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Albuminated PLGA nanoparticles containing bevacizumab intended for ocular neovascularization treatment

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Abstract: Bevacizumab, an anti-VEGF antibody, has demonstrated trustworthy effects in treatment of retinal and choroidal neovascularization that both are crucial sight threatening conditions. However, the weak point is the short half-life of the drug in vitreous which necessitates frequent intravitreal injections. Accordingly employing controlled-release drug delivery systems such as polymeric nanoparticles (NPs) has been suggested. In this study albuminated-PLGA-NPs containing bevacizumab were prepared and studied intended for reducing the number of injections. NPs were formulated by double-emulsion method and a single dose of NPs was intravitreally injected to rabbits. The drug concentrations in vitreous and aqueous humor were assayed in different time intervals using ELISA and intraocular pharmacokinetic parameters were calculated. Moreover, coumarin-6 loaded albuminated-PLGA-NPs were employed to evaluate the distribution and persistence of the NPs in the posterior segment. Results revealed that the bevacizumab vitreous concentration maintained above 500 ng mL^{-1} for about 8 weeks and 3.3 times elevation was observed in the drug vitreous MRT compared with the control. According to coumarin-6 NP tests, fluorescence emissions in posterior tissues were observed for 56 days which confirmed the nanoparticles persistence in ocular tissues during the test span. Therefore our prepared formulation may offer improvements in treatment of eye posterior segment neovascularization. © 2015 Wiley Periodicals, Inc. J Biomed Mater Res Part A: 103A: 3148–3156, 2015.

Key Words: albuminated PLGA nanoparticles, bevacizumab, controlled release, intraocular pharmacokinetic, choroidal neovascularization

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INTRODUCTION

In recent years vascular endothelial growth factor (VEGF) inhibitors have shown beneficial effects on ocular neovascularizations and effectively decreased neovascular activity as well as vascular permeability in various ocular tissues.[1,2] Retinal and choroidal neovascularization (CNV) are regarded as the leading causes of vision loss in different posterior segment ocular diseases including proliferative diabetic retinopathy, retinal vein occlusions, retinopathy of prematurity and age-related macular degeneration (AMD). Generally abnormal neovascularization is the result of imbalance between angiogenesis stimulators and inhibitors. Despite the progression in understanding the angiogenesis phenomena, the exact mechanisms have remained unclear in some parts. However, the role of VEGF family as a prominent stimulator of neovascularization has been clearly demonstrated.[3,4]

Bevacizumab (Avastin) is a humanized whole antibody against VEGF. Although FDA has approved the drug for the treatment of metastatic colorectal cancer it has been widely used by ophthalmologists for treatment of retinal and choroidal neovascularization during recent years.[5,6] However, one of the major restrictions of intravitreal anti-VEGF treatment is the repetitive injection requisites due to short half-life of these drugs in vitreous. In addition to the difficulties and costs, there are serious concerns about the adverse effects of these injections including intravitreal hemorrhage, endophthalmitis, retinal detachment, and cataract.[7,8] To address this problem administration of controlled drug delivery systems have been suggested. Polymeric nanoparticulate carriers have shown great promise in this area.[9,10] Poly(lactic-*co*-glycolic) acid (PLGA) is one of the most widely used biodegradable and biocompatible polymers in the field.[11,12] Having a wide

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range of degradation rate makes it an attractive polymer in the field of controlled drug delivery systems. PLGA nanoparticles (NPs) have been widely investigated in protein delivery studies.[13] However, the often deleterious preparation conditions necessary to entrap proteins in PLGA NPs, especially in double emulsion solvent evaporation method, lead to substantial protein aggregation, denaturation and subsequently inactivation. To overcome such problems employing of stabilizers have been recommended.[14] Among the various investigated stabilizers, albumin has shown immense potentials in this area.[15,16] As indicated in our recent study, the proper concentration of albumin could effectively preserve bevacizumab activity during the NP preparation.[17]

Therefore, in this study albuminated PLGA NPs containing bevacizumab were prepared, their release profiles following the intravitreal injections in rabbits were investigated and the related pharmacokinetics were calculated with the aim of achieving an appropriate controlled delivery system and thus decreasing the number of bevacizumab intravitreal injections.

MATERIALS AND METHODS

Materials

Bevacizumab (Avastin) was from Genentech/Roche (San Francisco, CA). Recombinant human $VEGF_{165}$ was purchased from R&D Systems (Minneapolis, MN). PLGA (RG502H, glycolide:lactide ratio of 50:50) was from Boehringer Ingelheim (Ingelheim, Germany). Human serum albumin (HSA) and Coumarin-6 were purchased from Sigma Aldrich (St. Louis, MO). Biotin conjugated rabbit anti-human IgG, Fc fragment specific, was bought from Thermo scientific (Waltham, MA) and horseradish peroxidase conjugated streptavidin (HRP-streptavidin) was from Invitrogen (Grand Island, NY). 3,3′,5,5′-Tetramethylbenzidine (TMB) was from PishtazTeb-Zaman (Tehran, Iran). Poly vinyl alcohol (PVA 22 kDa) eye drop (Sinatears) was from Sina Darou (Tehran, Iran). Ketamin and xylazine were from Alfasan (Woerden, Holland). All other solvents and materials were of analytical grade.

Assessment of bevacizumab concentration

Bevacizumab concentration assessment was conducted by enzyme-linked immunosorbent assay (ELISA) as described previously.[17] In brief human recombinant $VEGF_{165}$ was immobilized on Maxisorp plates (Nunc, Roskilde, Denmark), at a concentration of 0.125 μg mL^{-1} in 0.05 mol L^{-1} carbonate buffer, pH 9.6, overnight at 4°C (100 μL per well). Wells were then washed three times with phosphate-buffered saline (PBS) containing 0.05% Tween-20 (PBS/T) and blocked with 2% BSA solution in PBS/T for 4 h at 4°C (300 μL per well). Following three times washing, the wells were finally dried at room temperature and stored at 4°C for later use.

During the drug concentration assessment, samples with suitable dilutions in 0.1% BSA-PBS/T solution were incorporated into the wells (100 μL per well) and incubated at 37 °C for 1.5 h. A standard curve was prepared with bevacizumab ranging from 2.5 to 80 ng mL^{-1}. The detection of bound bevacizumab was performed by incubating the plates with biotin conjugated rabbit anti- human IgG (1/80000 in 1% BSA-PBS/T) for 1.5 h at 37°C, followed by 20 min incubation with HRP-conjugated streptavidin (1/10000 in PBS) at room temperature. The color was developed with TMB and the reaction was stopped by adding 20% sulfuric acid. Optical density (OD) was measured using a plate reader (ELX800, BioTec, Winooski, VT) at 450 nm.

Preparation of nanoparticles

Bevacizumab was entrapped in NPs using a modified double-emulsion solvent evaporation technique according to the results of our previous study.[17] In brief, 100 μL of 1% w/v bevacizumab solution in PBS (0.15*M*, pH 7.4) containing 8% w/v HSA was emulsified in 1 mL of dichloromethane bearing 4.5 mg mL^{-1} of PLGA by sonication using probe sonicator (45 s, 9.5 W, 0 cycle, microtip 419, Misonix, S-4000, Newtown, CT). Then 2 mL of diluted PVA eye drop (1% w/v in PBS) was added to the primary emulsion and the mixture was sonicated for 90 s more (9.5 W). All sonications were performed in an ice bath. The resulting double-emulsion system was stirred overnight (500 rpm) at 4°C to allow evaporation of dichloromethane and hardening of the NPs. Ultimately, NPs were collected by centrifugation (20,000*g*, 4°C, 15 min), washed three times with excess deionized water and dispersed in PBS. The nano-suspension was then mixed with 2% w/v mannitol and freeze-dried. All preparation procedures were performed in clean conditions.

Characterization of bevacizumab-loaded nanoparticles

Surface morphology of freeze-dried NPs was evaluated by scanning electron microscopy (SEM, S4160, Hitachi, Tokyo, Japan). The size, distribution and zeta potential were determined by Zetasizer (Nano ZS, Malvern, Worcestershire, UK).

The amount of entrapped bevacizumab in NPs was determined by an indirect method.[18] Therefore, following the formation of NPs, the nano-suspension was centrifuged (20,000*g*, 4°C, 15 min) and the clear supernatant was collected, diluted as necessary, and analyzed by ELISA tests. The entrapment efficiency (EE) was calculated as follows: $EE\% = \frac{(\text{total employed bevacizumab(mg)} - \text{bevacizumab in supernatant (mg)})}{\text{total employed bevacizumab (mg)}} \times 100$. Freeze dried NPs were weighed and ultimately the loading efficiency (LE) was calculated using the following equation $LE\% = \frac{(\text{total employed bevacizumab(mg)} - \text{bevacizumab in supernatant (mg)})}{\text{Nanoparticles mass (mg)} \times 100} \times 100$.

Evaluation of the pharmacokinetic of bevacizumab released from nanoparticles in rabbit vitreous

To investigate the *in vivo* release of bevacizumab from NPs, pre-determined amount of NP suspension, equivalent to 1 mg bevacizumab, in sterile PBS was injected to the vitreous of New Zealand albino rabbits, weighing 2–2.5 kg (test groups). Intravitreal injection was performed using 29-gauge needle 2 mm behind the limbus. Rabbits were first sedated through intramuscular injection of ketamine (10%, 50 mg kg^{-1}) and xylazine mixture (2%, 2 mg kg^{-1}). In addition another group of rabbits ($n = 3$) received 1 mg of Avastin intravitreally, as control. During the *in vivo* test span rabbits were monitored repeatedly and to prevent ocular

infection chloramphenicol eye drop (Chlobiotic 0.5%, Sina Darou, Tehran, Iran) was utilized QID for 1 week.

At specific times following the injections, 4 h, 1, 3, 7, 21, 42, and 56 days, rabbits ($n = 3$ in each time group) were sacrificed and their vitreous and aqueous humor samples were taken and analyzed by ELISA test. The procedure of animal killing was conducted according to the guidelines of the Animal Care and Ethics Committee of Farabi Eye Hospital (totally 35 rabbits were used for the study). Collected samples were centrifuged (5000 rpm, 5 min) and diluted as necessary before the drug concentration assessment ($n = 3$). Finally the bevacizumab vitreous concentrations were plotted versus time in both groups (test and control). The pharmacokinetic parameters of the drug in vitreous and aqueous humor were calculated utilizing noncompartmental method. Trapezoidal area method was used to determine the area under the concentration–time curve (AUC0-t) and the area under the first moment concentration-time curve (AUMC0-t).[19]

$AUC_{0\text{-}\infty}$ and $AUMC_{0\text{-}\infty}$ were obtained from the curves extrapolation according to the following equations: $AUC_{0\text{-}\infty} = AUC_{0\text{-}t} + (C_t/K)$ and $AUMC_{0\text{-}\infty} = AUMC_{0\text{-}t} + C_t t/K + C_t/K^2$, respectively, in which C_t is the bevacizumab concentration obtained from the final vitreous sample ($t = 56$ days), t is the corresponding time, and K is the terminal phase elimination rate constant. Mean residence time (MRT) was calculated as follows: $MRT = AUMC_{0\text{-}\infty}/AUC_{0\text{-}\infty}$ and the elimination half-life ($t_{1/2}$) was obtained from the following equation: $t_{1/2} = 0.693/K$. The vitreous clearances (Cl) were calculated as follows: $Cl = \text{dose}/AUC_{0\text{-}\infty}$.

Evaluation of intraocular toxicity of the prepared nanoparticles

The probable ocular toxicity of NPs was evaluated by electroretinography (ERG) and histology tests.

ERG test was performed by Metrovision Unit (Mon Pack3, Pérenchies, France), in mesopic conditions and in accordance with International Society for Clinical Electrophysiology of Vision (ISCEV) protocol. Dencott contact lenses (Paris, France) that employed as active (positive) electrodes were placed in eyes, skin ELE12 electrodes which used as reference were placed posterior to lateral canthus and earth was placed on forehead to reduce noises.

A basic ERG test was performed before any treatments on all rabbits using white flashes with luminosity of 100 cd m^{-2} provided by a flasher with 33 cm distance from the eyes. ERG tests were repeated after 3, 21, and 56 days following the NPs injections. The ERG data (implicit time and amplitude of a-wave and b-wave) of each treated group was compared with those of basic tests ($n = 3$). Finally the results were statistically analyzed using SPSS 17.0 software. p values <0.05 were defined statistically significant.

For histology tests the eyes were enucleated, the globes were opened vertically and placed on 10% buffered formalin for 24 h. Specimen dehydration and paraffin block formation were performed using standard pathology methods. Then slices with 0.3 μm thickness were prepared and stained by H&E method and finally the slides were examined under light microscope.

Nanoparticle distribution in posterior segment tissues

To evaluate the distribution and persistence of the NPs in the posterior segment tissues the fluorescent dye, coumarin-6, loaded albuminated NPs were similarly prepared but coumarin-6 (10^{-6} g) was loaded in NPs instead of bevacizumab. On the other hand 1 mg HSA was added to formulation and thus the total amount of incorporated protein in the NPs was 9 mg. Prepared fluorescence dye loaded NPs were then injected intravitreally (10^{-6} g dye/100 μL PBS) and 7, 21, 42, and 56 days after the injections eyes were enucleated and 6 μm cryosections were prepared using microtome (Cryocut 1800, Leica, Nussloch, Germany) and evaluated using confocal laser scanning microscopy (Ecilips Ti, Nikon, Tokyo, Japan). Intact and coumarin-free NP injected eyes served as controls and in order to have an accurate mean intensity comparison, similar microscopic settings in both software and hardware were set for all samples. NIS-Elements AR software (Nikon, Tokyo, Japan) was employed to estimate the mean pixel intensity of the fluorescence emission in the confocal images.

Additionally, to differentiate between the persistence and distribution of the coumarin loaded NPs and that of free coumarin in ocular tissues the coumarin-6 solution in dimethyl sulfoxide (DMSO), 10^{-6} g/100 μL, was intravitreally injected to a group of rabbits, the cryosections were prepared and ultimately examined under the fluorescence microscope with the same condition in all samples (BX51, Olympus, Tokyo, Japan).

RESULTS

Characterization of bevacizumab loaded albuminated nanoparticles

According to the Zetasizer results the size of bevacizumab loaded albuminated NPs was 190 ± 29 nm with polydispersity index (PDI) of 0.17 ± 0.05 and zeta potential of -24.5 ± 3.1. The calculated EE and LE were $84.1\% \pm 4.2\%$ and $7.4\% \pm 0.4\%$, respectively. As shown in SEM images (Fig. 1) particles were spherical with smooth surfaces and their size distributions were almost homogenous.

The pharmacokinetic of bevacizumab released from nanoparticles in rabbit vitreous

The vitreous and aqueous humor concentrations of free bevacizumab (in semi-log form) were plotted against times following the intravitreal injection of NPs containing bevacizumab and Avastin (equal to 1 mg drug in both groups) in rabbits (Fig. 2). The pharmacokinetic parameters of bevacizumab in both vitreous and aqueous humor were calculated using noncompartmental method. Table I summarizes the attributed values for both test and control groups. The maximum bevacizumab concentration in vitreous was obtained a week after the NP injection, however, in the control group Cmax was observed immediately following the Avastin injection. The aqueous humor maximum concentrations were observed 1 and 7 days after the injections in control and test groups, respectively. AUMC in NP injected rabbits in both vitreous and aqueous humor were significantly higher than those in the Avastin injected rabbits ($p < 0.05$). Obtained MRTs were

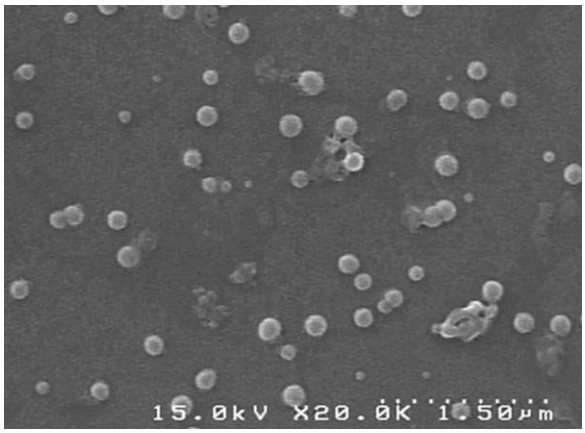

FIGURE 1. Scanning electron microscopy image of prepared nanoparticles containing bevacizumab.

4.6 and 15.1 days in control and test groups, respectively, showing a remarkable increase in the NP injected group ($p < 0.05$). The calculated vitreal $t_{1/2}$ through terminal phase elimination rate in the test group was 8.4 days which was significantly higher ($p < 0.05$) than that in the control group (5.2 days).

Likewise, AUC in the test group was remarkably increased in comparison with that in the control group ($p < 0.05$) and accordingly the drug clearance was significantly reduced following the administration of the NPs in comparison with that in Avastin ($p < 0.05$).

The ocular toxicity of the prepared nanoparticles

ERG results are summarized in Table II where no significant differences were observed either in implicit times or amplitudes of a and b waves after 3, 21, and 56 days following the NP injections compared to pre-injection data (before injection). Additionally no significant difference was observed in obtained ERG curves, as well [Fig. 3(A)].

In histology tests all H&E stained slides examined with light microscopic method revealed unremarkable histomorphologic changes in architecture of retina and choroid. No necrosis, hemorrhage, leukocytic infiltration or granulation tissue formation was noted. No foreign body reaction or evidence of fibrosis was found 3, 21, and 56 days after the injections in none of the specimens. Figure 3(B) shows the related microscopic images of retina sections in intact (B-1), Avastin treated (B-2), and NP treated (B-3) eyes after 56 days.

Altogether according to the histology and ERG results no significant ocular toxicity was observed during the observation period post intravitreal injection of the NPs in rabbits.

Nanoparticle distribution in posterior segment tissues

Coumarin-6 loaded albuminated PLGA NPs were employed to visualize the distribution and persistence of prepared formulation in ocular tissues. As indicated in the confocal images [Fig. 4(A)] the emitted green fluorescence shows the presence and distribution of the NPs in the retina, choroid and sclera. As can be observed the color intensity was reduced from 7 to 56 days after the NP injection and thus the NP concentration was decreased with time in such a way that this trend was faster in retina and choroid. Figure 4(B) indicates estimated mean pixel fluorescence intensities in retina and sclera the days after the intravitreal injection of NPs.

Coumarin-6 solution in DMSO was intravitreally injected to a group of rabbits to distinguish the difference between the dyes in the free and entrapped (in NPs) form. As can be seen in Figure 5, the fluorescence color fades faster in free coumarin received samples and therefore the free dye was more rapidly cleared from the retina, choroid and sclera than the entrapped dye in NPs.

DISCUSSION

Long lasting formulations of bevacizumab have been investigated in a number of papers.[20–27] The bevacizumab encapsulated PLGA NPs prepared by Pan et al. using double emulsion technique, showed no significant reduction in the neovascular area in rat retinas which can be explained by not considering the drug stability during the particles preparation.[20] Our previous study demonstrated the efficient protection ability of a proper concentration of albumin during the NP preparation. According to our *in vitro* studies the

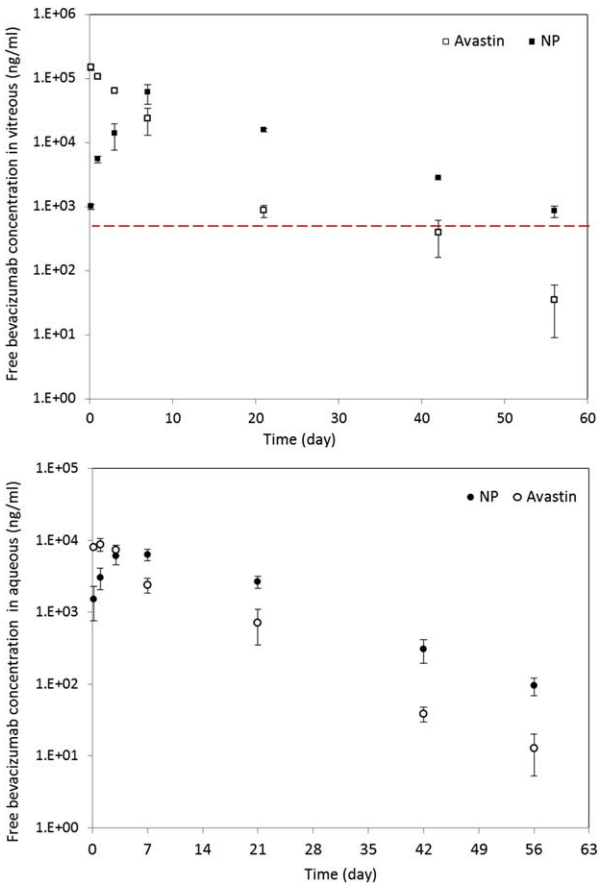

FIGURE 2. Semi-log plot of free bevacizumab concentration (mean ± SD) versus time in rabbit vitreous (A) and aqueous (B), following the intravitreal injection of the prepared NPs and Avastin. The dashed red line indicates the minimum concentration of bevacizumab that completely blocks the VEGF-induced angiogenesis (500 ng mL^{-1}). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

TABLE I. Pharmacokinetic Parameters in Rabbit Vitreous and Aqueous Humor Following the Intravitreal Injection of NPs (Test Group) and Avastin (Control Group)

Sample	Test Group		Control Group	
PK Parameters	VH[a]	AH[b]	VH	AH
C_{max}(μg mL^{-1})[c]	59.55 ± 20.31[d]	6.35 ± 1.14	145.05 ± 5.01	8.74 ± 1.71
t_{max} (day)[e]	7	~7	0.17	1
$AUC_{0-\infty}$ [f] (μg mL^{-1} day^{1})	851.93 ± 64.46	129.46 ± 26.10	613.87 ± 106.16	67.03 ± 16.89
$AUMC_{0-\infty}$[g] (μg mL^{-1} day^{2})	12802.66 ± 992.90	1682.36 ± 353.93	2823.60 ± 630.08	515.38 ± 189.51
MRT (day)[h]	15.07 ± 1.34	12.98 ± 0.23	4.57 ± 0.25	7.54 ± 1.03
Cl (mL day^{-1})[i]	1.18 ± 0.09	7.95 ± 1.69	1.66 ± 0.30	15.67 ± 4.48
$t_{1/2}$ (day)[j]	8.42 ± 0.06	7.79 ± 0.26	5.19 ± 0.24	5.82 ± 0.28

[a]Vitreous humor.
[b]Aqueous humor.
[c]Maximum concentration.
[d]Mean ± SD ($n = 3$)
[e]Time to attain C_{max}.
[f]Area under the concentration-time curve.
[g]Area under the first moment concentration-time curve.
[h]Mean residence time.
[i]Clearance
[j]Half-life

ratio 8:1 of HSA to bevacizumab could remarkably reduce the antibody instabilities caused by double emulsion preparation method.[17] It should be noted that analyzing the antibody concentration which relies on the binding of bevacizumab to $VEGF_{165}$ (e.g., ELISA) not only can specifically assay bevacizumab concentration values but can also properly portray the drug activity which unfortunately has been disregarded in some studies. For instance Li et al. reported the *in vitro* release of bevacizumab from PLGA NPs assayed by UV spectroscopy and thus the probable instabilities or inactivation of the drug were not considered.[21]

Liposomal formulation of bevacizumab prepared by Abrishami et al. showed prolonged residency of the drug for 42 days in rabbit eyes wherein the vitreous concentration was 1 and 5 times higher in comparison with the control (bevacizumab solution) at days 28 and 42, respectively.[22] According to our results following the NP injections the bevacizumab vitreous concentrations were 18, 7, and 24 times higher than the controls at days 21, 42, and 56 days, respectively.

Some other polymers have also been utilized as sustained release vehicles in research. According to Lu et al. report, the VEGF mRNA expression in retina of diabetic rats significantly declined for about 50%, 8 weeks after the intravitreal injection of bevacizumab loaded chitosan NPs compared with bevacizumab solution. However, this reduction was not significant 1 and 4 weeks after the injection of NPs in comparison with the drug solution.[23] In an *in vitro* study the electrochemically prepared mesoporous silicon oxide nanostructure could sustain the release of bevacizumab for more than 1 month.[24]

TABLE II. ERG Wave Data[a] Before and 3, 21, and 56 Days After Injection of the NPs

	Wave	Before Injection	After Injection	*p* Values[b]
			3 days after injection	
	a	14.80 ± 0.73	16.38 ± 2.21	0.18
	b	39.57 ± 1.72	38.82 ± 4.16	0.72
			21 days after injection	
Wave time	*a*	15.35 ± 1.42	15.367 ± 1.31	0.99
(ms)	*b*	39.27 ± 1.53	40.27 ± 1.33	0.33
			56 days after injection	
	a	15.20 ± 1.28	15.65 ± 1.04	0.08
	b	39.40 ± 3.06	41.17 ± 1.20	0.21
			3 days after injection	
	a	−20.2 ± 10.43	−17.83 ± 14.59	0.61
	b	104.1 ± 14.59	118.73 ± 57.77	0.31
Amplitude			21 days after injection	
(μV)	*a*	−17.97 ± 7.05	−17.85 ± 7.38	0.97
	b	109.62 ± 21.83	111.72 ± 29.06	0.79
			56 days after injection	
	a	−20.43 ± 9.40	−20.97 ± 7.63	0.94
	b	128.20 ± 34.42	157.92 ± 57.04	0.08

[a]Mean ± SD, $n = 3$.
[b]Resulted from the comparison between before and after injection data.

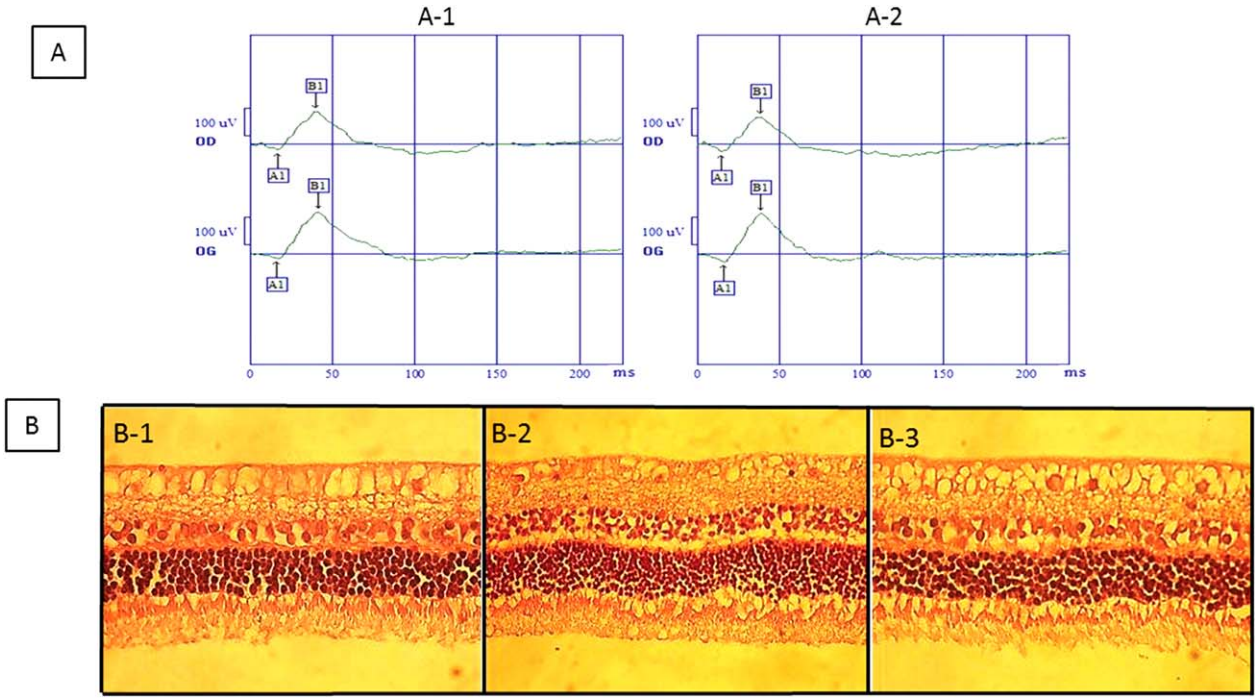

FIGURE 3. (A) Examples of electroretinogram recorded response before intravitreal injection of NPs (A-1) and 21 days after the injections (A-2). (B) Microscopic images of H&E stained retina sections in intact eye (B-1), control group, Avastin treated (B-2), and test group, NP treated (B-3), 8 weeks after intravitreal injections (×40). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

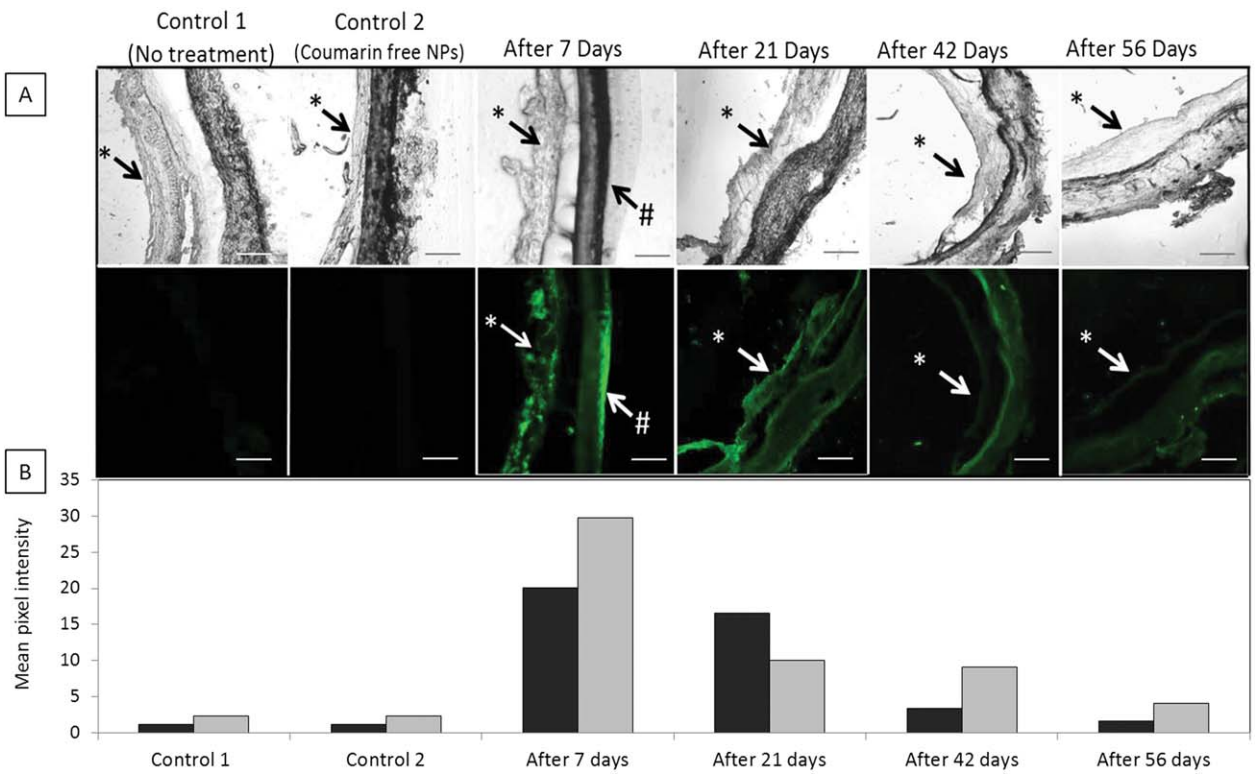

FIGURE 4. (A) Confocal images of posterior segments following the intravitreal injection of coumarin-6 loaded albuminated PLGA NPs. Intact and coumarin free NP injected eye sections were employed as control 1 and 2, respectively. *Retina and choroid, #sclera, the magnification bar corresponds to 500 μm. (B) The mean pixel fluorescence intensity in retina and choroid (black bars) and sclera (gray bars) following the intravitreal injection of coumarin loaded NPs. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

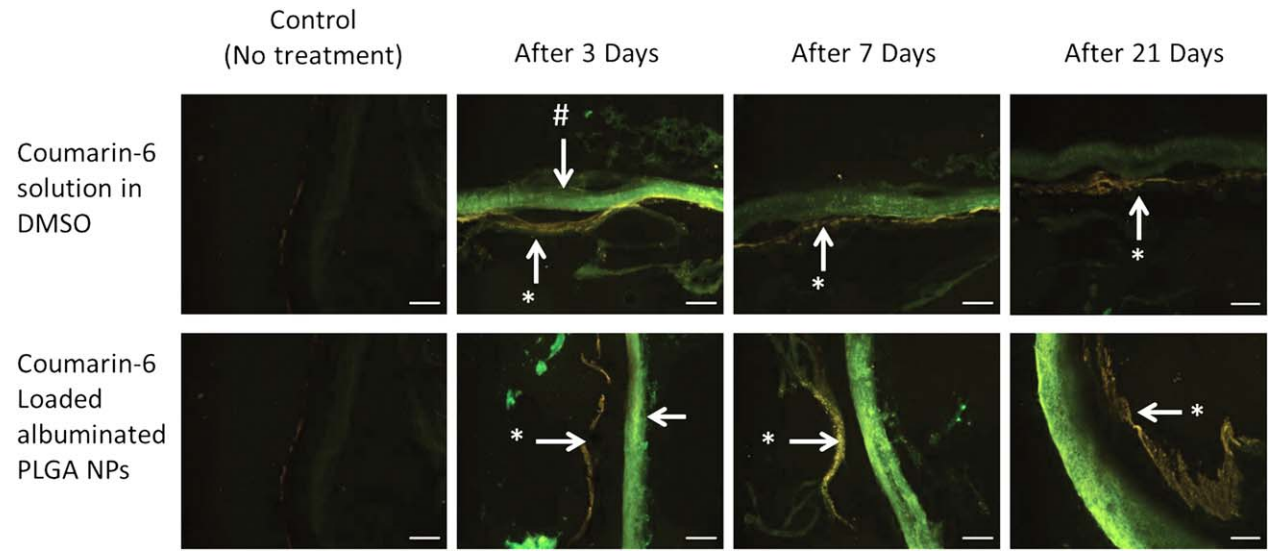

FIGURE 5. Fluorescence microscopic images of posterior segments following the intravitreal injection of coumarin-6 solution in DMSO and coumarin-6 loaded albuminated PLGA NPs. Intact eye section was used as control *Retina and choroid, #sclera, the magnification bar corresponds to 500 μm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

A number of papers have studied *in situ* forming hydrogels to provide extended release bevacizumab delivery systems. For instance cross-linked polysaccharides[25] and triblock copolymer of poly(2-ethyl-2-oxazoline)-*b*-poly(ε-caprolactone)-*b*-poly(2-ethyl-2-oxazoline)[26] have shown promising results in this area. Rauck et al. developed a thermoresponsive hydrogel by poly(ethylene glycol)-poly-(serinol hexamethylene urethane), ESHU, that could extend the release of bevacizumab for over 9 weeks with 4.7 times higher average concentration compared with the eyes receiving bolus bevacizumab injections.[27]

The pharmacokinetic of intravitreal injected bevacizumab in rabbits was evaluated by Bakri et al. using non-compartmental analysis and the resulted half-life of free bevacizumab concentration was 4.32 and 4.88 days in vitreous and aqueous humor, respectively.[28] According to the Nomoto et al. study on rabbits the resulted half-life of bevacizumab in vitreous was 5.95 days.[29] Accordingly, as can be observed in Table I, our data support previous reports ($t_{1/2}$ in vitreous was 5.19 days and in aqueous was 5.82 days). In various papers the vitreous half-life of drugs (e.g., bevacizumab and triamcinolone acetonide) in human has been reported to be more than that in rabbits which may be due to the difference in the eyes anatomy including a larger vitreous volume.[30]

Our obtained results indicated 3.3 and 1.6 times elevation in bevacizumab vitreous MRT and half-life, respectively, following the intravitreal injection of prepared NPs in comparison with the control group. Significant increase in the AUC in the test group may show the capability of designed NP in protecting the drug from the enzymatic degradations.[31] Because the kinetics of drugs released from novel controlled delivery formulations is commonly unknown, utilizing non- compartmental approach in assessment of pharmacokinetic parameter is preferably recommended in most studies.[32] Therefore, in such cases non-compartmental data (e.g., MRT) are more trustable for comparing drug pharmacokinetic in encapsulated and free forms.

Wang et al. *in vitro* studies on the endothelial cell proliferation demonstrated that the minimum concentration of bevacizumab required to completely inhibit the VEGF-induced endothelial cell growth was 500 ng mL^{-1} while the drugs IC_{50} was 22 ng mL^{-1}.[33] According to our results following the NP injection the bevacizumab vitreous concentration remained above 500 ng mL^{-1} over 8 weeks in the test group, while, it felled below 500 ng mL^{-1} about 6 weeks after Avastin injection in the control group [Fig. 2(A)].

Fluorescent coumarin-6 has been used in various studies to indicate the distribution and penetration of particulate drug delivery systems in animal tissues.[34,35] Hirota et al. employed coumarin-6 loaded PLGA microspheres to examine the distribution of microparticles in lung following the insufflations.[36] In the current study emitted florescence in the eye tissue sections indicated the distribution of albuminated PLGA NPs containing coumarin-6 in posterior segment. According to higher fluorescence intensity in retina and choroid 7 days after the intravitreal injection of coumarin loaded NPs (Figs. 4 and 5), it can be concluded that the dye entrapped in NP was more successful in distribution in retina and choroid than its solution form. However, more investigations are required to prove this hypothesis. Regardless of the fact that this emission is related to the entrapped dye or released coumarin, the difference between the images of coumarin solution and dye loaded NP images (Fig. 5) demonstrated the elongated persistence of coumarin-6 in NP injected eyes. Despite the unquestionable differences between bevacizumab and coumarin-6, our recent experiment can somewhat simulate the behavior of albuminated PLGA NPs containing bevacizumab in ocular tissues. However, it should be noted that large biomolecules

(>70 kDa) such as antibodies (~150 kDa) may not penetrate the blood-retinal barrier and sclera.[37]

According to Sakurai et al. study the fluorescence emitted from nanospheres was detectable in the vitreous cavity for more than 1 month following the intravitreal injection of fluorescein derivative loaded nanospheres. Their histological studies indicated that the nanospheres with a diameter of 200 nm and less were observed in retina as well as vitreous cavity and trabecular meshwork, while larger particles (2 μm) were not detected in retina which indicated the favorable potentials of nanospheres in drug targeting to retina.[38]

CONCLUSION

Altogether it can be concluded that the prepared NPs provided a sustained release formulation of bevacizumab for about 2 months and the drug vitreous concentration remained above 500 ng mL^{-1}, known as the minimum concentration that completely blocks the VEGF-induced angiogenesis,[33] for >8 weeks. Accordingly, our prepared formulation may offer improvements in treatment of eye posterior segment neovascularization and related vitreoretinal diseases by reducing the number of intravitreal injections and thus its associated concerns.

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