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Light activated, In situ Forming Gel for Sustained Suprachoroidal Delivery of Bevacizumab

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Abstract

Purpose—To develop a light activated polycaprolactone dimethacrylate and hydroxyethyl methacrylate based gel network that sustains the release of stable, active bevacizumab (an anti-VEGF antibody used to treat choroidal neovascularization) and to assess sustained ex vivo delivery in rabbit eyes and in vivo delivery in rat eyes following in situ gel formation in the suprachoroidal space.

Methods—Polycaprolactone dimethacrylate (PCM) was synthesized from polycaprolactone diol (PCD) and evaluated using NMR spectroscopy. PCM was used to cross-link hydroxyethyl methacrylate (HEMA) in the presence of 365 nm UV light and 2, 2-dimethoxy-2-phenylacetophenone (DMPA) as a photoinitiator. Bevacizumab was entrapped in the gel using 3 different cross-linking durations of 3, 7, and 10 minutes. In vitro release of bevacizumab in PBS pH 7.4 at 37°C during a 4 months study was quantified using a VEGF-binding based ELISA. Stability of released bevacizumab was monitored by size exclusion chromatography (SEC) and circular dichroism. Alexa Fluor® 488 dye conjugated bevacizumab mixed with polymers was injected suprachoroidally in rabbit eyes to study the effect of different cross-linking durations on the spread of the dye conjugated bevacizumab. In vivo delivery was assessed in Sprague Dawley (SD) rats by injecting Alexa Fluor® 488 dye conjugated bevacizumab mixed with polymers followed by cross-linking for 10 minutes. Spread in the rabbit eyes and in vivo delivery in rat eyes was monitored noninvasively using a fundus camera and Fluorotron Master™.

Results—Formation of PCM was confirmed by the disappearance of hydroxyl peak in NMR spectra. Cross-linking duration of 10 minutes resulted in a burst release of 21 % of bevacizumab. Other cross-linking durations had 62 % burst release. Bevacizumab release from 10 minute cross-linked gel was sustained for ~ 4 months. Release samples contained 96.1 % of

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bevacizumab in the monomeric form as observed in SEC chromatograms. Circular dichroism confirmed that secondary β -sheet structure of bevacizumab was maintained after release from the gel. As the cross-linking duration was increased to 10 minutes, the gel/antibody was better confined at the injection site in excised rabbit eye suprachoroidal space. Delivery of Alexa Fluor® 488 dye conjugated bevacizumab was sustained for at least 60 days in the suprachoroidal space of SD rats.

Conclusion—PCM and HEMA gel sustained bevacizumab release for 4 months and maintained the stability and VEGF-binding activity of bevacizumab. Light activated PCM and HEMA gel is suitable for in situ gel formation and sustained protein delivery in the suprachoroidal space.

Keywords

Bevacizumab; protein delivery; sustained delivery; suprachoroidal delivery; stimuli responsive gel

1. Introduction

Intravitreal injections of bevacizumab, an anti-VEGF full-length antibody, are being used off-label for the treatment of choroidal neovascularization. Bevacizumab as well as the FDA approved intravitreal therapy with ranibizumab, a Fab fragment of bevacizumab, require monthly injections due to the chronic nature of the disease. However, repeated intravitreal injections can elevate the risk of infection, retinal detachment¹, and hemorrhage². Thus, there is a need to develop a slow release system for therapeutic proteins such as bevacizumab, in order to maintain therapeutically relevant concentrations over extended periods. Numerous sustained delivery approaches including liposomes³, polymeric micro- and nano- particulate systems⁴⁻⁶, mesoporous silica films⁷, and gels⁸⁻¹⁰ have been assessed to sustain protein drug delivery. However, to our knowledge, there are no reports demonstrating sustained release of an antibody drug in its stable form for 4 months. This is because most techniques for preparing polymeric delivery systems adversely affect protein stability.

Formulation of sustained delivery systems such as microparticles and nanoparticles introduce organic solvent-water and air-water interfaces, that are major sources of protein denaturation and aggregation¹¹⁻¹⁶. Furthermore, sonication used during particle preparation can reduce protein stability¹⁷⁻¹⁹. Liposomes, especially small vesicles employ sonication, which can be detrimental for protein stability²⁰. In comparison to particulate systems and liposomes, gel systems are more attractive and offer a promising delivery platform due to their simplicity in formulation, and solvent free condition. Polymeric gels are useful in a wide range of applications such as drug delivery^{21,22}, dentistry²³, and tissue engineering²⁴. In situ forming gels based on a stimuli-response are particularly attractive for ophthalmic application²⁵. To form such gels in situ, a polymeric mixture can potentially be injected through a small gauge needle in a confined space within the eye, followed by activation using a stimulus such as light. This is beneficial since viscous gels might require large bore needles and relatively prolonged duration of injection.

We aimed to develop a light activated gel using a polycaprolactone-derivatized dimethacrylate (PCM) and hydroxyethyl methacrylate (HEMA) for sustaining the release of

bevacizumab (Avastin™, Genetech Inc, CA), a 145 kDa monoclonal antibody, in its active form. PCM has been used extensively in drug delivery systems²⁶⁻²⁸, primarily due to its slow degradation rate^{29, 30}. Other advantages of using PCM are that it does not create an acidic environment (a drawback of solid poly (lactic acid) and poly (glycolic acid) microspheres for protein drugs), is biocompatible, and can easily be blended with a wide variety of polymers. Similar to PCM, HEMA also has a number of biomedical applications, including manufacturing of soft contact lenses³¹, dental adhesives³², and drug delivery systems^{33, 34}, due to its hydrophilicity and high water uptake capacity. Furthermore, both polycaprolactone and HEMA are present in FDA approved products for human use.

Bevacizumab was chosen in our study because of its similar efficacy to ranibizumab, an FDA approved drug product, in the treatment of choroidal neovascularization (CNV) associated with wet age-related macular degeneration (AMD)³⁵. Wet AMD, the leading cause of blindness in the USA, is prevalent in 8 million people and is a lifelong condition. However, bevacizumab, which targets angiogenesis pathways by binding with vascular endothelial growth factor (VEGF), has a short vitreous half-life of 4.8 days³⁶, requiring repeated injections. Therefore, a sustained delivery system for bevacizumab would be of immense clinical significance.

In addition to the development of a sustained delivery gel system for bevacizumab, we also optimized its administration via suprachoroidal route in excised rabbit eyes and confirmed in vivo sustained delivery of the gel system in the suprachoroidal space of SD rats³⁷. Suprachoroidal delivery is a novel route that is less invasive to the retina, than intravitreal injections. Suprachoroidal injections localize the therapeutic agents adjacent to the choroid region, the target tissue affected in CNV³⁸. However, a drawback in suprachoroidal delivery is that due to abundance of blood vessels in the choroid region, bevacizumab is cleared very rapidly following suprachoroidal administration³⁹. Thus, the gel systems developed in this study are of potential value in sustaining bevacizumab levels in the choroid.

2. Materials and Methods

2.1 Materials

Polycaprolactone diol (Mn= 530), 2-isocyanatoethyl methacrylate, hydroxyethyl methacrylate (HEMA), dibutyltin dilaurate, 2,2-dimethoxy-2-phenylacetophenone (DMPA), and bovine serum albumin were purchased from Sigma Aldrich, St Louis, MO. Alexa Fluor® 488 protein labeling kit was purchased from Invitrogen Inc., Grand Island, NY. ELISA plates were purchased from Corning Incorporated, Tewksbury, MA. VEGF165 and secondary antibody for ELISA was purchased from R&D Systems, Inc, Minneapolis, MN.

2.2 Preparation and characterization of polycaprolactone dimethacrylate (PCM)

Polycaprolactone diol was reacted with 2-isocyanatoethyl methacrylate at 1:2 molar ratios in the presence of a trace amount of dibutyltin dilaurate as a catalyst to create polycaprolactone dimethacrylate. The product was characterized by ¹H NMR using a Varian Inova 500 MHz NMR (Agilent Technologies, Santa Clara, CA).

2.3 Preparation of PCM-HEMA gel

A co-monomer mixture was prepared with a ratio of 90:10 (45 μ l and 5 μ l of HEMA: PCM). Bevacizumab (50 μ l of a 25 mg/ml solution) was added to the above solution. Photoinitiator (DMPA) was added to the dispersion and vortexed for 1 minute. The copolymerization was initiated by irradiation with near-UV light at 365 nm using Acticure® spot curing system (EXFO Electro-Optical Engineering Inc, Richardson, TX), positioned 8 cm from the dispersion. Gels were prepared at three different durations of light exposure to yield different degrees of cross-linking (3, 7, and 10 minutes).

To assess the degree of cross-linking, conversion data were obtained using a Nicolet 6700 FT-IR spectrometer (Thermo Scientific, IL) operating in the near-infrared region. The liquid monomer sample was placed between two glass cover slips and placed in a block, with an entry and exit port for the infrared beam, and photopolymerized at room temperature. The thickness of the liquid monomer sample between the cover slips was 0.8 mm and the diameter of the sample exposed to infrared beam was 1 cm. The intensity of the band at around 6150 cm^{-1} ⁴⁰, arising from the reacting $=\text{CH}_2$ double bond, was used to quantify the degree of conversion, which correlates with the cross-link density in the gel.

2.4 In vitro sustained release of bevacizumab from gel

Gels prepared at different cross-linking durations were studied for release of bevacizumab at 37 °C. The method used to follow drug release from the gel was adopted from Zhang et al.⁴¹. Briefly, 1 ml of PBS buffer pH 7.4 was carefully layered over the surface of the gel matrix. The gel matrix volume was 100 μ l and the amount of bevacizumab entrapped in the gel matrix was 1.25 mg. At predetermined time intervals the entire 1 ml was removed from the surface of the gel and quantified for bevacizumab released. Fresh 1 ml PBS pH 7.4 was layered over the gel for continuation of the release study. Samples were evaluated for the amount of bevacizumab by using an ELISA. Percent release was calculated based on the initial amount of bevacizumab entrapped in the gel (1.25 mg). The comparisons of the mean between the three groups were performed using one-way ANOVA followed by Tukey's post hoc analysis (GraphPad Prism, GraphPad Software, Inc., CA). The differences were considered statistically significant at $P < 0.05$.

2.5 Quantification of bevacizumab released from gel

ELISA was used to quantify bevacizumab in the release samples. All release samples were diluted in PBS pH 7.4 buffer. A 96-well plate was coated with 100 μ l per well of 0.2 μ g/ml of VEGF165 and incubated for 14 h. The plates were later blocked by adding 300 μ l per well of reagent diluent (prepared by dissolving 1% bovine serum albumin in PBS pH 7.4). Bevacizumab standard or samples were added to each well and incubated for 2 hours at room temperature. The addition of 100 μ l of secondary antibody (diluted 1:5000 in 0.1 % bovine serum albumin) to each well was followed up by incubation for 2 h at room temperature. TMB substrate solution (100 μ l) was added to each well and plates incubated in the dark for 30 minutes at room temperature. Lastly, 50 μ l of stop solution (0.5 M HCl) was added to each well and plate gently tapped to ensure thorough mixing. The optical density of each well was immediately determined, using a microplate reader (Molecular Devices Corp., CA) set to 450 nm. Avastin formulation was used for preparing the standard curve. Three

washings were performed at each step, after addition of release sample, blocking buffer, VEGF standard, detection antibody, and streptavidin-HRP. The wash buffer was made by dissolving 0.05% Tween 20 in PBS pH 7.4 buffer.

2.6 Stability characterization by size exclusion chromatography (SEC)

A size exclusion column (TSK® Gel G3000SWX) was attached to high performance liquid chromatography (Waters Corporation, Milford, MA). A UV detector scanning over the wavelength of 210-400 nm was used to detect the output from the size exclusion column. The mobile phase was an aqueous solution of 0.182 M KH_2PO_4 , 0.018 M K_2HPO_4 and 0.25 M KCl at pH 6.2. Flow rate of the mobile phase was 0.50 ml/minute. A standard of bevacizumab was prepared by dilution from a stock solution of 25 mg/ml to 50 µg/ml, which is similar in concentration to the marketed formulation of bevacizumab. The solution was prepared using 60 mg/mL trehalose, 5.8 mg/mL NaH_2PO_4 , 1.2 mg/mL Na_2HPO_4 and 0.4 mg/mL polysorbate 20 at pH 6.2. The volume of the injection was 100 µl. All samples were centrifuged at 14,000 g for 10 minutes before use.

2.7 Secondary structure characterization by circular dichroism

Bevacizumab released from the cross-linked gel was studied in the “far-UV” spectral region (190-250 nm) by circular dichroism to evaluate the loss of the secondary structure of bevacizumab after entrapment and release from the gel. The spectra were obtained on an AVIV model 62 DS spectropolarimeter (AVIV Biomedical, Inc., Lakewood Township, NJ). Protein solutions were transferred into a 1 mm path length quartz cell, which was placed in a thermostatic cell holder. Data were collected at 1 nm intervals utilizing a 2 nm bandwidth. The spectrum for the appropriate formulation blank was collected and subtracted from each protein formulation spectrum.

2.8 Suprachoroidal injection in ex vivo rabbit eyes

To assess the retention of the polymer at different cross-linking durations, bevacizumab was conjugated to Alexa Fluor® 488 dye using Alexa Fluor 488® protein labeling kit. The procedure for conjugation was followed as per the vendor's protocol. A solution was prepared using PCM and HEMA monomers in a ratio of 90:10 (45µl and 5µl of PCM: HEMA). Alexa Fluor® 488 dye conjugated bevacizumab (50 µl) was added to the above solution. Photoinitiator was added to the dispersion and vortexed for 1 minute. The above dispersion (5 µl) was injected in the suprachoroidal space of ex vivo rabbit eyes (n=3 for each group) using a 50 µl Hamilton glass syringe attached to a 30G 5/4” needle. Eyes were subjected to near-UV light at 365 nm at three different cross-linking durations (3, 7, and 10 minutes). Fluorescence was studied up to 48 h after injection using Fluorotron Master™ (Ocumetrics, Inc, Mountain View, CA) and fundus camera (Genesis Df, Kowa Optimed Inc, Torrance, CA).

2.9 In vivo sustained delivery in SD rats

All animals were treated according to the Association for Research in Vision and Ophthalmology (ARVO) statement for the Use of Animals in Ophthalmic and Vision Research. Animal protocols followed during this study were approved by the Institutional

Animal Care and Use Committee of the University of Colorado Anschutz Medical Campus, Aurora, CO. Adult male Sprague Dawley rats (150–180 gm) were purchased from Harlan Sprague Dawley Inc. (Indianapolis, IN, USA). Rats were anesthetized using an intraperitoneal injection of a mixture of 80 mg/kg ketamine and 10 mg/kg xylazine.

A solution was prepared using PCM and HEMA monomers in a ratio of 90:10 (45 μ l and 5 μ l of PCM: HEMA). Alexa Fluor® 488 dye conjugated bevacizumab (50 μ l of a 1mg/ml solution) was added to the above solution. Photoinitiator was added to the dispersion and vortexed for 1 minute. Alexa Fluor® 488 dye conjugated bevacizumab along with the gel forming polymers (5 μ l) was injected in the suprachoroidal space of SD rats (n=4) using a 10 μ l Hamilton glass syringe attached to a 34G 1/2" needle. Eyes were subjected to near-UV light at 365 nm for 10 minutes. We used 10 minute exposure at an exposure intensity of 3.18 mW/cm² in our in vivo study. UV light at 365 nm exposure for 30 minutes at an exposure intensity of 3.0 ± 0.3 mW/cm² has been used in human clinical trials to treat keratoconus and bacterial keratitis⁴²⁻⁴⁴. Fluorescence was studied after injection using Fluorotron Master™ (Ocumetrics, Inc, Mountain View, CA) and fundus camera (Genesis Df, Kowa Optimed Inc, Torrance, CA).

2.10 In vitro toxicity

ARPE (human retinal pigment epithelial) cells (passage#24) were plated in a 96-well plate at a seeding density of 10,000 cells/well and allowed to adhere to the well for 24 hours. After 24 hours, cells were incubated with the media only (control), gel forming polymers (PCM, HEMA, and photoinitiator) without cross-linking, and gel forming polymers cross-linked for 3 minutes, 7 minutes, or 10 minutes. At the end of 24 hours, the media was aspirated out and 200 μ l fresh serum free medium was added to each well. MTT reagent (Sigma Aldrich, MO), i.e., 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrasodium bromide), was added (20 μ l of 5 mg/ml MTT dissolved in PBS pH 7.4) to each well and incubated at 37°C for 3 hours. The medium was aspirated out and the formazan crystals formed were dissolved in 200 μ l of dimethyl sulfoxide. The absorbance of the color developed was measured at 570 nm using a microplate reader.

2.11 In vivo toxicity

A solution was prepared using PCM and HEMA monomers in a ratio of 90:10 (45 μ l and 5 μ l of PCM:HEMA). Alexa Fluor® 488 dye conjugated bevacizumab (50 μ l of a 1mg/ml solution) was added to the above solution. Photoinitiator was added to the dispersion and vortexed for 1 minute. Alexa Fluor® 488 dye conjugated bevacizumab along with the gel forming polymers (5 μ l) was injected in the suprachoroidal space of SD rats (n=4 eyes) using a 10 μ l Hamilton glass syringe attached to a 34G 1/2" needle. Eyes were subjected to near-UV light at 365 nm for 10 minutes. At the end of 40 days of injection, rats were euthanized and eyes enucleated. Eyes were fixed in Davidson's fixative for 24 hours and 5 μ m thick sections were attained. The sections were stained using hematoxylin and eosin and examined under a light microscope (Olympus BX41 laboratory microscope) fitted with a camera (Diagnostics Instruments, Inc.).

3. Results

3.1 Preparation and characterization of polycaprolactone dimethacrylate (PCM)

Figure 1 shows the NMR spectra for polycaprolactone diol, isocyanatoethyl methacrylate, and polycaprolactone dimethacrylate (Figure 1A, 1B, and 1C, respectively). As the reaction between polycaprolactone diol and isocyanatoethyl methacrylate completed, the -OH group peaks, seen at 3.64 and 3.70 ppm, (Figure 1A) disappeared and the -NH peaks at 5.01 and 5.15 ppm appeared (Figure 1C). The methacrylate peaks from isocyanatoethyl methacrylate were also seen in the final spectrum at 5.60 and 6.12 ppm (Figure 1C).

3.2 Preparation of gel

Figure 2 shows the reaction scheme for the cross-linking of PCM with HEMA. In the presence of 365 nm UV light, DMPA photoinitiator was converted to benzoyl free radical, which further attacked the =CH₂ group in PCM and HEMA. This resulted in propagation via chain addition, which consumed the free monomer. FT-IR spectra confirmed the disappearance of ~ 78 % of the =CH₂ peak area (wavenumber of ~ 6150 cm⁻¹) at the end of 10 minutes.

3.3 In vitro sustained release of bevacizumab from cross-linked gel

Figure 3 shows the release of bevacizumab from the gel at different cross-linking durations. When reacted for either 3 or 7 minutes, the resulting gels exhibited a burst release of 82 % (± 6.8 %) and 62 % (± 3.5 %) of the drug, respectively. With the increase in the cross-linking duration to 10 minutes, the initial burst release was decreased to 21 % (± 0.4 %). With 10 minute cross-linking, bevacizumab activity/delivery in the release medium was sustained up to 4 months. Release at 0.25 hours from the gel cross-linked for 3 minutes was significantly different from the gel cross-linked for 10 minutes. Release from the gels cross-linked for 3, 7, and 10 minutes was significantly different from each other from 0.5 hours to 30 days. The release for the gel cross-linked for 10 minutes was significantly different from the other two gels from 40 to 90 days. However, there was no significant difference in release from 40 to 90 days between the gels cross-linked for 3 and 7 minutes. The release from the gels cross-linked for 3, 7, and 10 minutes was not significantly different from each other beyond 90 days.

3.4 Physical stability of bevacizumab released from cross-linked gel

Figure 4 shows size exclusion spectra of bevacizumab after release from the gel. The chromatogram of standard bevacizumab formulation showed a primary peak at retention time of ~ 8.5 minutes. A peak at ~ 7.2 minutes indicated dimers or trimers and accounted for 1.5 % of the total peak area in control sample. Peaks at ~ 8.5 and ~ 7.2 minutes were also observed in release samples at all time points (1, 2, 3, and 4 months). However, fragments of bevacizumab were observed in the release samples. A peak at ~ 10.2 minutes increased from 0.8 % in 1 month release sample to 1.8 % in the 4 month release samples.

3.5 Conformational stability of bevacizumab released from cross-linked gel

Figure 5 gives the ellipticity spectra of bevacizumab released from gel. The chromatogram was collected in the far UV-spectral range (190-250 nm). A negative band between 210 and 220 nm is a signature of β -sheet in bevacizumab. The sample released from the cross-linked gel also showed the characteristic band, indicating that bevacizumab did not lose its secondary β -sheet structure after entrapment in the cross-linked gel.

3.6 Retention of Alexa Fluor® 488 dye conjugated bevacizumab in ex vivo eyes

Alexa Fluor® 488 dye conjugated bevacizumab when entrapped in PCM and HEMA cross-linked for 10 minutes, maintained significantly higher levels at the site of injection (up to 48 h) as compared to 3 and 7 minutes cross-linking. Peak levels after 10 minutes cross-linking reduced by 36% in 60 minutes and then the levels were sustained till 48 hours. Fluorescence levels returned to the level of blank within 8 and 16 h for 3 and 7 minutes cross-linking durations, respectively. Fundus images (Figure 6A) depicted that cross-linked gel maintained its shape even at the end of 48 hours in the eyes with 10 minutes cross-linking. In comparison, fundus images of eyes with 3 and 7 minutes cross-linking did not show any fluorescence beyond 8 and 16 h, respectively.

3.7 In vivo sustained delivery in SD rats

Delivery of Alexa Fluor® 488 dye conjugated bevacizumab was sustained for 60 days after entrapment in gel network. Figure 7A shows representative fundus camera images of SD rat eyes that were injected with Alexa Fluor® 488 dye conjugated bevacizumab and gel forming polymers which were cross-linked in situ using 365 nm UV light. In comparison to eyes wherein the gel forming polymer was cross-linked, eyes without cross-linked gel lost the fundus camera signal by the end of day 5. Fluorescence of Alexa Fluor® 488 conjugated bevacizumab decreased by ~ 34 % from a concentration of 50.3 $\mu\text{g/ml}$ (± 15.7) immediately after injection to 33.4 $\mu\text{g/ml}$ (± 16.4) at the end of day 1 following entrapment in cross-linked gel. In comparison, ~ 85 % of the fluorescence was lost in the eyes wherein the gel forming polymers were not cross-linked and the fluorescence reached baseline levels by the end of day 5.

3.8 In vitro toxicity (Figure 8)

In the absence of cross-linking, mixture of gel forming polymers and photoinitiator were found to induce 27.6% cell death in ARPE cells when compared to control. Following cross-linked gel exposure, it was observed that the cell death reduced to 23.8, 20.3, and 5.3%, respectively, following incubation with gels cross-lined for 3, 7, and 10 minutes, respectively. This indicates that the toxicity is primarily caused by the uncross-linked polymers and free photoinitiator. However, as the individual polymers and photoinitiator was used up, the toxicity reduced.

3.9 In vivo toxicity (Figure 9)

According to histological examination of the anterior section of the SD rat eye at the end of 40 days of injection of gel forming polymers and Alexa Fluor® 488 conjugated bevacizumab followed by in situ cross-linking, no morphological or structural changes were observed.

This confirmed that the gel cross-linked for 10 minutes did not induce any gross toxicity in the ocular tissues.

4. Discussion

This is the first study to demonstrate sustained delivery of a therapeutic monoclonal antibody from a polycaprolactone dimethacrylate (PCM) and hydroxyethyl methacrylate (HEMA) gel. We have shown that 1) the gel sustained the *in vitro* release of bevacizumab for 4 months; 2) the gel maintained the monomeric form and activity of bevacizumab with little aggregation/degradation; and 3) bevacizumab entrapped in the light activated, *in situ* forming gel sustained *in vivo* delivery of bevacizumab following suprachoroidal injection in SD rats.

Gel sustained *in vitro* bevacizumab release and activity for four months

Sustained release of bevacizumab from PCM and HEMA gel was investigated using an ELISA method (enzyme-linked immunosorbent assay). Since the ELISA assay measures the active form of bevacizumab, any inactivated bevacizumab in the release samples was not quantified by this assay. An increase in cross-linking duration resulted in a decrease in burst release from 81 to 21%, with the 10 minute cross-linking resulting in the lowest burst release (Fig. 3). Following burst release, bevacizumab release was sustained for up to 4 months. It is generally desired that the burst release is minimized to ensure enough drug is retained for sustained release (39). However, the burst release may serve as the loading dose to obtain immediate effects, while the slow release phase serves as the maintenance dose to sustain drug effects. Bevacizumab is expected to neutralize VEGF⁴⁵ in tissues such as the choroid and retinal pigment epithelial (RPE) cells⁴⁶⁻⁴⁸ that overexpress VEGF in ocular diseases. The reported IC₅₀ of bevacizumab for inhibition of VEGF165 is 22 ng/ml (0.15nM)⁴⁹. The second phase of release (following burst) provided sustained bevacizumab levels of > 1 µg/ml in the release medium for up to 4 months. Therefore, persistent beneficial effects are anticipated with such a gel formulation. Since the bevacizumab ELISA we used is a VEGF binding assay, it only measures bevacizumab that retains its ability to bind to VEGF. Thus, our *in vitro* release indicates that the gel sustains the release and activity of bevacizumab up to 4 months.

Gel maintained the monomeric form and activity of bevacizumab with little degradation

Bevacizumab potentially can be rendered unstable during the preparation of the gel or during its residence and release. To obtain insight into the physical and conformational stability of gel-released bevacizumab, size exclusion chromatography and far UV circular dichroism were employed.

Size exclusion chromatography of released bevacizumab demonstrated the presence of high molecular weight aggregates (Figure 4) that likely correspond to dimers or trimers of bevacizumab^{50, 51}. Aggregates were observed in both control and release study samples. Furthermore, we observed the emergence of fragments during the release study. The appearance of fragments may be due to the degradation of the protein at the incubation

temperature or as a consequence of the formulation ingredients, the UV light and/or photoinitiator used in our study.

Bevacizumab had a distinct ellipticity at ~218 nm in the CD spectra (Figure 5). In our study, bevacizumab maintained its secondary structural features after entrapment and release from the gel. Furthermore, the CD spectrum of released bevacizumab closely resembled that of control bevacizumab, indicating lack of structural changes.

Gel retained bevacizumab levels in the suprachoroidal space for over 60 days in SD rats

An unmet need in the therapy of CNV is the development of slow release systems to sustain protein delivery in order to avoid the lifelong burden of monthly injections of anti-VEGF protein drugs. Since macromolecules such as bevacizumab clear rapidly from the suprachoroidal space³⁹, we developed a photo-responsive delivery system that forms a gel in suprachoroidal space for localized, sustained delivery to diseased tissue. We assessed the injectability and retention of our release system in the suprachoroidal space of excised rabbit eyes. Injectability is of importance for ocular delivery systems, as large bore needles such as 20 or 25 gauge have higher risks of endophthalmitis⁵² and hypotony⁵³ when used for ocular delivery by intravitreal injections. Furthermore, suprachoroidal injection using a large bore needle might cause hemorrhage⁵⁴. If a drug formulation can be delivered through a 30 gauge or smaller gauge needle, the system is expected to be safer than the larger gauge needle⁵⁵. During our experiments, we observed that our gel forming monomers were easily injected into the suprachoroidal space of excised rabbit eyes using a 30 gauge needle and later, these gels were cross-linked in-situ for sustained delivery. We also observed that after cross-linking for 10 minutes, Alexa Fluor® 488 dye conjugated bevacizumab was retained at the site of injection for at least 48 h in the excised rabbit eye. However, an initial rapid decrease in signal was observed, which might correspond to dilution or clearance of bevacizumab prior to the formation of the cross-linked gel or burst release of bevacizumab from the gel followed by clearance. Beyond the initial decrease, a near constant level was maintained in the suprachoroidal space. In agreement with our in vitro studies, we found that the gel system encapsulating bevacizumab was effective in sustaining the delivery of Alexa Fluor® 488 dye conjugated bevacizumab during a 60 day study in SD rats. To our knowledge this is the first report demonstrating the in vivo sustained delivery of bevacizumab for 60 days.

One limitation of our system was the use of a photoinitiator that breaks down in to a benzoyl free radical. Even though the free radical is immediately consumed in the cross linking process, it can induce some toxicity. Excessive free radicals are known to cause oxidative stress, and damage lipid, protein, and DNA in the eye⁵⁶.

5. Conclusions

A photo-responsive biodegradable gel was successfully synthesized and evaluated as a sustained release system for bevacizumab. The gel sustains the release of the entire loaded protein without affecting the stability of the protein. Further, our study demonstrates the significant potential of gels to sustain in vivo suprachoroidal delivery of macromolecules.

Thus, the novel gel formulation surpasses the current state of art for sustained release of proteins in the eye.

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References

1. Wu L, Martinez-Castellanos MA, Quiroz-Mercado H, Arevalo JF, Berrocal MH, Farah ME, Maia M, Roca JA, Rodriguez FJ. Twelve-month safety of intravitreal injections of bevacizumab (Avastin): results of the Pan-American Collaborative Retina Study Group (PACORES). *Graefes Arch Clin Exp Ophthalmol*. 2008; 246(1):81–7. [PubMed: 17674014]
2. Fung AE, Rosenfeld PJ, Reichel E. The International Intravitreal Bevacizumab Safety Survey: using the internet to assess drug safety worldwide. *Br J Ophthalmol*. 2006; 90(11):1344–9. [PubMed: 16854824]
3. Kim IS, Choi HG, Choi HS, Kim BK, Kim CK. Prolonged systemic delivery of streptokinase using liposome. *Arch Pharm Res*. 1998; 21(3):248–52. [PubMed: 9875439]
4. Sinha VR, Trehan A. Biodegradable microspheres for protein delivery. *J Control Release*. 2003; 90(3):261–80. [PubMed: 12880694]
5. Genta I, Perugini P, Pavanetto F, Maculotti K, Modena T, Casado B, Lupi A, Iadarola P, Conti B. Enzyme loaded biodegradable microspheres in vitro ex vivo evaluation. *J Control Release*. 2001; 77(3):287–95. [PubMed: 11733096]
6. Li Y, Pei Y, Zhang X, Gu Z, Zhou Z, Yuan W, Zhou J, Zhu J, Gao X. PEGylated PLGA nanoparticles as protein carriers: synthesis, preparation and biodistribution in rats. *J Control Release*. 2001; 71(2):203–11. [PubMed: 11274752]
7. Andrew JS, Anglin EJ, Wu EC, Chen MY, Cheng L, Freeman WR, Sailor MJ. Sustained Release of a Monoclonal Antibody from Electrochemically Prepared Mesoporous Silicon Oxide. *Adv Funct Mater*. 2011; 20(23):4168–4174. [PubMed: 21274422]
8. Aimetti AA, Machen AJ, Anseth KS. Poly(ethylene glycol) hydrogels formed by thiol-ene photopolymerization for enzyme-responsive protein delivery. *Biomaterials*. 2009; 30(30):6048–54. [PubMed: 19674784]
9. Koutsopoulos S, Zhang S. Two-layered injectable self-assembling peptide scaffold hydrogels for long-term sustained release of human antibodies. *J Control Release*. 2012; 160(3):451–8. [PubMed: 22465676]
10. Guzewicz N, Best A, Perez-Ramirez B, Kaplan DL. Lyophilized silk fibroin hydrogels for the sustained local delivery of therapeutic monoclonal antibodies. *Biomaterials*. 2011; 32(10):2642–50. [PubMed: 21216004]
11. Cleland JL, Jones AJ. Stable formulations of recombinant human growth hormone and interferon-gamma for microencapsulation in biodegradable microspheres. *Pharm Res*. 1996; 13(10):1464–75. [PubMed: 8899836]
12. Kim HK, Park TG. Microencapsulation of human growth hormone within biodegradable polyester microspheres: protein aggregation stability and incomplete release mechanism. *Biotechnol Bioeng*. 1999; 65(6):659–67. [PubMed: 10550772]
13. Alonso MJ, Gupta RK, Min C, Siber GR, Langer R. Biodegradable microspheres as controlled-release tetanus toxoid delivery systems. *Vaccine*. 1994; 12(4):299–306. [PubMed: 8178550]
14. Sah H. Protein behavior at the water/methylene chloride interface. *J Pharm Sci*. 1999; 88(12):1320–5. [PubMed: 10585229]
15. Sah H. Protein instability toward organic solvent/water emulsification: implications for protein microencapsulation into microspheres. *PDA J Pharm Sci Technol*. 1999; 53(1):3–10. [PubMed: 10343502]
16. Lu W, Park TG. Protein release from poly(lactic-co-glycolic acid) microspheres: protein stability problems. *PDA J Pharm Sci Technol*. 1995; 49(1):13–9. [PubMed: 7757453]

17. Suslick KS. The site of sonochemical reactions. *IEEE Trans Ultrason Ferroelectr Freq Control*. 1986; 33(2):143–7. [PubMed: 18291763]
18. Bittner B, Morlock M, Koll H, Winter G, Kissel T. Recombinant human erythropoietin (rhEPO) loaded poly(lactide-co-glycolide) microspheres: influence of the encapsulation technique and polymer purity on microsphere characteristics. *Eur J Pharm Biopharm*. 1998; 45(3):295–305. [PubMed: 9653634]
19. Zambaux MF, Bonneaux F, Gref R, Dellacherie E, Vigneron C. Preparation and characterization of protein C-loaded PLA nanoparticles. *J Control Release*. 1999; 60(2-3):179–88. [PubMed: 10425324]
20. Lentz BR, Madden S, Alford DR. Transbilayer redistribution of phosphatidylglycerol in small, unilamellar vesicles induced by specific divalent cations. *Biochemistry*. 1982; 21(26):6799–807. [PubMed: 7159562]
21. Lu S, Anseth KS. Photopolymerization of multilaminated poly(HEMA) hydrogels for controlled release. *J Control Release*. 1999; 57(3):291–300. [PubMed: 9895416]
22. Cai S, Liu Y, Zheng Shu X, Prestwich GD. Injectable glycosaminoglycan hydrogels for controlled release of human basic fibroblast growth factor. *Biomaterials*. 2005; 26(30):6054–67. [PubMed: 15958243]
23. Stansbury JW, Dickens SH. Network formation and compositional drift during photo-initiated copolymerization of dimethacrylate monomers. *Polymer*. 2001; 42(15):6363–6369.
24. Lee KY, Mooney DJ. Hydrogels for tissue engineering. *Chem Rev*. 2001; 101(7):1869–79. [PubMed: 11710233]
25. Christie JG, Kompella UB. Ophthalmic light sensitive nanocarrier systems. *Drug Discov Today*. 2008; 13(3-4):124–34. [PubMed: 18275910]
26. Aishwarya S, Mahalakshmi S, Sehgal PK. Collagen-coated polycaprolactone microparticles as a controlled drug delivery system. *J Microencapsul*. 2008; 25(5):298–306. [PubMed: 18465301]
27. Goodwin CJ, Braden M, Downes S, Marshall NJ. Release of bioactive human growth hormone from a biodegradable material: poly(epsilon-caprolactone). *J Biomed Mater Res*. 1998; 40(2):204–13. [PubMed: 9549615]
28. Wakis V, Jonnalagadda S. Novel poly-DL-lactide-polycaprolactone copolymer based flexible drug delivery system for sustained release of ciprofloxacin. *Drug Deliv*. 2011; 18(4):236–45. [PubMed: 21189060]
29. Lam CX, Savalani MM, Teoh SH, Hutmacher DW. Dynamics of in vitro polymer degradation of polycaprolactone-based scaffolds: accelerated versus simulated physiological conditions. *Biomed Mater*. 2008; 3(3):034108. [PubMed: 18689929]
30. Kweon H, Yoo MK, Park IK, Kim TH, Lee HC, Lee HS, Oh JS, Akaike T, Cho CS. A novel degradable polycaprolactone networks for tissue engineering. *Biomaterials*. 2003; 24(5):801–8. [PubMed: 12485798]
31. Alvarez-Lorenzo C, Hiratani H, Gomez-Amoza JL, Martinez-Pacheco R, Souto C, Concheiro A. Soft contact lenses capable of sustained delivery of timolol. *J Pharm Sci*. 2002; 91(10):2182–92. [PubMed: 12226845]
32. Moraes RR, Garcia JW, Wilson ND, Lewis SH, Barros MD, Yang B, Pfeifer CS, Stansbury JW. Improved dental adhesive formulations based on reactive nanogel additives. *J Dent Res*. 2012; 91(2):179–84. [PubMed: 22019910]
33. Dziubla TD, Torjman MC, Joseph JJ, Murphy-Tatum M, Lowman AM. Evaluation of porous networks of poly(2-hydroxyethyl methacrylate) as interfacial drug delivery devices. *Biomaterials*. 2001; 22(21):2893–9. [PubMed: 11561895]
34. Hsue GH, Guu JA, Cheng CC. Poly(2-hydroxyethyl methacrylate) film as a drug delivery system for pilocarpine. *Biomaterials*. 2001; 22(13):1763–9. [PubMed: 11396879]
35. Fine HF, Zhitomirsky I, Freund KB, Barile GR, Shirkey BL, Samson CM, Yannuzzi LA. Bevacizumab (avastin) and ranibizumab (lucentis) for choroidal neovascularization in multifocal choroiditis. *Retina*. 2009; 29(1):8–12. [PubMed: 18784620]
36. Bakri SJ, Snyder MR, Reid JM, Pulido JS, Singh RJ. Pharmacokinetics of intravitreal bevacizumab (Avastin). *Ophthalmology*. 2007; 114(5):855–9. [PubMed: 17467524]

37. Tyagi P, Kadam RS, Kompella UB. Comparison of suprachoroidal drug delivery with subconjunctival and intravitreal routes using noninvasive fluorophotometry. *PLoS One*. 2012; 7(10):e48188. [PubMed: 23118950]
38. Patel SR, Lin AS, Edelhauser HF, Prausnitz MR. Suprachoroidal drug delivery to the back of the eye using hollow microneedles. *Pharm Res*. 2011; 28(1):166–76. [PubMed: 20857178]
39. Olsen TW, Feng X, Wabner K, Csaky K, Pambuccian S, Cameron JD. Pharmacokinetics of pars plana intravitreal injections versus microcannula suprachoroidal injections of bevacizumab in a porcine model. *Invest Ophthalmol Vis Sci*. 2011; 52(7):4749–56. [PubMed: 21447680]
40. Hill DJT, Perera MCS, Pomery P. Copolymerization of methyl methacrylate and diethylene glycol bis(allyl carbonate). *Polymer*. 1997; 38(3):695–702.
41. Zhang L, Parsons DL, Navarre C, Kompella UB. Development and in-vitro evaluation of sustained release poloxamer 407 (P407) gel formulations of ceftiofur. *J Control Release*. 2002; 85(1-3):73–81. [PubMed: 12480313]
42. O'Brart DP, Kwong TQ, Patel P, McDonald RJ, O'Brart NA. Long-term follow-up of riboflavin/ultraviolet A (370 nm) corneal collagen cross-linking to halt the progression of keratoconus. *Br J Ophthalmol*. 2013; 97(4):433–7. [PubMed: 23385632]
43. Viswanathan D, Males J. Prospective longitudinal study of corneal collagen crosslinking in progressive keratoconus. *Clin Experiment Ophthalmol*. 2012; 40(11):1111–1117. [PubMed: 22035120]
44. Makdoui K, Mortensen J, Sorkhabi O, Malmvall BE, Crafoord S. UVA-riboflavin photochemical therapy of bacterial keratitis: a pilot study. *Graefes Arch Clin Exp Ophthalmol*. 2012; 50(1):95–102. [PubMed: 21874347]
45. Ferrara N, Hillan KJ, Novotny W. Bevacizumab (Avastin), a humanized anti-VEGF monoclonal antibody for cancer therapy. *Biochem Biophys Res Commun*. 2005; 333(2):328–35. [PubMed: 15961063]
46. Kliffen M, Sharma HS, Mooy CM, Kerkvliet S, de Jong PT. Increased expression of angiogenic growth factors in age-related maculopathy. *Br J Ophthalmol*. 1997; 81(2):154–62. [PubMed: 9059252]
47. Bhutto IA, McLeod DS, Hasegawa T, Kim SY, Merges C, Tong P, Luty GA. Pigment epithelium-derived factor (PEDF) and vascular endothelial growth factor (VEGF) in aged human choroid and eyes with age-related macular degeneration. *Exp Eye Res*. 2006; 82(1):99–110. [PubMed: 16019000]
48. Marneros AG, Fan J, Yokoyama Y, Gerber HP, Ferrara N, Crouch RK, Olsen BR. Vascular endothelial growth factor expression in the retinal pigment epithelium is essential for choriocapillaris development and visual function. *Am J Pathol*. 2005; 167(5):1451–9. [PubMed: 16251428]
49. Wang Y, Fei D, Vanderlaan M, Song A. Biological activity of bevacizumab, a humanized anti-VEGF antibody in vitro. *Angiogenesis*. 2004; 7(4):335–45. [PubMed: 15886877]
50. Liu L, Ammar DA, Ross LA, Mandava N, Kahook MY, Carpenter JF. Silicone oil microdroplets and protein aggregates in repackaged bevacizumab and ranibizumab: effects of long-term storage and product mishandling. *Invest Ophthalmol Vis Sci*. 2011; 52(2):1023–34. [PubMed: 21051703]
51. Kahook MY, Liu L, Ruzyski P, Mandava N, Carpenter JF, Petrash JM, Ammar DA. High-molecular-weight aggregates in repackaged bevacizumab. *Retina*. 2010; 30(6):887–92. [PubMed: 20458261]
52. Kunimoto DY, Kaiser RS. Incidence of endophthalmitis after 20- and 25-gauge vitrectomy. *Ophthalmology*. 2007; 114(12):2133–7. [PubMed: 17916378]
53. Acar N, Kapran Z, Unver YB, Altan T, Ozdogan S. Early postoperative hypotony after 25-gauge sutureless vitrectomy with straight incisions. *Retina*. 2008; 28(4):545–52. [PubMed: 18398355]
54. Low SW, Ng YJ, Yeo TT, Chou N. Use of Osteoplug polycaprolactone implants as novel burr-hole covers. *Singapore Med J*. 2009; 50(8):777–80. [PubMed: 19710975]
55. Marsh DA. Selection of Drug Delivery Approaches for the Back of the Eye: Opportunities and Unmet Needs in Drug Product Development for the Back of the Eye. Springer Sciences; New York: 2011.
56. Qu J, Wang D, Grosskreutz CL. Mechanisms of retinal ganglion cell injury and defense in glaucoma. *Exp Eye Res*. 2010; 91(1):48–53. [PubMed: 20394744]

Figure 1.

NMR spectra for (A) polycaprolactone diol, (B) isocyanatoethyl methacrylate, and (C) polycaprolactone dimethacrylate. Formation of polycaprolactone dimethacrylate was confirmed by the appearance of the NH peaks at 5.01 and 5.15 ppm. Methacrylate peaks were evident at 5.60 and 6.12 ppm.

Figure 2.

Reaction scheme for the preparation of cross-linked gel. Polycaprolactone dimethacrylate was cross-linked to hydroxyethyl methacrylate in the presence of 365 nm UV light and photoinitiator (2, 2 dimethoxy-2- phenyl-acetophenone).

Figure 3.

Cumulative release of bevacizumab from the gel at three different cross-linking durations. Burst release from the gel prepared by 10 minute cross-linking was 21%. Other cross-linking durations had 62% burst release. Data is expressed as mean $\pm$ S.D. for n=3.

Figure 4.

Size exclusion chromatograms of bevacizumab released from the gel prepared by 10 minute cross-linking. Marketed formulation of bevacizumab (Avastin™, Genentech Inc., CA) was diluted to 50µg/ml and used as control.

Figure 5.

Circular dichroism spectra for of bevacizumab released from the gel prepared by 10 minute cross-linking. The marketed formulation of bevacizumab (Avastin™, Genentech Inc., CA) was diluted to 50μg/ml and used as control.

Figure 6.

Injection and in situ cross-linking of gel forming polymers mixed with Alexa Fluor® 488 dye conjugated bevacizumab is feasible after injection in suprachoroidal space. (A) Fundus camera images depicting the effect of cross-linking duration on spread of Alexa Fluor® 488 dye conjugated bevacizumab in excised rabbit eyes. (B) Ten minute cross-linking duration resulted in sustained levels of Alexa Fluor® 488 dye conjugated bevacizumab for more than 48 hours, as assessed by ocular fluorophotometry. In comparison, 3 and 7 minute cross-linking duration could only confine Alexa Fluor® 488 dye conjugated bevacizumab for 8 and 16 hours, respectively. Five μ l of the gel forming polymers containing Alexa Fluor® 488 conjugated bevacizumab was injected in the suprachoroidal space of ex vivo rabbit eyes using a 50 μ l Hamilton glass syringe attached to a 30G needle. Eyes were subjected to near UV light at 365 nm at 3, 7, and 10 minute cross-linking durations. Data is represented as mean $\pm$ S.D. for n=3.

Figure 7.

Alexa Fluor® 488 dye conjugated bevacizumab is retained in the suprachoroidal space of rats up to 60 days following entrapment in a gel. (A) Fundus camera images of SD rat eyes at different time points after injection of Alexa Fluor® 488 dye conjugated bevacizumab and gel forming polymers in the suprachoroidal space. Gels were further cross-linked in situ using 365 nm UV light. (B) In comparison, Alexa Fluor® 488 dye conjugated bevacizumab levels decreased rapidly and no signal was attained beyond 5 days, wherein gel forming polymers were not cross-linked. (C) Ocular fluorophotometry (Fluorotron Master™) was used to determine the levels of Alexa Fluor® 488 dye conjugated bevacizumab following injection of Alexa Fluor® 488 dye conjugated bevacizumab and gel forming polymers in the suprachoroidal space and cross-linking. After an initial decrease, constant levels of Alexa Fluor® 488 dye conjugated bevacizumab were maintained in the suprachoroidal space where gel forming polymers were cross-linked. Data is expressed as mean $\pm$ S.D. for $n = 4$. Blank eye levels (dashed line) were measured once before the study and extrapolated to remaining time points.

Figure 8.

The gel cross-linked for 10 minutes was not toxic in vitro. In vitro toxicity was assessed by exposing human retinal pigment epithelial (ARPE) cells to the gel forming polymers either without cross-linking or after cross-linking for 3, 7, or 10 minutes.

Key: PCM -polycaprolactone dimethacrylate, HEMA - hydroxyethyl methacrylate, DMPA - 2,2 dimethoxy-2-phenylacetophenone. PCM+HEMA+DMPA is a mixture of the gel forming polymers without cross-linking (2nd bar). Time in brackets indicates the time for cross-linking in the three groups. For control, only media was used and considered as 100% viability. Data is represented as mean $\pm$ S.D. for n=4.

Figure 9.

Gel cross-linked for 10 minutes did not induce any retinal detachment, atrophy, or hypertrophy. In vivo toxicity was assessed following (A) suprachoroidal injection of the gel forming polymers in SD rats and further cross-linking in situ using 365 nm UV light. (B) Rat eyes without any injection were used as control. At the end of 40 days rats were euthanized and eyes enucleated.

Sections (5 μ m thick) were obtained and stained using H&E. Eyes from SD rats without any injection were used as control. Magnification of the eye is 4 $\times$. Magnification is at 20 $\times$ and shows the site of injection in (A). A similar area was magnified in the control eye (B).