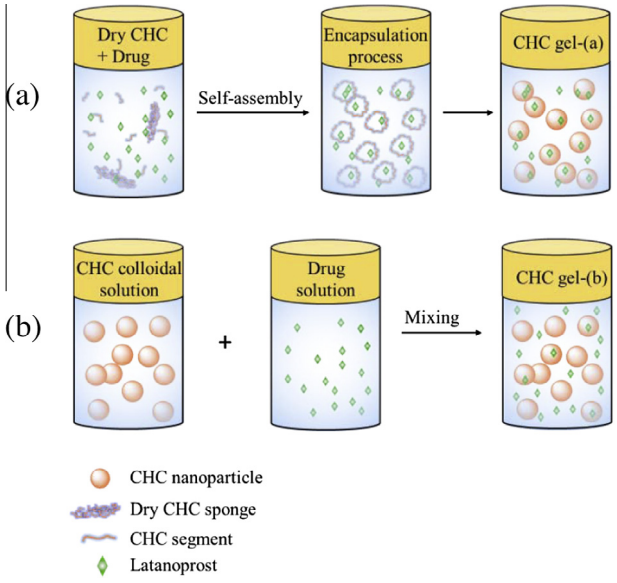
Fig. 2. Release profiles of latanoprost (50 μg ml^{-1} gel) from colloidal CHC gels: (a) with different concentrations of BAK (0 and 0.02%); (b) with 0.02% BAK, prepared with different gelling kinetics by controlling the temperatures (4 and 37 °C); and (c) with 0.02% BAK, prepared using different drug-encapsulation methods (CHC gel-(a), prepared using protocol (a), and CHC gel-(b), prepared using protocol (b); see the text). Values are the mean from analysis of different samples; error bars indicate the standard deviation (n = 3).

3.4. Effect of gelation temperature on drug release

For thermogelling systems, such as the present CHC gels, the gelation rate is dependent on the temperature. Higher temperatures generally correspond to increased gelation rates [20,31,32]. For the colloidal CHC gels the gelation is virtually an aggregation of dispersed nanocapsules, and the gelation kinetics could thus influence the structure of the formed network. The nano/microstructure could in turn influence the release of drugs from the gels. Therefore the influence of gelation kinetics on latanoprost release was investigated. Pre-gelation solutions (containing 0.02% BAK) were gelled at 4 °C or 37 °C and the drug release was investigated. The gelation took about 4 min at 37 °C and about 32 min at 4 °C. The drug release (evaluated at 37 °C) from the gel formed at 4 °C was slower than that from the gel formed at 37 °C, as shown in Fig. 2b. However, the difference was relatively small. Interestingly, the difference became more pronounced at later times, and after 30 days it seemed that the release had levelled off, with roughly 10% less drug released from the gel formed at 4 °C than from the gel formed at 37 °C.

3.5. Effect of drug distribution on drug release

Only a fraction of the drug seemed to be released within the experimental time-frame, with the other fraction being released very slowly. Therefore, the correlation of drug release with fraction of drug loaded in the nanocapsules during preparation was investigated. It is known that the EE depends on the loading procedure [15,16]. To achieve different EEs, latanoprost was loaded into the CHC nanocapsules by two different procedures prior to preparation of the thermogelling solutions (see Scheme 2). In procedure (a), dry CHC was dissolved in latanoprost-containing solution, resulting in an EE of 63 ± 7.8% (mean ± SD, n = 3). In procedure (b), latanoprost and CHC solutions were prepared separately and subsequently mixed, resulting in an EE of 51 ± 5.3% (n = 3), i.e. less latanoprost was loaded in the nanocapsules by procedure (b). As can be seen in Fig. 2c, the latanoprost release was initially somewhat faster from the gels prepared using protocol (a) than from the gels prepared using protocol (b). However, after a release of about 35%



Scheme 2. Schematic illustration of the processes of preparing CHC colloidal gels. (a) The dry CHC sponge was dissolved in the solution containing latanoprost. (b) Separate CHC containing solution and latanoprost-containing solution were combined and thoroughly mixed.

(4 days) the opposite trend was observed, with more latanoprost being released from the gel prepared using protocol (b). The release then levelled off for both gels. After 30 days, 61% of drug had been release from the gel prepared using protocol (b), while for the gel prepared using protocol (a) only 49% had been released. It seems that a higher EE of drug into the nanocapsules correlates with a reduction of the drug fraction with fast release kinetics, as expected.

3.6. Effect of injection on drug release

In applications the colloidal CHC gels will be pushed through a syringe upon injection into the target site. As the gel transforms into a quasi-liquid under the high shear during injection, the final structure of the gel may be different from the pre-injection structure. To investigate the effect of the injection on the drug release from a clinically relevant formulation, the release was compared with and without passing through a gauge 30 syringe. The investigated formulation was the same as was used in the evaluation of the in vivo therapeutic effect (2.4% CHC, 500 μg ml^{-1} latanoprost, 0.02% BAK). As seen in Fig. 3, the release from the gel passed through the syringe was somewhat accelerated compared to the release from the as-prepared gel; however, for both gels the release levelled off around 70%. Even if the release was accelerated by passing through a syringe, it is still very slow, especially when considering that 10% DMSO was present in the dissolution medium to accelerate the release.

3.7. Cytotoxicity of the CHC colloidal gels

To further prove the potential of the latanoprost-loaded colloidal CHC gels for biomedical applications, different formulations were investigated for cytotoxicity by MTT assay, using SIRC cells. CHC gels without latanoprost and BAK exhibited very low cytotoxicity, as shown in Fig. 4a. The cell viability over the whole range of investigated concentrations was above 80% after 48 h of treatment. The gel with 2.4% CHC concentration was further investigated when loaded with BAK and/or latanoprost. As seen from Fig. 4b, the loading of BAK and/or latanoprost into the gels reduced cytotoxicity compared to free latanoprost or BAK. After 48 h the gel containing only latanoprost displayed a cell viability of about 70% and the gel containing both BAK and latanoprost displayed a cell viability of about 30%, compared to cell viabilities of about 15% for free latanoprost or BAK.

Given that the presented gels would allow for a very low frequency of administration, it may be economically and clinically possible to prepare sterile doses that can be stored and handled without the preservative effect of BAK. Therefore, the cytotoxicity was further investigated for gels with 2.4% CHC containing

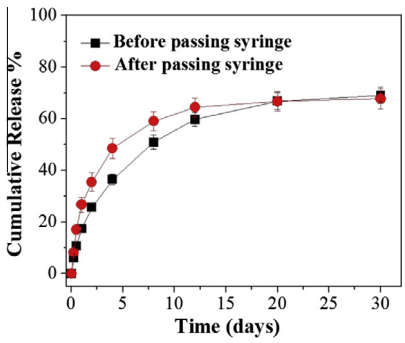


Fig. 3. Effect of injection on release profile. Release of latanoprost (500 μg ml^{-1} gel) from colloidal CHC gels containing 0.02% BAK, with and without passing through a gauge 30 syringe. Values are the mean from analysis of different samples; error bars indicate the standard deviation ($n = 3$).

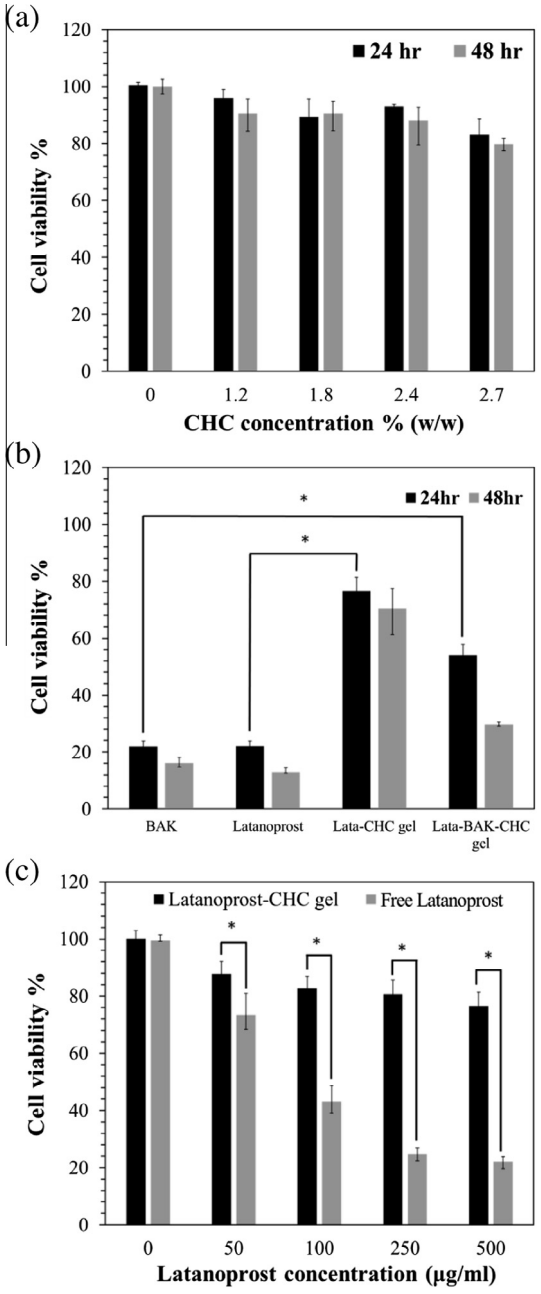


Fig. 4. Cell viability of SIRC cells exposed to the colloidal gels and free latanoprost/BAK. (a) Gels of different CHC concentrations at 24 and 48 h; (b) 2.4% CHC colloidal gel containing 500 μg ml^{-1} latanoprost and 0.02% BAK, as well as pure CHC gel and the corresponding amounts of free latanoprost and BAK; and (c) different concentrations of free latanoprost and latanoprost in 2.4% CHC gel at 24 h. Values are presented as the median from analysis of different samples; error bars indicate the interquartile range ($n = 4$). Significance was tested for using the Wilcoxon sum rank test; * indicates significantly reduced cytotoxicity ($p \leqslant 0.05$).

different latanoprost concentrations but no BAK. The cell viability at 24 h was compared to the corresponding amount of free latanoprost. As seen in Fig. 4c, the cell viability was as low as 22% with 500 μg ml^{-1}free latanoprost. However, when the same amount of latanoprost was loaded in a CHC gel, the corresponding cell viability was 78%.

3.8. In vivo therapeutic effect in glaucoma model

To evaluate the therapeutic efficacy (pharmacodynamics effect) of latanoprost-loaded CHC gels for reducing IOP, triamcinolone

acetonide (TA), a synthetic corticosteroid, was delivered into the rabbits' right eyes by intravitreal injection (0.1 ml of 4% TA) to induce an abnormally high IOP level (two injections; days 0 and 7). The left eyes were used as normal controls (without any treatment). As shown in Fig. 5, the IOP of the control group (without TA) was about 17 mmHg and that of the rabbits injected with TA gradually increased. Fourteen days after the second TA injection the IOP value levelled off around an average of 30 mmHg. Subsequently (day 21), 500 μl of latanoprost-loaded CHC gel (2.4% CHC, 500 μg ml^{-1} latanoprost, 0.02% BAK) or latanoprost free control was injected into the subconjunctival space of the right eyes. The abnormally high IOP values were decreased to 26 mmHg 2 days after the latanoprost–CHC gel injection, 23 mmHg 10 days after injection and 20 mmHg at the end of the test (39 days after injection). Compared to the CHC gel control, the latanoprost–CHC gel achieved a significantly greater reduction of IOP from day 25 and onwards, i.e. 4–39 days after injection. This is in contrast to latanoprost eye drops, where the IOP lowering effect only remains for 2–3 days after administration in rabbits [33].

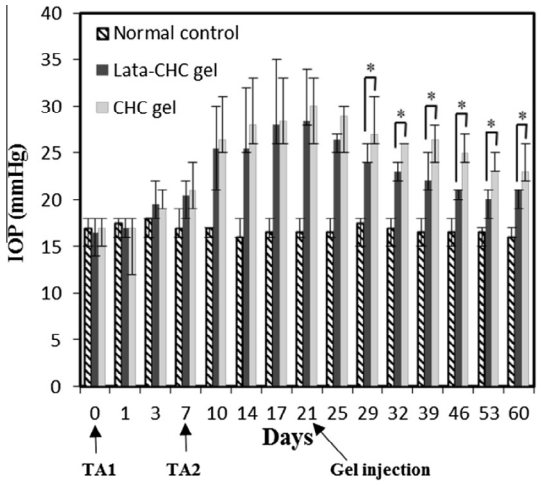


Fig. 5. IOP lowering effect of latanoprost-carrying CHC colloidal gel. The IOP levels of the rabbits' eyes injected subconjunctivally with latanoprost-carrying CHC colloidal gel (lata-CHC gel, 2.4% CHC, 500 μg ml^{-1} latanoprost, 0.02% BAK), gel without latanoprost (CHC gel) and normal control. In the eyes injected with lata-CHC gel and CHC gel, increased IOP had been induced by intravitreal injection of triamcinolone acetonide at day 0 (TA1) and day 7 (TA2). For each animal the IOP was determined as the average of five measurements at each time point. Values are presented are the median IOP from analysis of different animals; error bars indicate the interquartile range ($n = 6$). Significance was tested for using the Wilcoxon sum rank test; * indicates significantly reduced IOP ($p \leqslant 0.05$).

3.9. In vivo biosafety

To further investigate the biosafety and tissue reaction, latanoprost-loaded CHC colloidal gels (2.4% CHC, 500 μg ml^{-1} latanoprost, 0.02% BAK) were directly injected into subcutaneous and subconjunctival sites of rabbits. Based on the histological survey, there was no significant inflammation, hemorrhagic angiogenesis or scarring formation in the subcutaneous or subconjunctival sites 1 month after injection (Fig. 6).

4. Discussion

An injectable shear-reversible latanoprost-loaded colloidal CHC gel with sustained release for glaucoma treatment was successfully developed. The formulation presents not only thermo-induced gelation, but also shear-reversible gelation, as presented in Section 3.2 and Fig. 1. The shear-induced structural breakdown of the colloidal network means that the formulation can be injected from the gel state. This feature removes the requirement of strict temperature control during the handling of the formulation, which is a complicating factor for normal thermogelling systems. Furthermore, the rapid recovery of the gel state upon removal of the high shear ensures restoration of the structural integrity of the gel at the target site.

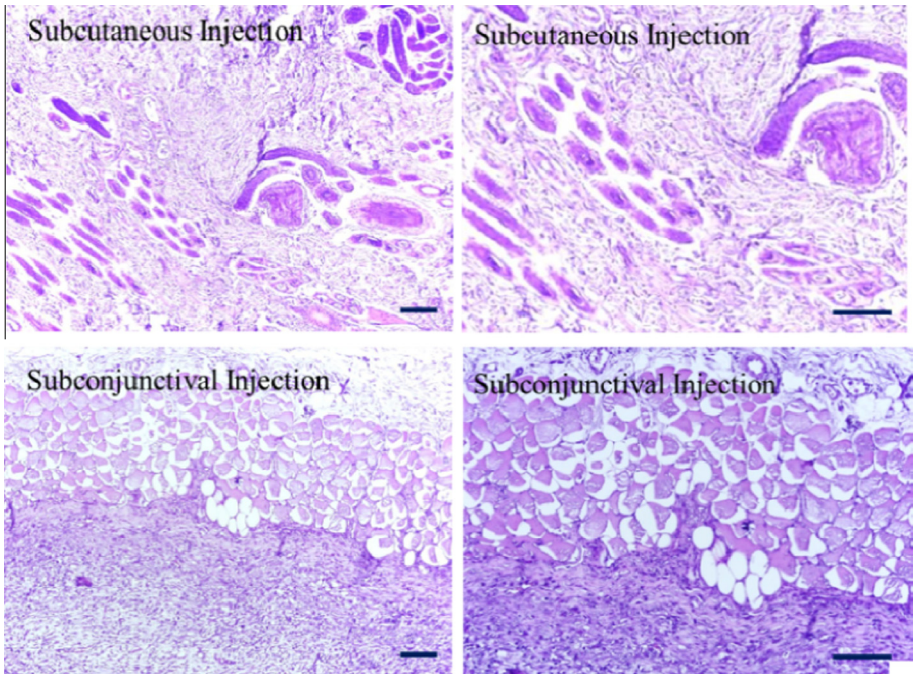


Fig. 6. In vivo biosafety by histological survey. Representative images of subconjunctival and subcutaneous regions 1 month after the injection of latanoprost-carrying CHC colloidal gel (lata-CHC gel, 2.4% CHC, 500 μg ml^{-1} latanoprost, 0.02% BAK). The scale bar is 150 μm. Six rabbits were investigated.

The in vitro drug release studies revealed that, for all formulations and preparation conditions, one fraction of the loaded drug was released over the 30 days of the experiment, while another fraction remained in the gels (Figs. 2 and 3). We hypothesize that the fraction of drug available for fast drug release was inversely dependent on the drug actually loaded into the nanocapsules. This is supported by the drug release profiles from gels prepared using different drug loading protocols (Fig. 2c). For the gels with a nanocapsule EE of 51%, the drug release levelled off at 61%, while for gels with an EE of 63% the drug release levelled off at 49%. It was also found that the total drug loading of the gels influenced the fraction of drug with a fast release rate. As seen in supporting Fig. S3, a tenfold increase in drug content increased the plateau value at day 30 from 60 to 70%. This behaviour is logical, as a higher drug content should lead to a reduced drug EE of the nanocapsules. The gelation temperature and presence of BAK were both found to slightly influence the release profiles, and a lower gelation temperature also led to a somewhat lower plateau value for the fraction of drug released at after 30 days (Fig. 2a and b). The exact mechanisms behind this behaviour are yet to be elucidated and merit further investigation. The drug release profile after injection is highly relevant for applications. As seen in Fig. 3, the injection process caused an accelerated initial release. However, the release was still extended, and after 30 days the release had levelled off at about 70% for both the gel pushed through the syringe and the as-prepared control. The results from the in vitro drug release clearly suggest that the formulations have the potential for extended release in vivo, especially when considering that the in vitro release was performed in the presence of 10% DMSO to accelerate the kinetics.

The formulations did not only present extended release of loaded latanoprost, the loading of BAK and latanoprost into the CHC gels reduced their cytotoxicity, as presented in Section 3.4 and shown in Fig. 4. A small decrease in cell viability was observed for the pure CHC gels, but the viability remained above 80% even for the highest investigated CHC concentration of 2.7%. The loading of BAK and latanoprost into the CHC gels did improve cell viability compared to the free substances, but the cell viability was still as low as about 30% after 48 h for gels containing both BAK and latanoprost (Fig. 4b). The low cell viability may be problematic for clinically relevant applications. However, it was observed that, for gels loaded only with latanoprost, the cell viability was greatly improved compared to the free substance (Fig. 4c). For a dose of 500 μg ml^{-1}, the viability was improved from 22% for free latanoprost to 78% when encapsulated in CHC. This may be a key feature for avoiding cytotoxicity in the development of formulations carrying high drug loads to be released over extended times. The observation that gels without BAK were much less cytotoxic indicates that a more clinically feasible formulation may be sterile-packed one-dose preparations that can be stored and handled without the preservative effect of BAK. For a depot formulation like in the present study, this would be a viable alternative, as the frequency of administration is very low.

In vivo experiments revealed excellent performance for the selected formulation (2.4% CHC, 500 μg ml^{-1} latanoprost, 0.02% BAK), despite the low cell viability observed in vitro. The extended therapeutic effect of the latanoprost-loaded colloidal CHC gel was proved in a rabbit glaucoma model, where a single administration lowered the IOP for up to 40 days (Fig. 5). It is worth mentioning that the IOP of the control animals, to which colloidal CHC gel without latanoprost was administered, also recovered from the peak value over time. However, this recovery was slower and levelled off at a higher value. This recovery of the control group is explained by the steroid-induced increase in IOP results in a peak value with slow recovery over time, as demonstrated by others [34]. The sustained lowering of IOP for the latanoprost-loaded CHC formulation is highly relevant as the effect of free latanoprost only remains for 2–3 days after administration in rabbits [33] and other IOP-lowering substances reduced IOP in steroid-induced rabbit models for less than 8 h [35]. In addition to the extended release, the drug-loaded gels also presented excellent biocompatibility, as determined by histological survey 1 month after administration (Fig. 6).

5. Conclusion

The drug used in this investigation, latanoprost, is currently one of the most powerful ocular hypotensive drugs available and the patent expired in March 2011. Improved glaucoma treatments are highly relevant, but no new classes of glaucoma drugs have emerged since the introduction of latanoprost. There are, however, a number of ongoing developments, the depot latanoprost delivery system being one approach [1,13,14]. Therefore, the results of this study, a new biocompatible injectable formulation with local sustained release of latanoprost and proven efficiency in vivo, are extremely interesting. The presented formulation allows for easy administration, easy preparation without volatile organic solvents, no-burst release and localization to site of injection. The results of this study suggest that the formulations should be further investigated with a view to clinical use. It is also easily foreseen that the use of these novel gels, or the same concept with different components, could be extended to the treatment of a number of critical conditions, from chronic disorders to cancer. The highly desirable properties suggest that the presented shear-reversible thermogelling colloidal CHC gels could offer improved clinical performance in a number of localized therapies.

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Appendix A. Figures with essential colour discrimination

Certain figures in this article, particularly Figs. 1, 3 and 6 are difficult to interpret in black and white. The full colour images can be found in the on-line version, at http://dx.doi.org/10.1016/j.actbio.2014.03.016.

Appendix B. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.actbio.2014.03.016.

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