Improved Anti-Inflammatory Effects in Rabbit Eye Model Using Biodegradable Poly Beta-Amino Ester Nanoparticles of Triamcinolone Acetonide

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PURPOSE. Results of previous studies on the benefits of ocular drug delivery using polymeric mucoadhesive nanoparticles suggested longer presence and better penetration of nanoparticles, and, thus, increased effect and bioavailability of drugs entrapped in nanoparticles. In this study, a novel polymer, poly β-amino ester, was used for the preparation of triamcinolone acetonide–loaded nanoparticles using a modified emulsification/solvent diffusion method.

METHODS. Mucoadhesiveness studies, in vitro drug release, x-ray powder diffraction, differential scanning calorimetry, and scanning electron microscopy were used for physicochemical characterization of nanoparticles. Thirty-six hours after inducing uveitis by intravitreal injection of a lipopolysaccharide, sampling from the aqueous humor was done and inflammatory factors, such as cell, protein, nitric oxide, and prostaglandin E2, were compared.

RESULTS. Nanoparticles with a mean size of 178 nm and drug loading of 5.3% were prepared and used for in vivo studies in rabbits with uveitis. Higher anti-inflammatory effect was observed for polymeric nanoparticles of triamcinolone acetonide compared with microparticles of prednisolone acetate and triamcinolone acetonide, and an equal effect compared with subconjunctival injection of triamcinolone acetonide in terms of inhibiting inflammation and inflammatory mediators.

CONCLUSIONS. It can be concluded that polymeric nanoparticles of triamcinolone acetonide will provide as good an anti-inflammatory effect as the subconjunctival injection method and are better compared with other drug delivery systems.

Keywords: nanoparticles, triamcinolone acetonide, poly beta-amino ester, mucoadhesive, uveitis, nitric oxide, ocular drug delivery

Topical drug delivery has long been considered as the easiest method with highest patient compliance. However, due to existing defense mechanisms in the eye, when microsuspensions and topical solutions are administered, the residence time in the precorneal space and penetration to ocular tissue are both reduced to 5 to 6 minutes and 1% to 3%, respectively.1,2 A new mucoadhesive polymeric carrier, cross-linked poly acrylic acid, Durasite (Inspire Pharmaceuticals, Durham, NC), has been used to increase the residence time in the precorneal space and improve the effect of topical medication.3 Despite this, the use of mucoadhesive polymers in the form of solution leads to the complete hydration of the polymer, and therefore, its mucoadhesivity is greatly reduced.1

In recent years, we have witnessed a new era for the applications of nanoparticles (NPs). Due to their interaction with the mucosal layer, NPs can increase drug residence time in the precorneal space up to 20 minutes.1 Also, they can significantly increase rates of bioavailability, corneal penetration, and conjunctival uptake of the drug loaded in their structure.4 Naturally, the therapeutic effect of drugs loaded in polymeric NPs is significantly increased.5 Usage of mucoadhesive polymers in the preparation of NPs can increase drug residence in the precorneal area from several hours up to 1 day, which can guarantee a better penetration and improve the effect of the loaded drug in mucoadhesive controlled-release NPs.1 Polymeric NPs with positive surface charge (Eudragit S; methyl methacrylate-methacrylic acid copolymer, Chitosan) have demonstrated their mucoadhesive properties by the ionic interaction between the positive-charge amine groups and
negative-charge mucine sialic acid residues on the ocular surface. This can prolong the presence of these NPs on the ocular surface, and maximizes bioavailability and corneal penetration of drugs encapsulated in such polymeric NPs.6,7

Apart from these features, it is proven that the use of cationic mucoadhesive polymers, such as chitosan, increases transepithelial absorption by dissociating tight junctions, and functions as enhancers.8

In this study, we aim to improve ocular drug delivery in eyes with uveitis, using mucoadhesive polymeric NPs. In this regard, we examined the in vitro and in vivo characteristics of polymeric NPs prepared from the poly-β amino ester (PbAE), a cationic polymer, and triamcinolone acetonide (TA). For a comparison of the effects of these NPs and commercially available medications, we used rabbits with endotoxin-induced uveitis (EIU).

PbAE is a biodegradable and biocompatible polymer with positive surface charge due to its amine groups.9

There are reports concerning NPs prepared with this polymer for drug delivery to cancer tumors and as gene delivery,9–11 but to date, their mucoadhesive properties have not been used. There are no reports of the use of PbAE for topical drug delivery to the eyes.

For in vivo studies, we used uveitis, which is a severe inflammation of the anterior chamber.13 This disease is induced in rabbits by intravitreal injection of lipopolysaccharide (LPS).13,14 LPS causes breaks in the vessel wall and infiltration of cells and protein into the anterior chamber aqueous humor.13 This initiates a series of ocular inflammatory reactions along with activation of cyclooxygenase-2 (COX-2) and inducible NO synthase (iNOS) enzyme, which leads to a sudden increase of inflammatory factors, such as nitric oxide (NO)15 and prostaglandin E2 (PGE2), in the anterior chamber.16 Ohgami et al17 measured these factors in the aqueous humor and presented a suitable baseline to compare the anti-inflammatory effect of different drugs.

**Materials and Methods**

**Materials**

TA was purchased from Crystal Pharma (Valladolid, Spain); 4,4-trimethylene dipiperidine and triethylamine were purchased from Sigma-Aldrich (Diegem, Belgium); 1,4-butanediol diacrylate was purchased from Alfa Aesar Organics (Karlsruhe, Germany); mucin type I-S was obtained from Sigma-Aldrich (St. Louis, MO); polyvinyl alcohol (PVA, 88% hydrolyzed, MW 7500; Cecil Instruments, Cambridge, England), and then a calibration curve was derived.

**NP Preparation**

Poly beta-amino ester was synthesized according to a method reported previously.18 NPs were prepared using a modified emulsification/solvent diffusion method.19 First, 110 mg of the drug/polymer mixtures at different ratios of 4:10 and 1:10 was dissolved in 2 mL of dichloromethane-acetone (8.2 vol/vol). The solution was then injected at 0.5 mL/min rate to 20 mL aqueous medium containing PVA (1% wt/vol) as emulsifier and homogenized through sonication in an ice-water bath at 50% power for 5 minutes using a probe sonicator (S-4000-010; Misonix, Newtown, CT). To prepare NPs, the prepared emulsion was stirred at 500 rpm overnight on a magnetic stirrer so that its organic solvents would diffuse and evaporate. The nonencapsulated TA crystals were eliminated by successive filtration through six 3-μm (Whatman, Buxinhamshire, UK) and 1.2-μm (Sartorius, Goettingen, Germany) cellulose filters. To separate the NPs from the continuous-phase and residual solvent, the NPs were first centrifuged (5K30; Sigma, Ostrode, Germany) at 30,427g and 108C for 30 minutes. The supernatant was then discarded, and the NPs washed again as described above. Finally, the sample was lyophilized (alpha 2-4 LD plus; Christ, Ostrode, Germany).

**NP Characterization**

Mean particle size, size distribution, and zeta potential (ζ-potential) were measured using photon correlation spectroscopy (Zetasizer nano ZS; Malvern, Worcestershire, UK). To measure particle size, samples were diluted with a ratio of 1:20 with deionized water, and moved to the device after a gentle bath sonication to prevent clumping. To determine ζ-potential, NPs were first diluted with a ratio of 1:25; incubated for 0.5 hour at 37°C in deionized water and pH levels of 7.4, 6.8, and 6.3 (prepared with phosphate buffer); and moved to the device for surface charge assessment after gentle bath sonication. These two processes were repeated three times for each sample.

To examine the morphology of NPs, two drops of undiluted NP suspension was placed on the pan surface, and left to dry in a closed container at room temperature. Then, it was coated with gold using a sputter coater (SCD 005; Bal-Tec, Basel, Switzerland), and transferred to the scanning electron microscopy (SEM XL30; Philips, Amsterdam, Netherlands) for evaluation.

**NP Mucoadhesiveness Measurement**

A periodic acid/Schiff (PAS) was used for colorimetric method to assess mucoadhesiveness of NPs.20,21

In the first step, to prepare a standard mucin calibration curve, 2-mL samples of standard mucin type I-S were prepared in different concentrations of 0.25 mg/2 mL, 0.75 mg/2 mL, and 1 mg/2 mL; 0.2 mL periodic acid reagent was added to each sample, and they were incubated in a water bath for 2 hours at 37°C with gentle shaking. Then, 0.2 mL Schiff reagent was added to each sample, and they were left at room temperature for 30 minutes before measuring the adsorption of different mucin concentrations with a spectrophotometer (CE 7500; Cecil Instruments, Cambridge, England), and then a calibration curve was derived.

In the second step, 10 mg of the NPs was dispersed in 6 mL of solutions prepared with buffer phosphate at pH levels of 6.3, 6.8, and 7.4, which contained 0.5 mg/mL mucin type I-S. The resulting nanosuspensions were then incubated in a water bath for 2 hours at 37°C with gentle shaking. To separate the mucin adsorbed by the NPs, samples were centrifuged at 30,427g for 10 minutes, and then 2 mL of the supernatant was extracted to calculate the free mucin content using a method similar to that for standard mucin adsorption. Eventually, the amount of mucin adsorbed by the NPs at different pH levels was calculated using the following equation:

\[
\text{Original Mucin} - \text{Mucin in Supernatant} / \text{Original Mucin} \times 100
\]
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their physical mixture of different ratios using the DSC823 (Mettler Toledo, Neu-Isenburg, Switzerland) equipped with STARe software 9.01 (Mettler Toledo, Neu-Isenburg, Switzerland) and Julabo Thermocryostate (Julabo, Allentown, PA). For this purpose, 6 mg of the sample was sealed in 400 μL aluminum pans and scanned at 10°C per minute from 25 to 300°C. X-ray powder diffraction (XRPD) was measured using a Siemens 85 (Munich, Germany) with Cu Kα radiation.

NP Entrapment Efficiency, Drug Loading, and HPLC Analysis

To measure entrapment efficiency (EE) and drug loading (DL), 20 mg lyophilized NPs was added to 10 mL acetonitrile, the mixture was stirred at 500 rpm for 30 minutes, and 20 mL ethanol was added while stirring after a short sonication, and then filtered through a 0.22-μm filter to separate the polymer and precipitates. The filtered sample was tested for the drug content in NPs using HPLC method. The EE was determined as the amount of drug in NPs divided by the original amount of drug used for the preparation of NPs. The DL was defined as the amount of drug in NPs divided by the total weight of the prepared NPs.

Drug Release Experiment

In this stage, an appropriate amount of NPs containing 2 mg TA was added to vessels with 500 mL balanced salt solution (BSS, pH 7.4) while being paddle stirred at 100 rpm at 37°C. By sampling of 2 mL at suitable times, the cumulative drug release from NPs was studied and plotted against the time line. Each sample was immediately centrifuged at 33,902g to separate NPs, and the supernatant was injected into the HPLC device to measure the amount of released drug. Precipitated NPs were redispersed in 2 mL BSS and moved back to the sampling vessel. All procedures were conducted in light-protected conditions.

Animals and Induction of Uveitis by LPS

In this study, we used 24 adult pigmented New Zealand female rabbits weighing between 2 and 3 kg. These rabbits were purchased from the animal house of Tehran University of Medical Sciences. The rabbits were housed in separate cages under a cycle of 12 hours of light and 12 hours of darkness. Animal management protocols were based on ARVO guidelines, which were also approved by the Ethics Committee of the Pharmaceutical Research Centre, Tehran University of Medical Sciences, which was in adherence with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Measurement of NO, Protein, and PGE2 Concentration and Infiltrated Cell Numbers in Aqueous Humor

Sampling from the rabbits’ anterior chamber was done 36 hours after LPS injection when uveitis reaches its peak. After puncturing the anterior chamber with a 30-gauge insulin needle, 80 μL of aqueous humor was extracted and mixed immediately with an equal amount EDTA 0.1% and stored at −80°C for further assessment.

All rabbit eyes were examined at the slit lamp before entering the study to exclude cases with inflammation that might create false results.

Animal Groups and Medications

Studies were carried out on six groups of four rabbits each and both eyes were included in the experiment. Two groups were used as controls; one as the healthy group, and the other as an EU group receiving no treatment. The third group received 50 μL of topical PbAE-TA 1% NPs (appropriate amount of PbAE-TA NPs dispersed in BSS, pH 7.4) every 4 hours. The fourth group received one drop of TA 1% micropususpan (Micronized TA dispersed in BSS, pH 7.4) every 4 hours. The fifth group received one drop of prednisolone acetate (PA) 1% micropususpan (Pred Forte 1%; Allergan, Irvine, CA) every 4 hours. All topical administrations were started immediately after LPS injection and continued until 36 hours with 4-hour intervals. The last group received a single subconjunctival injection of TA 0.4 mg/0.1 μL (Triamhextal; Hexal AG, Holzkirchen, Germany) immediately after LPS injection. The animal study followed guidelines approved by the ethical committee of the Pharmaceutical Research Centre, Tehran University of Medical Sciences.

Statistical Analysis

Differences among groups were evaluated using ANOVA on ranks, followed by Student-Newman-Keuls multiple comparison tests. A probability value of less than 0.05 was considered to represent a statistically significant difference. These computations were performed using the program SigmaPlot version 11 (Systat Software, San Jose, CA).

RESULTS

NP Preparation

TA loaded PbAE NPs prepared in this study, which appeared spherical with a smooth surface according to SEM images (Fig. 1). The Table shows the NP formulations and characterization. We studied the effect of changes in the solvent system on the size and polydispersity index (PDI) of NPs. Using dichloromethane alone as the solvent system gave better results than the use of acetone alone. Simultaneous use of the two acetone...
and dichloromethane solvents together, as the solvent system, provides for a better size and PDI compared with the use of each one alone, and at higher proportions of dichloromethane to acetone, a better PDI and size was achieved. The use of higher-power sonication led to smaller NPs. It was found that the best method for preparing NPs is using 20:80 ratio of acetone-dichloromethane and 30% sonicator power for 7 minutes. These NPs were passed successively from 6.0-, 3.0-, and 1.2-μm filters before EE and in vitro release studies to separate sediment particles of pure drug. Previous studies have shown that the use of this method for preparing NPs from corticosteroids can cause the formation of drug crystals along with NPs, and lead to a false increase in DL and an abnormal increase in burst effect.19 This study showed a 5.3% DL and 53% EE in a 1:10 drug polymer ratio for 178-nm NPs.

NP Characterization

As presented in the Table, z-potential of PbAE-TA NPs increased at lower pH levels; it reached +17 at a pH of 6.3 and continued to increase at lower levels (data not demonstrated). At all three common ocular pH levels, we observed a positive surface charge for PbAE-TA NPs.

We examined mucin adsorption by these NPs at 3 normal eye pH values of the eye using the colorimetric method. As the pH decreases from 7.4 to 6.3, the mucin adsorption percentage of PbAE-TA NPs significantly increases (P < 0.05) from 22% to 78%, which is concordant with the z-potential observed for these NPs at given pH levels.

The PbAE thermogram in Figure 2 shows a wide-based endothermic peak at approximately 290°C, which corresponds with the melting point and the glassy transition point for PbAE at 70°C.

Figure 3 shows the x-ray diffraction (XRD) pattern of NPs prepared with PbAE at 4:10 and 1:10 ratios and their physical mixture. Intact TA shows limited intrinsic crystal peaks at 2θ of 9°, 14°, 17°, and 24°, which is indicative of the crystal structure of the drug. According to the intrinsic peaks at 2θ of 18°, 19°, and 21°, the polymer has a partially crystalline structure, which corresponds to results of DSC and the wide melting peak at approximately 290°C (Fig. 2). As for the

![Figure 2. The DSC spectrums TA, PbAE, PbAE-TA NPs, and the physical mixture (PM) at drug-polymer ratio of 4:10.](image-url)
Physical mixture of TA and PbAE, each had their apparent peak, which indicates their crystalline structure when they are mixed. Nonetheless, the intensity of the peaks was quite reduced with PbAE-TA NPs, which indicates decreased crystallinity of these NPs as an effect of the production process on the polymer and drug.

**In Vitro Drug Release**

As can be seen in Figure 4, the drug release from PbAE-TA NPs with drug-polymer ratios of 1:10 and 4:10, reached 80% in 12 hours. The burst release for both ratios was 30% to 40% at 1-hour drug release time.

**In Vivo Studies**

The amount of protein in the aqueous humor of rabbits with EIU was 18 times higher than that in healthy rabbits. Although therapy with both topical PbAE-TA NPs and subconjunctival injection of TA successfully reduced the amount of protein and inflammation, statistically, the effects were similar in the two groups (Fig. 5A). The two groups of TA injection and topical PbAE-TA NPs showed similar results in terms of reducing cells and inflammatory mediator NO (Figs. 5B, 5C). The reduction seen with topical PbAE-TA NPs was approximately twice that with topical TA and PA microparticles. As for PGE2, the variation seen in the results, it appears that the differences in

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**Figure 3.** The XRPD spectrums of TA, PbAE, PbAE-TA NPs, and the PM at drug-polymer ratios of 4:10 and 1:10.

**Figure 4.** Release profiles of different ratios of PbAE-TA NPs. Data represent mean value of three replications ± SD.

**Figure 5.** Effect of PbAE-TA NPs in comparison with other formulations on LPS-induced protein (A), cell (B), NO (C), and PGE2 (D) concentrations in the aqueous humor of rabbits. Mean ± SE of data from four rabbits is shown. *P < 0.05.
DISCUSSION

In this project, the modified emulsification/solvent diffusion method was applied to prepare mucoadhesive NPs for the ocular delivery of TA. There are many reports of preparing NPs with this method, leading to the preparation of 100 to 200 nm NPs, which is considered a suitable size in terms of drug delivery.\textsuperscript{24,25} 

TA loaded PbAE NPs prepared in this study were spherical with a smooth surface according to SEM images (Fig. 1).

Mucin is a common glycoprotein in mucus layers of the body,\textsuperscript{26} to predict NP adsorption by the ocular mucus layer, mucoadhesiveness of the NPs were determined. The adsorption mechanism of PbAE-TA NPs is based on an ionic noncovalent interaction between amino groups of the PbAE polymer with positive charge and the sialic acid residues of the mucin with negative charge; thus, increased mucin adsorption at lower pH levels can be attributed to maximized ionization of PbAE amino groups at these pH levels. Similar results have been reported on the mucoadhesive properties of cationic polymers, such as chitosan and the effect of hydrogen-ionic noncovalent bonds.\textsuperscript{6}

The DSC thermogram for TA showed an endothermic peak corresponding to the melting point of approximately 300°C, which is indicative of a crystalline anhydrous state for TA. With PbAE-TA NPs, there were no peaks around the TA melting point, and the thermogram was not different from that of pure PbAE. This can be attributed to drug solubilization or the molecular distribution of the drug in polymeric matrix.\textsuperscript{19,27}

Regarding the in vitro drug release, we found a burst release of 30% to 40% in the first hour that drug release was observed; however, NPs with drug-polymer ratio of 1:10 showed a slower drug-release profile. This can be attributed to the adsorption of hydrophobic drug molecules to the surface of polymeric NPs during the preparation of NPs.\textsuperscript{28} The fast drug release from PbAE-TA NPs can be attributed to faster drug diffusion from the polymeric network with positive charge and faster erosion of the PbAE at a pH of 7.4.\textsuperscript{9}

In this study, we compared the effect of topical mucoadhesive PbAE-TA NPs versus conventional methods of treatment, such as ocular injection of TA, topical PA, and TA microparticles. This was done by comparing the amount of inflammatory factors in the aqueous humor.\textsuperscript{17} The presence and injection of endotoxin causes blood aqueous barrier breakage, therefore, the presence of cells, protein, and inflammatory factors in the aqueous humor.\textsuperscript{29} Topical TA and PA microparticles and PbAE-TA NPs demonstrated significantly different results in reducing the amount of aqueous humor protein, which indicates better efficiency of NPs in inhibiting inflammation compared with microparticles (Fig. 5A), and the reason could be sought in the longer residence time and higher uptake of mucoadhesive NPs in comparison with microparticles. Comparing these factors in eyes treated with TA injections and rabbits treated with topical PbAE-TA NPs showed no significant difference; this indicates a similar therapeutic effect of these two types of drug delivery in the treatment of uveitis. The reason for the insignificant difference of different methods on PGE2 can be sought in the mechanism and duration of PGE2 formation, which is dependent on the activity of the COX-2 enzyme. The COX-2 activating agent is NO, which is an inflammatory mediator.\textsuperscript{17,30} The NO in all four groups is inhibited by PA and TA drugs from the very first moments. This can affect the increasing time of PGE2 and delay it. For better conclusions about PGE2, we need to analyze aqueous humor samples taken at different times. However, because our pilot studies had shown that sampling from the anterior chamber per se is associated with increased inflammatory factors because of the puncturing process, sampling at different times was not done.

CONCLUSIONS

This study showed the usefulness of poly β-amino esters as a mucoadhesive polymer as an ocular drug delivery system. NPs made from this polymer are as effective as the injection method and more effective than microparticles in the treatment of uveitis in rabbits. These observations can be attributed to the slow-release properties, prolonged presence in preocular area, and better penetration of NPs into the ocular barrier compared with common procedures.

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