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Nanoparticles in Porous Microparticles Prepared by Supercritical Infusion and Pressure Quench Technology for Sustained Delivery of Bevacizumab

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Abstract

Nanoparticles in porous microparticles (NPinPMP), a novel delivery system for sustained delivery of protein drugs, was developed using supercritical infusion and pressure quench technology, which does not expose proteins to organic solvents or sonication. The delivery system design is based on the ability of supercritical carbon dioxide (SC CO_2) to expand poly(lactic-co-glycolic) acid (PLGA) matrix but not polylactic acid (PLA) matrix. The technology was applied to bevacizumab, a protein drug administered once a month intravitreally to treat wet age related macular degeneration. Bevacizumab coated PLA nanoparticles were encapsulated into porosifying PLGA microparticles by exposing the mixture to SC CO_2. After SC CO_2 exposure, the size of PLGA microparticles increased by 6.9 fold. Confocal and scanning electron microscopy studies demonstrated the expansion and porosification of PLGA microparticles and infusion of PLA nanoparticles inside PLGA microparticles. In vitro release of bevacizumab from NPinPMP was sustained for 4 months. Size exclusion chromatography, fluorescence spectroscopy, circular dichroism spectroscopy, SDS-PAGE, and ELISA studies indicated that the released bevacizumab maintained its monomeric form, conformation, and activity. Further, in vivo delivery of bevacizumab from NPinPMP was evaluated using noninvasive fluorophotometry after intravitreal administration of Alexa Flour 488 conjugated bevacizumab in either solution or NPinPMP in a rat model. Unlike the vitreal signal from Alexa-bevacizumab solution, which reached baseline at 2 weeks, release of Alexa-bevacizumab from NPinPMP could be detected for 2 months. Thus, NPinPMP is a novel sustained release system for protein drugs to reduce frequency of protein injections in the therapy of back of the eye diseases.

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Keywords

Supercritical fluid; Bevacizumab; PLGA; Intravitreal; Sustained release; Noninvasive fluorophotometry

INTRODUCTION

Age-related macular degeneration (AMD), a degenerative eye disease that typically affects the geriatric population, is the leading cause of vision loss worldwide[1]. Among the two forms of AMD (dry and wet), wet AMD causes blurred central vision as a consequence of vascular hyper-permeability and abnormal blood vessel growth behind macula, the central part of the retina at the back of the eye [1, 2]. Vascular endothelial growth factor (VEGF) is a protein that plays a critical role in angiogenesis and vascular hyper-permeability associated with wet AMD.

The introduction of anti-VEGF therapy in 2004 transformed the treatment paradigm of wet AMD and currently drugs such as pegaptinib sodium (Macugen™, Eyetech Inc. New York, NY), ranibizumab (Lucentis™, Genentech, Inc. San Francisco, CA), and aflibercept (Eylea™, Regeneron Pharmaceuticals, Inc., Tarrytown, NY) are approved by the FDA [3-5]. Further, bevacizumab (Avastin, Genentech Inc. San Francisco, CA), a full-length recombinant monoclonal antibody against VEGF has been thoroughly investigated as a potential alternative to Lucentis, a Fab fragment against VEGF, for wet AMD treatment [6]. A randomized clinical study showed that intravitreal injection of bevacizumab results in a significant decrease in macular edema and improvement of visual activity [7-9]. While these advancements in AMD treatments offer significant benefits to the patients, optimal treatment is hindered by frequent monthly injection required for present therapies. Apart from the economic burden associated with frequent treatment visits to the eye clinic necessary to sustain protection against AMD progression, the high frequency of intravitreal injections has been associated with injection-related complications such as retinal detachment, endophthalmitis, hemorrhage, and cataractogenesis [10]. Hence, a key unmet need for AMD therapy is the reduction in dosing frequency. In this regard the development of sustained release drug delivery systems that maintain a therapeutically relevant concentration of protein drug for extended periods is advantageous for effective treatment of wet AMD.

Biodegradable and biocompatible polymers such as poly(lactide) (PLA) and poly(lactide-co-glycolide) (PLGA) are approved by the FDA in drug products and have been extensively investigated for the delivery of therapeutic proteins and peptides [1112, 13]. Numerous methods have been developed for the preparation of protein encapsulated microparticles using these polymers[14-16]. Even though the emulsion solvent evaporation method is commonly used for microparticle preparation, organic solvents used in this process are known to affect protein stability [15]. During microparticle preparation, organic solvents such as dichloromethane, ethyl acetate, and methanol can cause changes in protein conformation and possibly biological activity [17, 18]. These conformational changes may also enhance protein

immunogenicity [19-21]. Therefore, alternative methods of microparticle preparation that preserve the protein stability need to be developed.

Supercritical fluid (SCF) technology with its unique features is suitable for pharmaceutical processing and for the development of microparticle based formulations for both small and large molecules [22-24]. Supercritical fluids above their critical point have fluid-like densities and gas-like diffusivity, allowing efficient mixing under supercritical conditions. Supercritical carbon dioxide (SC CO_2) is widely used in preparing pharmaceutical products because it is non-toxic, economical, can be recycled, and more importantly, requires low temperature (31°C) and pressure (72 bar) for critical conditions. Further, our earlier studies indicated that SC CO_2 exposure reduces residual dichloromethane in PLGA microparticles to less than 25 ppm [25]. Another interesting feature of SCF technology is its ability to modify polymers. Exposure of SC CO_2 followed by rapid pressure drop can be used to induce expansion and porosification of PLGA microparticles. Our previous studies demonstrated the expansion and pore formation in PLGA microparticles but no morphological changes in PLA polymer matrix [25].

Considering these polymer morphological changes with supercritical CO_2, in the current study we assessed the delivery of the protein drug, bevacizumab, from preformed porous PLGA microparticles and compared its performance with porous PLGA microparticles formed in the presence of bevacizumab. In addition, we developed a novel supercritical infusion and pressure quench technology for the preparation of bevacizumab coated PLA nanoparticles in porous PLGA microparticles (NPinPMP), as a novel delivery system. The prepared microparticles were characterized for PLGA microparticle expansion and PLA nanoparticle infusion by mean particle size analysis, confocal microscopy, and scanning electron microscopy (SEM). The in vitro cumulative release of bevacizumab was evaluated and the protein content estimated by micro BCA and ELISA methods. The stability of bevacizumab in the release samples was further analyzed by size exclusion chromatography (SEC), gel electrophoresis, fluorescence spectroscopy, and circular dichroism (CD) spectroscopy. The in vivo delivery of fluorescent dye labeled bevacizumab from NPinPMP was evaluated non-invasively using ocular fluorophotometry in rats after intravitreal injection.

EXPERIMENTAL SECTION

MATERIALS

Bevacizumab (Avastin, 25 mg/ml; Genentech, CA) was purchased from a local pharmacy. In all experiments except preliminary methods 1 and 2 for particle preparation, the excipients in the commercial bevacizumab (Avastin 25 mg/ml) formulation were removed by dialysis against phosphate buffered saline at 4°C for 6 h. The process was repeated thrice. Polymers, L-PLA of inherent viscosity 1.0 dL/g and PLGA 50:50 with acid end group and inherent viscosity of 0.67 dL/g were obtained from Durect Corporation, Birmingiham, AL, USA. Poly vinyl alcohol (average molecular weight 70 kDa; cold water soluble), Nile red, and 6-coumarin were procured from Sigma Chemical Co. (St. Louis, MO, USA). Bicinchoninic acid (BCA) kit was obtained from Thermo Scientific (Pierce Biotechnology, IL, USA) and ELISA kit components were procured from R&D systems (Minneapolis, IN,

USA). Dichloromethane (DCM) and other solvents are of HPLC grade and obtained from Fisher Scientific (Pittsburgh, PA, USA). Carbon dioxide (99.95%) obtained from Airgas company (Denver, CO, USA) was used as the supercritical fluid.

METHODS

Preparation of Plain PLGA Microparticles and PLA Nanoparticles

Plain polymeric PLGA microparticles and PLA nanoparticles were prepared using an emulsion solvent evaporation method. Plain PLGA microparticles were prepared by dissolving 100 mg of polymer in 1 ml of DCM followed by dispersion of polymer solution into 10 ml of 2% aqueous poly vinyl alcohol solution under homogenization at 10,000 rpm for 1 min (Virtishear Cyclone®, USA). The prepared O/W emulsion was further transferred into 100 ml of 2% aqueous poly vinyl alcohol solution under homogenization at 15,000 rpm for 5 min using a Virtishear Cyclone® Homogenizer. The organic solvent was then evaporated from the final preparation by stirring for 3 hr at room temperature. Subsequently, the microparticles were separated by centrifugation (Beckman, USA) at 12,000 rpm for 15 min at 4°C. The microparticle pellet was suspended in 50 ml of distilled water and washed for three times. Finally, the washed micro particles were lyophilized (Labconco Triad, USA) for 24 hr to obtain dry microparticles. Plain PLA nanoparticles were prepared by dissolving 100 mg of polymer in 1 ml of DCM and 200 μl of water was dispersed into polymer solution and sonicated (Misonix Inc., USA) for 1 min at a power level of 10 W on ice. This was later dispersed in 50 ml of 2% aqueous polyvinyl alcohol and sonicated for 5 min at 30 W. Subsequently, the organic solvent was removed by stirring for 3 hr at room temperature and the hardened PLA nanoparticles were collected by centrifugation (Beckman, USA) at 12,000 rpm for 15 min at 4°C. The PLA nanoparticles were washed three times by suspending in 50 ml of distilled water followed by centrifugation (Beckman, USA) at 12,000 rpm for 15 min at 4°C. After washing, the PLA nanoparticles were lyophilized for 24 hrs.

Preparation of Bevacizumab Encapsulated Sustained Release Porous PLGA Microparticles using Supercritical Pressure Quench Technology (PMP1 And PMP2)

In the present study bevacizumab encapsulated porous PLGA microparticles were prepared by different approaches and evaluated for bevacizumab in vitro release. In the first approach, supercritical fluid pressure quench technology was used for expansion and porosification of blank PLGA microparticles and then bevacizumab was filled into porous microparticles. Briefly, 50 mg of plain PLGA microparticles were placed in a high pressure vessel and exposed to SC CO_2 at a pressure of 1150-1200 psi and a temperature of 33°C for 30 min. After completion of the SC CO_2 exposure, the pressure was released over a minute and the particles were collected. Subsequently, bevacizumab was filled into pores by incubating 100 μl of bevacizumab solution equivalent to 2.5 mg for 30 min with 50 mg of plain PLGA microparticles and lyophilized overnight (PMP1). In a second approach, bevacizumab was encapsulated into PLGA microparticles by first coating 2.5 mg of bevacizumab in 100 μl on 50 mg of plain PLGA microparticles through lyophilization followed by their exposure to supercritical CO_2 as explained above (PMP2). Figure 1 shows a scheme for the preparation of PMP1 (Fig. 1A) and PMP2 (Fig. 1B). Un-encapsulated protein was removed by suspending SC CO_2 treated particles in 1 ml of PBS for 30 min under constant shaking at

4°C and collecting the particles by centrifugation at about 16,000 g for 15 min at 4°C. Bevacizumab loading in particles was estimated by subtracting the drug quantity in supernatant from the initial amount exposed to particles. The pellet was re-dispersed in 500 μl of PBS and lyophilized for 24 hr and used for drug release studies in vitro in PBS, pH 7.4. Based on inadequate control of drug release, a third, new approach was developed.

Development of Supercritical Infusion and Pressure Quench Technology for Bevacizumab Encapsulation (NPinPMP)

We developed a novel supercritical infusion and pressure quench technology for preparing nanoparticles in porous microparticles (NPinPMP) in order to sustain bevacizumab release. In this technology, plain PLA nanoparticles were coated with bevacizumab by lyophilization and further mixed with plain PLGA microparticles and exposed to supercritical CO_2. Briefly, 100 μl of bevacizumab solution (2.5 mg) was added to 50 mg of PLA nanoparticles and incubated at 4°C for 30 min and lyophilized overnight (B-PLA NP). Later, bevacizumab coated PLA nanoparticles were mixed with plain PLGA microparticles and placed in a high pressure vessel. The particles were exposed to SC CO_2 at a pressure of 1150-1200 psi and a temperature of 33°C for 30 min. After SC CO_2 exposure, the pressure was released over a minute and the particles were collected. The efficiency of B-PLA NP infusion inside PLGA MP was evaluated by conducting SC CO_2 infusion studies at different weight ratios of B-PLA NP to PLGA MP. 100 mg of this mixture containing 5 mg, 10 mg, 15 mg, and 25 mg of bevacizumab coated PLA NP was placed in high pressure vessel and exposed to SC CO_2. Particles were washed for 30 min and drug loading was estimated as described above for PMP1 and PMP2. Particles were lyophilized overnight and used for characterization and drug release studies. Figure 2 shows the scheme for preparation of NPinPMP.

CHARACTERIZATIONS

Mean Particle Size

Plain PLGA microparticles, PLA nanoparticles, bevacizumab coated PLA nanoparticles, and bevacizumab coated PLA nanoparticles infused porous PLGA microparticles were characterized for mean particle size using dynamic light scattering (Malvern Zetasizer ZS, Malvern, UK) and micro-flow imaging techniques (DPA4100 MFI, Protein Simple, Ottawa, Canada). The particles were dispersed in distilled water and measurements were taken in triplicate.

In vitro Cumulative Release of Bevacizumab

Bevacizumab encapsulated microparticle formulations were evaluated for in vitro release in PBS pH 7.4. SC CO_2 treated particles (10 mg) were weighed and dispersed in 1 ml of PBS pH 7.4 and incubated at 37°C under shaking at 200 rpm (Max Q shaker incubator, Thermo scientific, Asheville, NC, USA). Since we used centrifugal force to separate released drug from encapsulated drug, we cannot rule of that centrifugation itself did not enhance in vitro release. At pre-determined time points, the suspended particles were centrifuged at 13,000 g for 15 min and the supernatant was collected. The pellet comprising particles was re-suspended in 1 ml of fresh PBS pH 7.4 and incubated. The bevacizumab content in the samples was estimated using micro BCA assay as per the manufacturer's instructions (Pierce

Biotechnology, IL, USA). Activity of bevacizumab released from NPinPMP was evaluated by a sandwich ELISA method.

Activity Evaluation of Bevacizumab in Release Samples by ELISA

An ELISA plate (BD life sciences, USA) was coated with 100 µl of 0.1 µg/ml of VEGF-165 in 50 mM sodium carbonate buffer, pH 9.6 and incubated overnight at 4°C. After overnight incubation, the plate was washed with wash buffer thrice, blotted, and air dried. Afterwards, 300 µl of blocking solution (0.5% BSA and 0.05% Tween 20 in PBS, pH 7.4) was added to each well and incubated in the dark for 1 hr. Then, the plate was washed with wash buffer thrice, blotted, and dried. Bevacizumab standards were prepared (0.5-50 ng/ml) in dilution buffer and incubated in dark for 2 hr. Subsequently, 100 µl of each released sample was added to the respective wells. After 2 hr incubation, the plate was washed with wash buffer thrice, blotted, and dried. The secondary goat anti-human IgG (FC) antibody was diluted (1:10000) in TBS (Tris-buffered saline) pH 7.6-7.8 with 1 % BSA and 100 µl of this solution was added to the plate and incubated in dark for 2 hr. After incubation, the plate was washed thrice with wash buffer and dried. To each well, 100 µl of TMB substrate (3, 3', 5, 5"3 tetramethylbenzidine) was added and left for color development. After 30 min incubation, 50 µl of stop solution was added and absorbance was recorded at 450 nm. A similarly processed standard curve was used to quantify bevacizumab in the released samples.

Stability Evaluation of Bevacizumab in Release Samples by Size Exclusion Chromatography (SEC)

Stability of bevacizumab after SC CO_2 treatment and in the in vitro release samples was studied using high performance size exclusion chromatography method, and compared with native bevacizumab. The silica based size exclusion column (TSK® Gel G3000SWX) having 5 µm particle diameter, with dimensions of 7.8 mm × 30 cm, and pore size of 250 Angstroms was used. 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 per minute. A UV detector scanning over the wavelength of 210-400 nm was used to detect the eluents from the size exclusion column.

Evaluation of Bevacizumab Degradation by Gel Electrophoresis

The degradation of native bevacizumab, SC CO_2 treated bevacizumab and bevacizumab from in vitro release samples was assessed by both reducing and non-reducing gel electrophoresis and compared with fresh stock of bevacizumab. Gel electrophoresis was performed using 4-20% SDS-PAGE precast gradient gel. Samples were prepared by taking 30 µl of each sample equivalent to 10 µg of bevacizumab and 15 Pl of 2X loading dye followed by boiling for 5 min and then centrifuged at 15,000 rpm for 5 min (Beckman Avanti 30, Beckman Coulter, Inc. USA). Each sample (40 µl) was loaded on precast gel and electrophoresed for 2 hr at 20 mA. Subsequently, the gel was stained with Coomassie Blue R-250, de-stained, and visualized under Gel-DOC system (Bio-Rad Laboratories, USA).

Conformational Stability Evaluation of Bevacizumab in Release Samples by Circular Dichroism (CD)

The change in the secondary structure of bevacizumab after SC CO_2 exposure and in the in vitro release samples was determined using CD spectroscopy (Photophysics, USA) and compared with freshly prepared bevacizumab. The samples at equal protein concentrations were taken in a stain free quartz cuvette with a path length of 1 mm and spectra were recorded at 25 °C. The data was collected at 1 nm step size in the 200-260 nm wavelength regions. For comparative evaluation, the CD spectra of native and SC CO_2 treated bevacizumab were also recorded.

Stability Evaluation of Bevacizumab by Fluorescence Spectroscopy

The protein folding and stability of native bevacizumab, SC CO_2 treated bevacizumab, and bevacizumab from in vitro release samples were evaluated using fluorescence spectroscopy (Spectramax Plus, Molecular Devices, USA) and compared with freshly prepared bevacizumab. Bevacizumab sample equivalent to 50 μg/ml was excited at 280 nm and emission spectrum was recorded in the 290-400 nm range. Measurements were performed in triplicate using 10 mm path length quartz cuvette.

Confocal Microscopy of NPinPMP

Confocal microscopy study was used to confirm the infusion of bevacizumab coated PLA nanoparticles inside the porous PLGA microparticles by SC CO_2 treatment. Nile red loaded PLA nanoparticles and 6-coumarin loaded PLGA microparticles were prepared using emulsion solvent evaporation method similar to the procedure described above. Nile red and 6-coumarin (100 μg/100 mg polymer) were dissolved in polymer solution of PLA and PLGA, respectively, before the particle preparation. 6-coumarin loaded PLGA microparticles and the mixture of Nile red loaded PLA NP and 6-coumarin loaded PLGA MP at a weight ratio of 1:9 were subjected to SC CO_2 similar to the procedures described above. The SC CO_2 treated particles were observed under confocal microscopy (Leica Microsystems, USA) at different magnifications (10, 20 & 100X). Further, Z-stack confocal imaging was adopted to capture the localization of Nile red in the 6-coumarin loaded PLGA microparticles. Images were captured at an interval of 0.25 μm. Nile red excitation was done at 561 nm and fluorescence images were captured using a red filter. Similarly, 6-coumarin excitation was done at 488 nm and the fluorescence image was captured using a green filter.

Scanning Electron Microscopy of NPinPMP

The infusion of bevacizumab coated PLA nanoparticles inside the porous PLGA microparticles by SC CO_2 treatment was confirmed using scanning electron microscopy. Surface morphology of gold coated NPinPMP was visualized using a scanning electron microscope (JSM-6510, Jeol USA, Inc., CA) at different magnifications ranging from 1000X to 5000X.

In Vivo Delivery of Bevacizumab from NPinPMP in 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, USA.

In vivo delivery of bevacizumab was evaluated following intravitreal administration of Alexa Fluor 488 conjugated bevacizumab in NPinPMP in a rat model. Bevacizumab was conjugated with Alexa Flour® 488 dye according to the kit suppliers (Alexa Fluor 488 protein labeling kit, Invitrogen, USA). The Alexa-conjugated bevacizumab, along with unlabeled bevacizumab, was encapsulated into NPinPMP similar to the procedures described above at a PLA NP to PLGA MP ratio of 1:9. Rats were anesthetized by intraperitoneal injection of ketamine (35 mg/kg)/xylazine (5 mg/kg) and once the rats were under anesthesia, betadine solution was applied on the eye surface, and intravitreal injections were made using a 30-G needle. The right eyes were injected with bevacizumab encapsulated NPinPMP (1.8 μg of Alexa conjugated bevacizumab and 5.4 μg unlabeled bevacizumab/5 μl; 300 mg particles/1 ml PBS pH 7.4) and left eyes were injected with bevacizumab solution (1.8 μg of Alexa conjugated bevacizumab and 5.4 μg unlabeled bevacizumab/5 μl). Ocular fluorescence due to the release of Alexa-bevacizumab was monitored periodically using ocular fluorophotometry (Fluorotron Master™, Ocumetrics, CA, USA) until the fluorescence reached the lower detection limit or baseline. Ocular fluorophotometry is a non-invasive procedure for measuring the light emitted by sodium fluorescein or similar fluorophores (e.g., Alexa 488) along the visual axis of the eye. The optical system of the instrument uses a blue light to excite the fluorophore and collects the emitted light using the same system. The instrument is calibrated to scan and report sodium fluorescein equivalent concentrations of the fluorophore at several 0.25 mm intervals. These intervals do not correspond to physical distances along the visual axis. The instrument scans and reports the fluorophore concentrations beginning at the back of the eye and ending at the front of the eye. Based on the background auto-fluorescence signal and previously reported fluorescence intensity peak locations for various eye tissues [26], peaks can be assigned to tissues within the eye including vitreous humor. Ocular fluorophotometry offers a simple tool for non-invasive, repeated fluorophore measurements in the eye of the same animal at different time points.

Baseline fluorescence values of eyes were monitored before injecting the formulations. At each time point, three fluorometric scans were taken and mean value was used. Standard curve for Alexa-bevacizumab at different concentrations was obtained using a cuvette and ocular fluorophotometry with a rat lens adapter. The standard curve was plotted in the range of 0.76 to 25 μg/ml and used to convert fluorescein equivalent concentrations provided by fluorophotometer to corresponding bevacizumab concentration in each study group. At the end of study, animals were sacrificed, eyes were enucleated and ocular tissues were separated. The bevacizumab was extracted and estimated using ELISA as described below.

Extraction and Estimation of Bevacizumab from NPinPMP Injected Rat Eyes

Rat eyes were enucleated and stored at −80° C. Ocular tissues including sclera, choroid-retinal pigment epithelium, retina, vitreous humor, lens, aqueous humor, cornea and conjunctiva were separated on a ceramic tile that was pre cooled by placing it on a container

filled with dry ice. The Isolated ocular tissues were taken into pre-weighed tubes and re-weighed. A lysis buffer was prepared by dissolving 1 M of NaCl, 20 mM of Tris-HCl, 5 mM of EDTA, and 1 % v/v of insect protease inhibitor cocktail in purified water. On the day extraction, tissues were thawed at room temperature for 15 minutes. The samples were homogenized in 250 µl buffer (150 µl lysis buffer and 100 µl of standards' diluent) using a tissue disrupter for 30 sec. To each sample, 250 µl of diluent was added, and the total volume was made up to 500 µl. Samples were bath sonicated on an ice bath for 15 seconds. The samples were centrifuged at 13,000 g for 15 minutes at 4°C, and the supernatant was collected separately into appropriately labeled tubes. To each pellet, 500 µl of diluent was added and homogenized using tissue disruptor for 30 sec. The samples were centrifuged at 13,000 g for 15 minutes at 4°C, and the supernatant was collected and pooled. The total 1 ml pooled samples were used for bevacizumab estimation using ELISA. ELISA procedures similar to those described was above were used. ELISA estimated drug levels may be an underestimate since the tissue extraction procedures may result in less than complete recovery or sonication may potentially reduce protein activity.

Statistical Analysis

All data present in this study is expressed as Mean ± SD. Statistical significance analysis was performed with one-way analysis of variance followed by Student's t-test for pair wise comparison of subgroups using SigmaPlot 11.0 (Systat Software, San Jose, California). P values < 0.05 were considered statistically significant.

RESULTS

Preparation of PMP1, PMP2, and NPinPMP and Optimization of Drug Release from NPinPMP

In vitro cumulative release of bevacizumab from PMP1 and PMP2 is shown in Figure 1. Bevacizumab release from both formulations was rapid with a high burst release. Complete release of bevacizumab from PMP1 occurred by end of day 7 with an initial burst release of 74% within 24 hr. Similarly, PMP2 showed total release of 92% by day 7, with a burst release of 68% in 24 hr.

To sustain bevacizumab release, we developed a supercritical infusion and pressure quench technology where bevacizumab-coated PLA nanoparticles were encapsulated inside the porous PLGA microparticles (Fig. 2A). When 25% w/w of bevacizumab coated PLA NP was used with 75% w/w blank PLGA MP in the SCF technique, 75% bevacizumab was released in 21 days, with an initial burst release of 52% by the end of day 1 (Fig. 2B). In contrast, the batches prepared with bevacizumab PLA NP at 5, 10, and 15% w/w showed sustained release of bevacizumab with a cumulative total release of about 71, 81, and 67%, respectively, at the end of 4 months. The burst release in 1 day was 24, 21, and 27%, respectively, for the 5, 10, and 15% w/w PLA NP formulations. Using a VEGF-165 binding sandwich ELISA assay, the activity of bevacizumab released from NPinPMP prepared using 10% w/w bevacizumab coated PLA NP was assessed. The results indicated that the VEGF-165 binding activity of bevacizumab was maintained during the 4 month study

(Figure 3).Batch prepared with 10% of bevacizumab coated PLA NP were further characterized for structural and conformational stability studies and in vivo delivery in rats.

Mean Particle Size Analysis

The mean particle size of plain PLGA MP, plain PLA NP, bevacizumab PLA NP and NPinPMP was determined using dynamic light scattering and microflow imaging techniques and the results are shown in Table 1. The mean particle size of plain PLGA MP and plain PLA NP were 1.6 μm, and 251 nm, respectively. After bevacizumab coating of PLA NP, the mean particle size was slightly increased to 265 nm. After SC CO_2 exposure of bevacizumab PLA NP and PLGA MP mixture, the resultant NP infused porous PLGA microparticle (NPinPMP) showed mean particle size of 11.6 μm. The higher in mean particle size of NPinPMP indicates the expansion of PLGA MP after SC CO_2 exposure.

Aggregation and Degradation of Bevacizumab Released from NPinPMP

There were no visible aggregates present in vitro released bevacizumab samples from NPinPMP until the end of 4 months. Analyses of SEC chromatograms of released bevacizumab showed virtually no change in the elution pattern, which was observed as a single, prominent peak similar to native bevacizumab at 8.1 min suggesting that physical and chemical stability of bevacizumab was maintained at 37°C during the 4 month release study (Figure 4A). We observed the formation of very low percentage (<1%) of high molecular weight soluble aggregates at the beginning of 2 months, which did not increase in abundance through the duration of the 4-month release study. This was also evident from non-reducing SDS-PAGE of released bevacizumab, where less than 1 % of multimeric forms of bevacizumab were observed for samples collected at 2 months and beyond. This fraction of multimeric forms did not increase through the end of 4 months. However, when examined by SDS-PAGE under reducing conditions, these multimeric forms were completely absent (Figure 4B). This indicates that the multimeric forms of released bevacizumab formed at 2 months and beyond likely consisted of inter-molecular disulfide linkage. No low molecular weight degradation products were observed in SEC chromatograms at all time points. This was further confirmed by reducing and non-reducing SDS-PAGE of released bevacizumab, which showed absence of degraded products (Figure 4C). Size exclusion chromatography and SDS-PAGE data indicated absence of significant aggregates and degraded fragments.

Conformational Stability of Bevacizumab by Circular Dichroism and Fluorescence Spectroscopy

The CD spectrum of native bevacizumab showed a peak at 218 nm, indicating the presence of higher percentage of beta sheet like structure, as expected for immunoglobulins, which are known to be rich in beta-sheet conformation (Figure 5A). Released bevacizumab from NPinPMP at 1, 2, 3 and 4 month time points gave CD spectra similar to native bevacizumab, indicating that the secondary structure was maintained for bevacizumab released from NPinPMP. Fluorescence emission spectra of released and native bevacizumab showed similar patterns with emission maxima at 338 nm indicated tertiary structural stability of bevacizumab (Figure 5B).

Confocal and Scanning Electron Microscopy Imaging of NPinPMP

Confocal images of expanded porous PLGA MP and NPinPMP formulations are shown in Figure 6, and Figure 7. Figure 6 indicated the expansion of PLGA microparticle with pore formation after SC CO_2 exposure. After SC CO_2 treatment, the red signal of Nile red was observed to be colocalized with the green signal of 6-Coumarin (Figure 6), indicating the infusion of PLA NP inside the expanded PLGA MP. The Z section images of NPinPMP formulation showed localization of Nile red loaded NP at various depths inside the expanded 6-Coumarin loaded PLGA microparticles.

Surface morphology of NPinPMP formulation was evaluated by SEM and images are shown in Figure 6. It was observed that SC CO_2 leads to the expansion of PLGA MP with pore formation. Also, from the SEM images it was evident that PLA NPs were encapsulated inside the expanded porous PLGA MP. While some pores in the MP were fused, others were open.

In Vivo Delivery of Bevacizumab from NPinPMP in Rat Model

After intravitreal injection of NPinPMP and solution containing both Alexa-bevacizumab and unlabeled bevacizumab at 1:3 ratio, the distribution of labeled bevacizumab along eye optical axis (at several data points in the anterior to posterior direction) was determined by measuring the Alexa fluorescence intensity distribution (equivalent of sodium fluorescein concentration). Fluorescein equivalent concentrations reported by Fluorotron Master were converted to Alexa-bevacizumab concentrations using a standard curve and finally corrected for the total bevacizumab dose administered (Figure 8C). Total bevacizumab concentration was determined by multiplying with factor of 4. The fluorescence scans revealed more prolonged delivery of Alexa-bevacizumab from NPinPMP compared to solution. The Alexa-bevacizumab solution injected group showed bevacizumab concentration of 72.84 μg/ml on day 1 and reduced to 9.36 μg/ml by day 15 indicating rapid elimination from vitreous region. In NPinPMP injected group the bevacizumab concentration in the vitreous on day 1 was found to be 21.1 μg/ml and the bevacizumab concentration was 13.96 μg/ml on day 45. However by end of day 60, the bevacizumab concentration reached baseline reading. It indicated the sustained delivery of bevacizumab from NPinPMP.

In addition to fluorophotometry data, sustained delivery of bevacizumab from NPinPMP was evident based on the presence of bevacizumab in ocular tissues at 2 months post-dosing, measured using an ELISA assay. ELISA based bevacizumab content in different ocular tissues is shown in the Figure 8. The data indicated that bevacizumab was present in sclera, choroid-retinal pigment epithelium (CRPE), vitreous humor, and lens with the highest quantity present in the vitreous humor, followed by the retina and choroid-retinal pigment epithelium. In aqueous humor, cornea, and conjunctiva, bevacizumab was below detection limits. In the bevacizumab solution injected group, bevacizumab was below detection limits in all the above ocular tissues. Since bevacizumab was quantified using an ELISA based on VEGF binding, it can be inferred that bevacizumab released from NPinPMP retained its VEGF binding activity in vivo during the two month study.

DISCUSSION

Commonly used emulsion-solvent evaporation methods for protein encapsulation in PLGA microparticles have great potential to affect protein stability and consequently drug release profile [27, 28]. In the current study, we developed a novel method and delivery system for sustained protein delivery. We used supercritical fluid infusion and pressure quench technology for the encapsulation of bevacizumab-coated PLA nanoparticles inside the porous PLGA microparticles. The mild processing conditions, which were free of organic solvents, minimized bevacizumab degradation and resulted in nearly complete and sustained release of encapsulated bevacizumab in fairly stable form for 4 months. Further, in vivo noninvasive ocular fluorophotometry indicated the superior ability of NPinPMP to sustain bevacizumab delivery when compared to plain solution.

Expansion of PLGA Microparticles and Infusion of PLA Nanoparticles by Supercritical CO_2

PLGA copolymers are available with different composition, molecular weight, and crystallinity. These differences in the physiochemical properties of the polymers affect the polymer behavior upon supercritical fluid exposure. In earlier studies we reported that amorphous PLGA polymers were more susceptible to plasticization by supercritical CO_2 compared to crystalline PLA polymers [25]. With their less ordered structures, amorphous polymers enable diffusion and interaction of CO_2 with polymers, altering polymeric inter-chain network and increasing chain mobility. Supercritical CO_2 diffuses into amorphous PLGA microparticles and plasticizes them, thereby reducing their glass transition temperature. Upon depressurization or pressure quenching, CO_2 rapidly expands, allowing microparticle expansion, pore generation, and finally vitrification of the microparticle. PLGA microparticles expanded 10-fold with pore formation [25]. On the other hand, crystalline PLA with its organized structure does not undergo expansion in supercritical CO_2. Considering these findings, in the present study we assessed 3 approaches to develop bevacizumab sustained release microparticles. With the first approach, utilizing preformed porous PLGA microparticles filled with bevacizumab, we observed high burst release and nearly complete release of protein by end of day 7. In the second approach, where bevacizumab coated PLGA microparticles were porosified, we observed similar burst release with 92% protein release occurring by end of 7 days. The reason for rapid release of bevacizumab with the first two approaches could be the presence of large number of open pores with no significant obstructions for the access of aqueous medium to the drug. This can result in rapid dissolution and solvation of hydrophilic molecules, like antibodies. In the third approach, we assessed a combination of non-expanding nanoparticles and expanding microparticles in supercritical CO_2 atmosphere, in order to load bevacizumab. With supercritical CO_2, while amorphous PLGA rapidly expands due to fast CO_2 diffusion and plasticization, crystalline PLA does not[25]. Based on this principle, we hypothesized that bevacizumab coated PLA nanoparticles remain unexpanded in CO_2 allowing their infusion into porosifying PLGA microparticles being plasticized, expanded, and porosified under supercritical CO_2 atmosphere. Since PLGA particels are larger to begin with when compared to PLA nanoparticles in this study, the entry of PLGA MP into PLA NP is unlikely. The unexpanded PLA nanoparticles might work as plugs to block some of the pores in the particles so that the release of bevacizumab can be retarded. Based on our

confocal microscopy studies with red labeled PLA NP and green labeled PLGA MP, it is evident that PLA NP were incorporated in PLGA MP. Further, our SEM pictures indicated some fused pores and some open pores in NPinPMP. Fused pores in PLGA MP might represent the entry points for NP under high pressure, while the open pores might represent the exit points for CO_2 during pressure quenching.

NPinPMP Sustain Bevacizumab Release

The increase in mean particle size of NPinPMP observed in this study is consistent with the expansion of PLGA microparticles following supercritical CO_2 exposure. Upon supercritical CO_2 treatment the mean particle size was increased by 7 fold (1.6 μm Vs 11.6 μm). In the development of NPinPMP formulations, the weight ratio of bevacizumab loaded PLA nanoparticles and PLGA microparticles was optimized for efficient encapsulation of bevacizumab as well as for sustaining release with minimum burst release. When 25% w/w of bevacizumab coated PLA NPs were used, higher burst release was observed. This might be due to incomplete infusion of PLA NP inside the expanded PLGA MPs and bevacizumab was released immediately from un-encapsulated PLA NPs. With other batches prepared with 5, 10, and 15% w/w of PLA NP, slow release was observed, suggesting efficient PLA NP encapsulation inside porous PLGA MP. We observed in vitro release of bevacizumab from NPinPMP for 4 months and no further release was observed from the system. PLA and PLGA polymers used in this study may take 12-14 months and 3-4 months, respectively, for complete degradation [29]. Since bevacizumab was coated on the surface of PLA nanoparticles, total duration of release is expected to be about 4 months.

Confirmation of PLGA Microparticles Expansion and Infusion of PLA Nanoparticles

SEM images have provided clear evidence for PLGA microparticle expansion and porosification and also they have shown infusion of PLA NP inside the pores. Further evidence for PLA nanoparticle encapsulation inside the expanded PLGA microparticles was provided by laser confocal microscopy images of Nile red loaded PLA nanoparticles inside 6-coumarin loaded PLGA microparticles. The laser confocal microscopy was a good tool in discerning the distribution and penetration of two or more different fluorescent signals at a single time. The confocal images revealed the presence of strong green fluorescence in the expanded and porous PLGA microparticles. After SC CO_2 treatment with Nile red loaded PLA nanoparticles, the red fluorescence of Nile red was distributed within the green fluorescence, indicating the infusion of nanoparticles inside the expanded microparticles. The Z stacking images have indicated the increasingly brighter red fluorescence along the Z axis, towards the core of the particles. This data confirmed the infusion of Nile red loaded nanoparticles inside PLGA microparticles.

SEC, CD, Fluorescence, and SDS-PAGE Confirmed the Stability of Bevacizumab

A major hurdle in developing the protein release formulations is maintaining the protein stability for long duration during storage and in release conditions. The conventional method used for protein encapsulation inside PLGA polymeric particles involves emulsification of organic polymeric phase and aqueous protein phase, where protein is exposed to organic solvent. Organic solvents and further harsh processing conditions may have adverse effects on protein stability and activity. In this study we tried to minimize the effects of these harsh

conditions and loss by adopting a different approach where protein was adsorbed on prepared NPs followed by SC CO_2 induced internalization of these NPs into porous microparticles. Size exclusion chromatography and CD spectroscopy showed that bevacizumab retained significant percentage of monomeric state (>99 %) and retained the secondary structure. We consider it is likely that oxidation of monomeric proteins led to the generation of a small fraction of soluble high molecular weight species peaks observed in SEC chromatogram from 2 months onwards in released samples. The oxidation of surface exposed cysteine residues on protein surface can lead to the formation of inter-molecular disulfide linkages. This was evident from differences in reducing and non-reducing SDS-PAGE profile of bevacizumab bands, where we observed the presence of multimers in non-reducing condition, which were absent in PAGE when conducted under conditions that would reduce disulfide crosslinks.

Noninvasive Monitoring by Ocular Fluorophotometry and ELISA Indicated Sustained Delivery of Bevacizumab

The initial fluorescence signal from the solution-injected eyes was very high compared to the NPinPMP injected eyes and it was eliminated rapidly. The fluorescence levels reached basal fluorescence within two weeks. However, the fluorescence intensities from the NPinPMP formulations were low to begin with but sustained for a prolonged period. The persistence of fluorescence intensities over 2 months indicates continuous release of bevacizumab. From NPinPMP the release was found to be slightly higher at one month compared to 15 days, possibly due to the initiation of polymer degradation. At two months, the fluorescence was close to baseline, suggesting lower release rates. Lack of detectable fluorescence signal beyond 2 months is consistent with a lack of sufficient sensitivity and/or elimination of the drug. It was however clear that the signal for NPinPMP could be detected for at least 4-times longer duration when compared to plain drug solution. In addition to fluorophotometry data, sustained delivery of bevacizumab from NPinPMP was evident based on the presence of bevacizumab in ocular tissues at 2 months post-dosing, measured using an ELISA assay. Our in vitro study indicates release up to 120 days. In vivo Fluorotron study could detect fluorescence signal reliably up to 60 days. At 60 days, ELISA based analysis indicated high drug levels in vitreous humor (Figure 8E). Thus, it is likely that the NPinPMP will sustain drug levels much further in vivo, which will be assessed in future studies.

CONCLUSIONS

With the growing interest in sustained release protein therapeutics for efficient treatment of chronic ocular diseases, a polymeric microparticle preparation technique that maintains protein stability is essential. Earlier studies have indicated the limitations of PLGA microparticle technologies in preparing sustained protein release formulations. However, in the present study we successfully developed a novel NPinPMP delivery system based on supercritical fluid infusion and pressure quench technology. The prepared porous particles sustained the release of monomeric bevacizumab for 4 months. Conformational and functional stability of bevacizumab after in vitro release was established by ELISA, SEC, CD and SDS-PAGE data. Infusion of PLA NP into expanded porous PLGA MP was proven

by confocal microscope and SEM. Sustained in vivo delivery of bevacizumab when compared to plain drug was demonstrated using noninvasive fluorophotometry following intravitreal injection in rats. Bevacizumab released from NPinPMP in vivo retained its VEGF binding activity. In conclusion, NPinPMP is useful in releasing stable bevacizumab for a few months.

Acknowledgments

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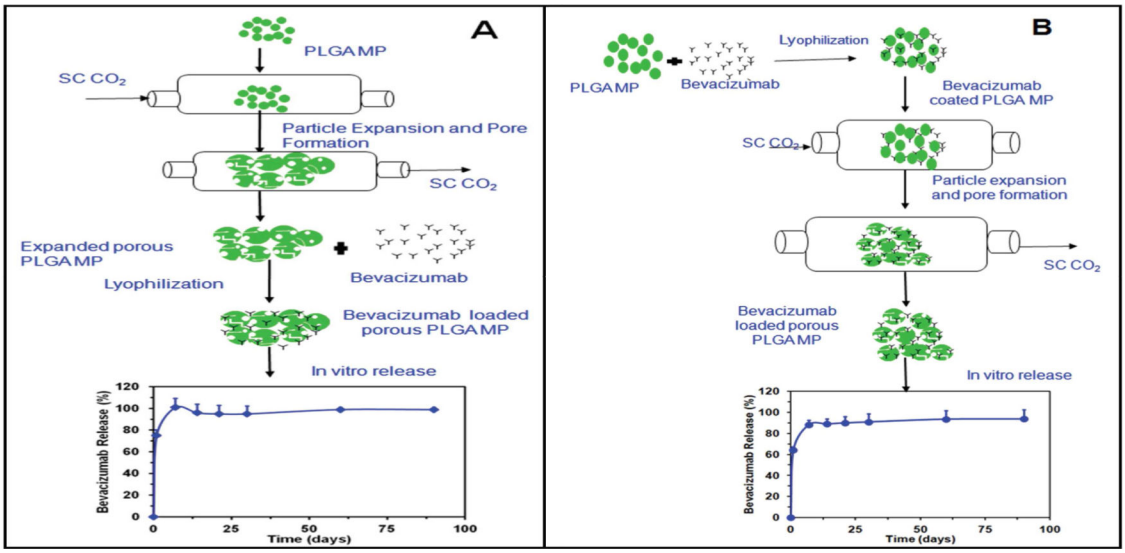

Figure 1.

Schematic representation for the preparation of bevacizumab encapsulated porous PLGA microparticles prepared by supercritical pressure quench technology. (A) Plain PLGA microparticles were made porous by supercritical pressure quench technology and bevacizumab was loaded (PMP1). (B) Bevacizumab was coated on plain PLGA microparticles and exposed to supercritical CO_2 (PMP2). The release was performed in PBS pH 7.4 and bevacizumab content was estimated using a micro-BCA kit. Results are expressed as mean ± SD for n=3.

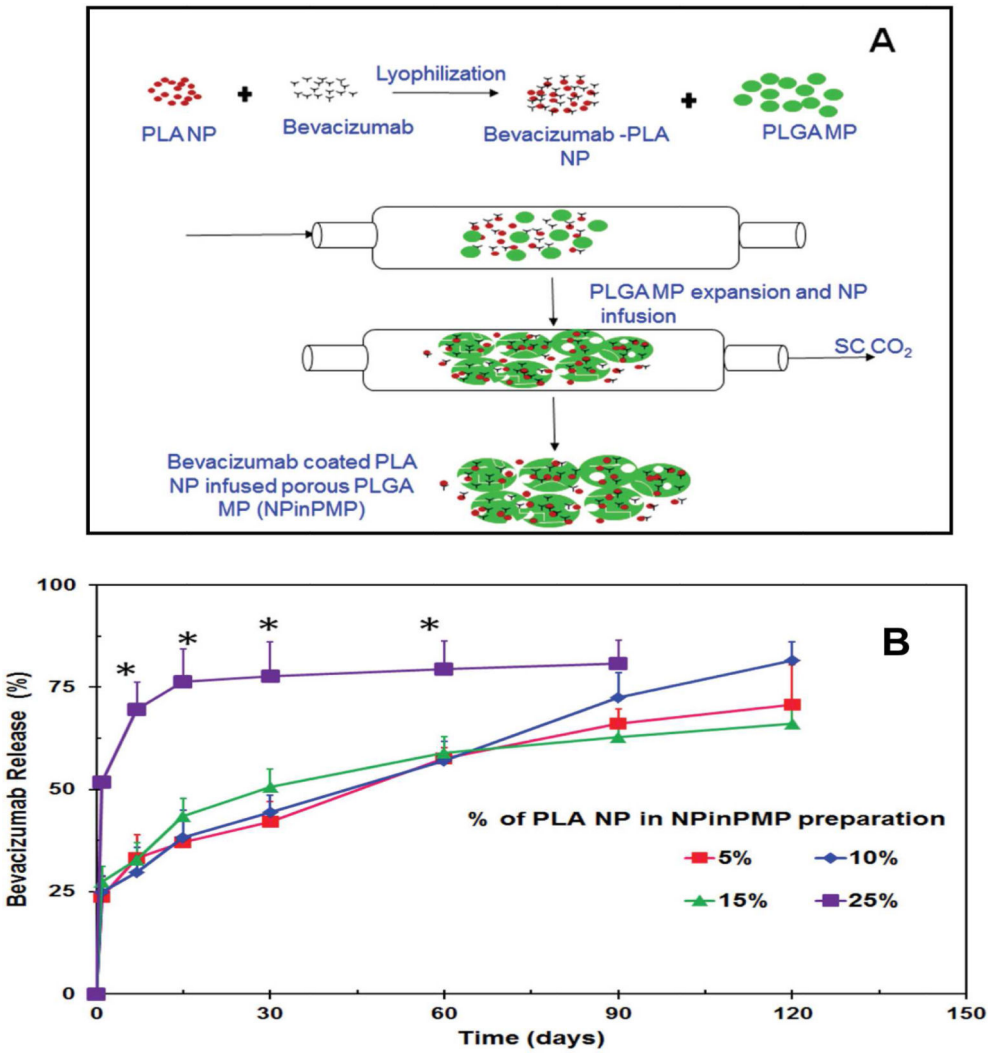

Figure 2.

(A) Schematic representation for the preparation of bevacizumab coated PLA NP infused porous PLGA MP (NPinPMP) using supercritical fluid technology. Bevacizumab coated PLA nanoparticles (NP) were lyophilized and mixed with plain PLGA microparticles (MP) and treated with SC CO_2 at 1200 psi/33 °C for 30 min. (B) In vitro cumulative release of bevacizumab from NPinPMP formulations. The release was performed in PBS pH 7.4 and bevacizumab content was estimated using a micro-BCA kit. Results are expressed as mean ± SD for n=3.

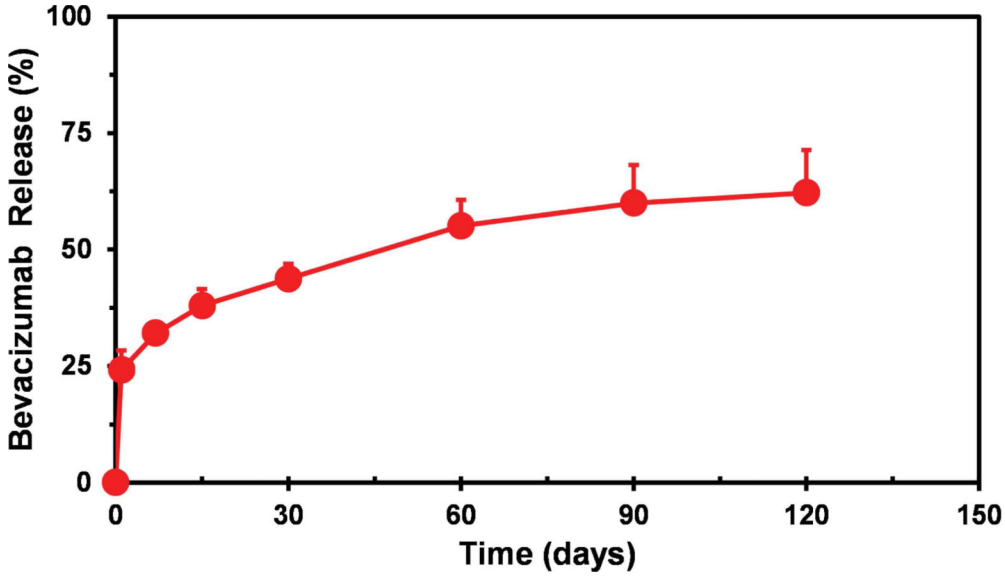

Figure 3.

In vitro cumulative release of bevacizumab from NPinPMP based on 10% w/w bevacizumab coated PLA nanoparticles in PBS, pH 7.4. Bevacizumab content was estimated by ELISA. Results are expressed as mean ± SD for n=3.

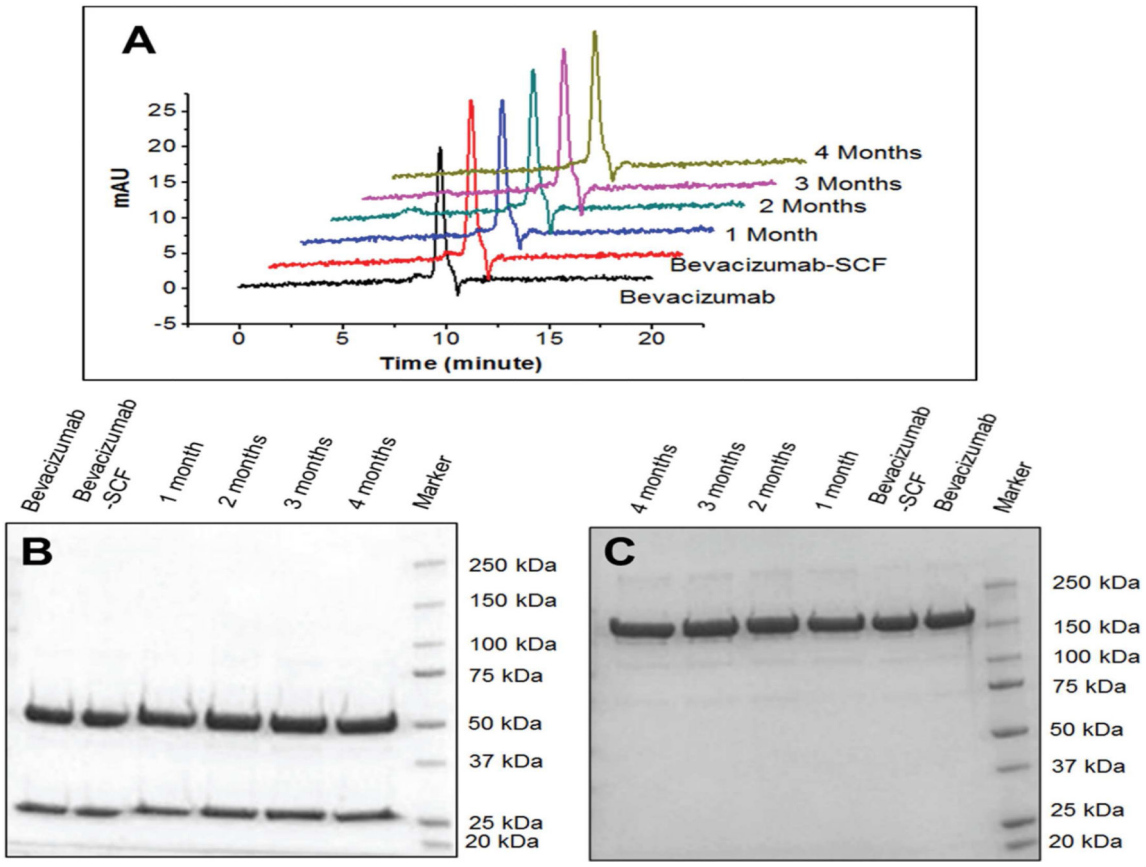

Figure 4.

Structural stability evaluation of bevacizumab by size exclusion chromatography and SDS-PAGE. (A) SEC chromatograms of native, SC CO_2 treated bevacizumab and in vitro release samples of bevacizumab from NPinPMP formulation. (B) Reducing, and (C) Non-reducing gel SDS-PAGE pictures of native, supercritical CO_2 treated, and in vitro release samples of bevacizumab after 1, 2, 3, and 4 months.

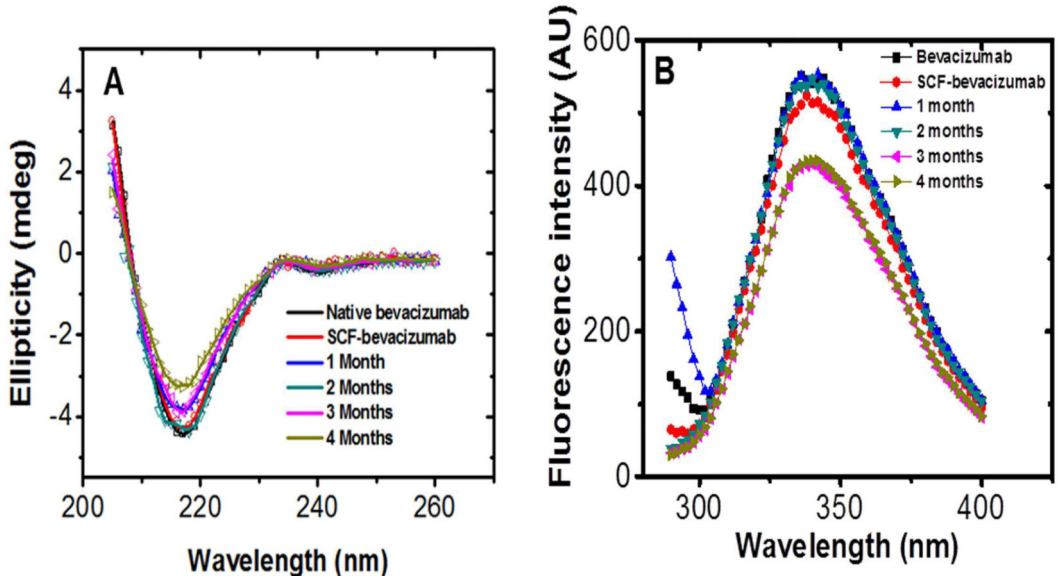

Figure 5.

Conformational stability evaluation of native bevacizumab, supercritical CO_2 treated bevacizumab, and 13, 23, 33, and 4-month in vitro release samples of bevacizumab by (A) circular dichorism (CD), and (B) fluorescence spectroscopy.

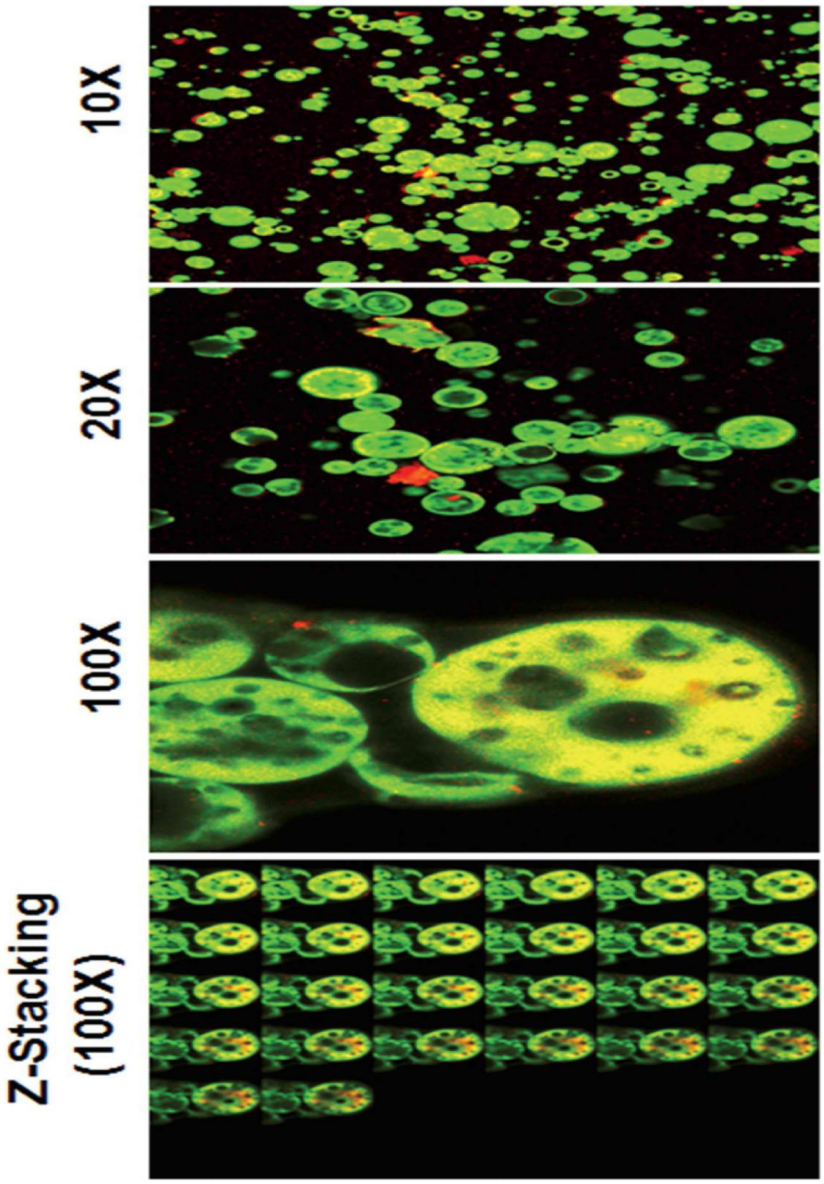

Figure 6.

Confocal microscopy images of Nile red loaded PLA nanoparticles infused 6-Coumarin loaded PLGA microparticles (NPinPMP) prepared by supercritical fluid technology. The Nile red loaded PLA nanoparticles were mixed 6-coumarin loaded PLGA microparticles at 1:9 ratio and treated with supercritical CO_2 at 1150-1200 psi/33°C for 30 min and images were recorded at different magnifications.

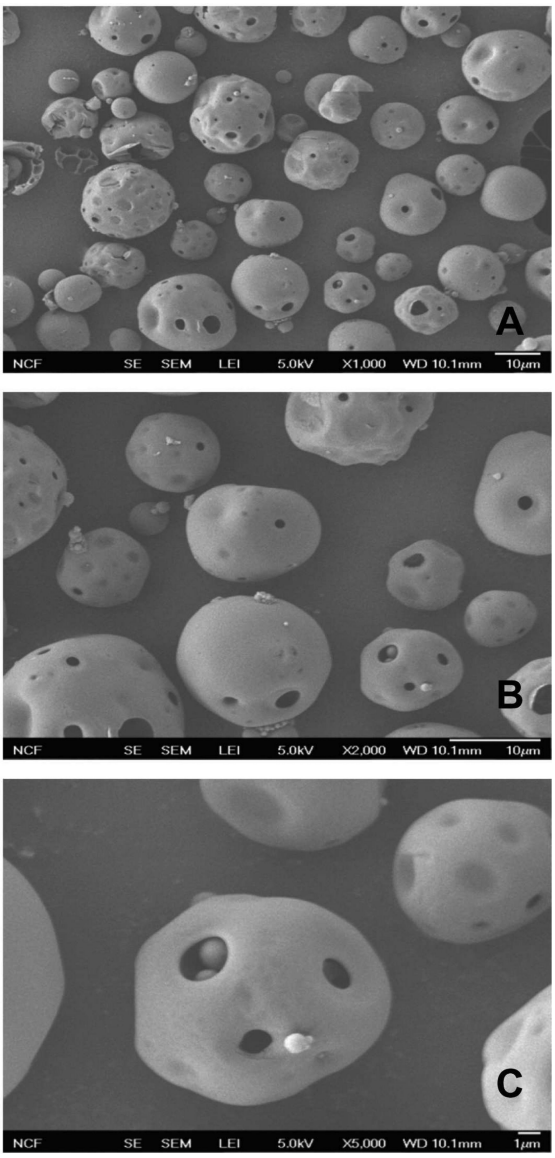

Figure 7.

Scanning electron microscopy images of PLA nanoparticles infused PLGA microparticles (NPinPMP) prepared by supercritical fluid technology. PLA nanoparticles and PLGA microparticles were used at a ratio of 1:9. Pictures taken at different magnifications: (A) 1000X, (B) 2000X, and (C) 5000X.

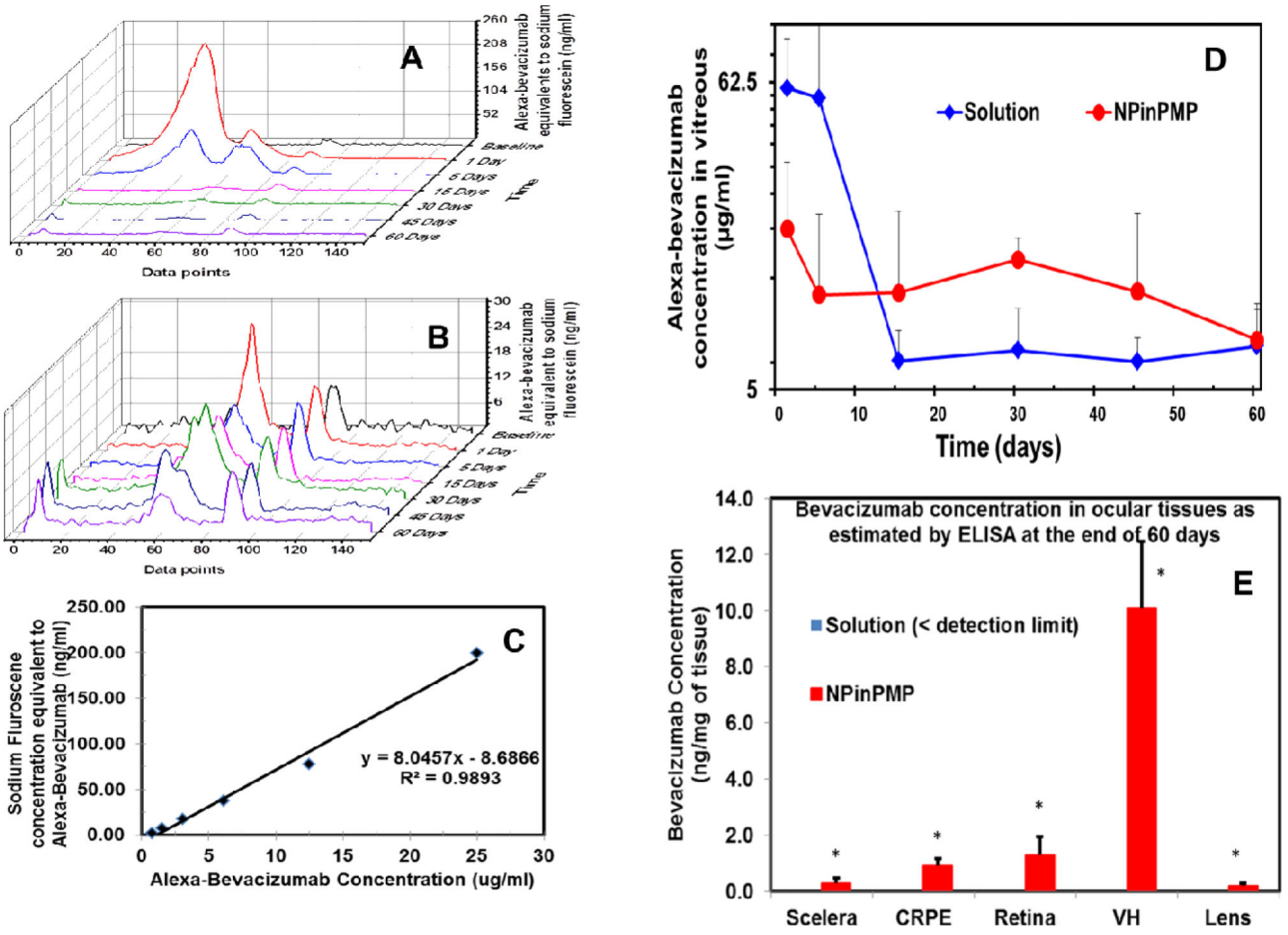

Figure 8.

Noninvasive ocular flurophotometry of rat eyes after intravitreal injection of (A) Alexa-bevacizumab solution, and (B) Alexa-bevacizumab loaded NPinPMP. (C) Standard curve for Alexa-bevacizumab generated using ocular fluorophotometry. The lower limit of quantification (LLOQ) and limit of detection LOD were observed to be 0.76 µg/ml and 0.35 µg/ml, respectively. (D) The fluorescence levels returned to basal level by 2 weeks in Alexa-bevacizumab injected rats, whereas the fluorescence levels in NPinPMP group were higher beginning about day 12 up to about 2 months. The data represented as mean±SD for n=4 E) Bevacizumab concentrations in rat ocular tissues after intravitreal injection of Bevacizumab NPinPMP. Bevacizumab NPinPMP and bevacizumab solution injected animals were euthanized at 2 months post-dosing and eyes were enucleated and flash frozen. Eye tissues including sclera, choroid-retinal pigment epithelium (CRPE), retina, vitreous humor (VH), lens, aqueous humor, cornea. and conjunctiva were separated and bevacizumab was extracted and measured using ELISA and normalized to tissue weight. In the bevacizumab NPinPMP group, for which data is shown above, bevacizumab was not detectable in aqueous humor, cornea, and conjunctiva. In bevacizumab solution group, bevacizumab was not detected in any of the tissues assessed. Data is presented as mean ± S.D. for n=4.

Table 1

Mean particle size and distribution of plain PLA nanoparticles, plain PLGA microparticles, and bevacizumab coated PLA nanoparticles infused into porous PLGA microparticle measured by dynamic light scattering (DLS) and micro flow imaging (MFI) techniques.

S.No	Particles	Mean Particle Size
1	Plain PLGA microparticles (PLGA MP)	1670±103 nm
2	Plain PLA nanoparticles (PLA NP)	251±15 nm
3	Bevacizumab coated PLA nanoparticles (B-PLA NP)	265±9 nm
4	B-PLA NP infused PLGA MP (NPinPMP)	11,610±1501 nm