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Size-dependent disposition of nanoparticles and microparticles following subconjunctival administration

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Abstract

The purpose of this study was to determine the retention and ocular distribution of subconjunctivally administered nanoparticles and microparticles. Fluorescent polystyrene particles (carboxylate modified, negatively charged) of various sizes (20 nm, 200 nm and 2 μm; Fluospheres, dose 400 μg) were administered to male Sprague-Dawley rats by subconjunctival injection under anaesthesia. The disposition of the particles in the periocular and ocular tissues was studied for up to 60 days by quantifying the particle amounts using liquid extraction followed by spectrofluorimetric analysis. The effect of dose on the particle disposition was investigated with a 40-μg dose of the particles. The effect of an increase in surface hydrophobicity was evaluated for the 20 and 200 nm particles at 1 day post administration. Following periocular administration, penetration into the ocular tissues was negligible for the carboxylate-modified microparticles as well as nanoparticles. Almost the entire dose of the 200 nm and 2 μm particles was retained in the periocular tissue at 60 days post-administration. The 20 nm particles disappeared rapidly from the periocular tissue with 15 and 8% of administered dose remaining after 1 and 7 days, respectively. The 20 nm particles could not be detected in the periocular tissue at 60-days post-administration. An increase in the surface hydrophobicity did not affect the periocular retention of 200 nm particles but elevated that of the 20 nm particles, at the end of day 1. It was concluded that subconjunctivally administered 200 nm and larger particles can be almost completely retained at the site of administration for at least two months. Periocular administration of particulate systems of this size would likely be useful as sustained drug delivery systems.

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Introduction

Topical application of drugs for the treatment of disorders of the posterior segment is inefficient due to a combination of reasons, including the permeability-limiting corneal and conjunctival barriers, long diffusional path lengths along the lens axis that limit drug entry into the vitreous, rapid precorneal drug drainage that limits conjunctival penetration of drugs into the eye and drug loss into the systemic circulation via the conjunctiva (Stjernschantz & Astin 1993; Jarvinen et al 1995; Kompella & Lee 1999; Geroski & Edelhauser 2000; Sunkara & Kompella 2003). Although the systemic routes, such as the oral route, can deliver drugs to the posterior segment of the eye, the fraction of dose delivered to the retina is very low. This is due to the presence of the inner blood–retinal barrier, which, analogous to the blood–brain barrier, severely limits drug entry into the extravascular space of the retina and into the vitreous. Consequently, to achieve therapeutic concentrations of the drugs in the retina, large systemic doses are required, leading to side effects. Intravitreal injections are clinically used to deliver drugs to the vitreo-retinal region. However, for chronic retinal disorders, repeated intravitreal injections are required, which can potentially induce retinal detachment, haemorrhage, endophthalmitis and cataracts (Maurice 2001).

Periocular modes of administration, including the subconjunctival, subtenon, juxtascleral, peribulbar and retrobulbar routes of administration, are now emerging as promising alternatives for retinal drug delivery (Raghava et al 2004). In all these routes, the drug is interfaced near the sclera. The sclera allows diffusion of low-

molecular-weight drugs as well as macromolecules (Ambati et al 2000b; Ambati & Adamis 2002). Periocularly administered drugs can gain entry into the retina and vitreous by diffusing across the sclera, choroid and retinal pigment epithelial layers. Periocular injections are less invasive to the globe and are potentially safer than intravitreal injections. With reduced dose requirements due to enhanced retinal drug delivery from the periocular routes (Ayalasomayajula & Kompella 2004), systemic drug exposure and side effects would potentially be reduced with the periocular route compared with the systemic route of administration.

Since many vitreo-retinal disorders, including diabetic retinopathy, macular oedema and age-related macular degeneration, are chronic in nature, several sustained delivery systems, including non-degradable and degradable implants, have been developed for localized delivery to the posterior segment of the eye (Metrikin & Anand 1994; Chetoni et al 1996; Bourlais et al 1998; Kunou et al 2000; Yasukawa et al 2000; Chiang et al 2001; Yasukawa et al 2001; Kompella et al 2003; Ayalasomayajula & Kompella 2005). These sustained delivery systems are made from either biostable (non-biodegradable, non-erodible) or biodegradable polymers. Indeed, a non-erodible intravitreal implant (Vitrasert; Bausch & Lomb) of ganciclovir is in clinical use for the treatment of cytomegalovirus retinitis (Dhillon et al 1998). This implant delivers sustained therapeutic levels of ganciclovir for a period of approximately 7½ months. Although Vitrasert is a very effective device for sustained drug delivery, it requires expensive surgery for placement and removal, and it has limitations similar to the intravitreal injections (Cadman 1997a, b). Scleral plugs and related biodegradable implant-like drug delivery systems are exposed to the vitreous (Yasukawa et al 2001). Such systems can contribute debris of the degrading implant to the vitreous, potentially leading to harmful side effects. Hence, there is a need to explore other non-surgical approaches for sustaining drug delivery to the posterior segment of the eye.

In recent times, nano- and microparticulate systems have generated considerable interest for sustaining drug delivery to the eye (Joshi 1994; Herrero-Vanrell & Refojo 2001). Nanoparticle suspensions have been shown to improve the residence time of topically applied ophthalmic formulations. Also, intravitreally administered microparticles have been shown to sustain drug delivery. However, intravitreal particulate systems can likely interfere with vision (Maurice 2001) and potentially induce proliferative retinal disorders (Algvere & Martini 1985, 1986; Algvere et al 1988; Martini 1992). Periocular administration of particulate systems is less likely to suffer from vision-interfering side effects.

We have previously demonstrated that the delivery of budesonide, a corticosteroid, to the intraocular tissues, including the retina, can be sustained better with subconjunctivally administered biodegradable microparticles (3.6 μm) compared with nanoparticles (345 nm) (Kompella et al 2003; Ayalasomayajula & Kompella 2005). One reason for this difference could be that the nanoparticles were removed more rapidly from the subconjunctival site of administration compared with the microparticles, due to their size differences. To determine this possibility and to understand the disposition of particulate systems based on their size, this study investigated the disposition of non-degradable nano- and micro-particulate systems after periocular (subconjunctival) injection.

Materials and Methods

Materials

Carboxylate-modified (negatively charged) fluorescent polystyrene nanoparticles (Fluospheres, 20 nm and 200 nm) and microparticles (Fluospheres, 2 μm) loaded with bodipy (yellow-green) fluorescent dye were purchased from Molecular Probes (Eugene, OR). In addition, bodipy containing amine-modified (positively charged) (200 nm) and sulfate- (200 nm) or aldehyde sulfate (20 nm)-modified (neutral and hydrophobic) polystyrene particles were purchased from Molecular Probes. The dye does not leach out from these particles when suspended in aqueous media. The manufacturer's reports indicate that there is $< 1\%$ loss of the dye from Fluospheres after 6 months of storage in the dark in aqueous medium and $< 10\%$ change in the fluorescent signal in xylene after storage in the dark for 6 months. The Fluospheres, as well as the dye, are inert to alkaline hydrolysis when the temperature is maintained below 60°C (http://fmrc.pulmcc.washington.edu/DOCUMENTS/FMRC499.pdf). In addition, Fluospheres allow the fluorometric quantification of intact particles. All other reagents used in this study were purchased from Sigma (St Louis, MO) and were of analytical grade.

Particle size and zeta-potential measurement

The particle size and the zeta-potential were measured using Zeta Plus zeta-potential analyzer (Brookhaven Instruments Ltd, New York, NY), which employs the dynamic light scattering technique for particle size measurement. The software calculates the effective diameter based on the Stokes–Einstein equation as a diameter of an equivalent hydrodynamic sphere from z-average diffusion coefficients. The particle size and zeta-potential measurements were carried out after 1:1000 dilution of 2% stock in phosphate-buffered saline (PBS; ionic strength 0.15 M).

Subconjunctival disposition of particulate systems

Subconjunctival administration of the particles was performed as previously described (Kompella et al 2003; Ayalasomayajula & Kompella 2005). Briefly, Sprague-Dawley rats (SASCO, Wilmington, MA), 180–200 g, were anaesthetized with an intraperitoneal injection of sodium pentobarbital (40 mg kg^{-1}). The eyelids were gently stretched and 20–30 μL of a suspension of 20 nm, 200 nm or 2 μm fluorescent particles (equivalent to 400 μg of particles) were administered in the posterior subconjunctival region using a 27G needle. At various intervals (immediately after administration and at 1, 7 and 60 days post administration), the rats were euthanized with a single intraperitoneal administration

of sodium pentobarbital (250 mg kg^{-1}), and both the dosed (ipsilateral) and undosed (contralateral) eye were enucleated and frozen (−70 °C). The eyes were stored at −70 to −80 °C until analysis. The blood was collected immediately after sacrifice by cardiac puncture. Each rat received only one type of particle and independent groups of rats were used for different particle types and intervals. The experiments were carried out in adherence with the Association for Research in Vision and Ophthalmology (ARVO) statement for the use of animals in ophthalmic and vision research. All the protocols utilized in this study, were approved by the institutional animal care and use committee (IACUC) at the University of Nebraska Medical Center.

Effect of particle dose on retention at the site of administration

The 20 nm and 2 μm carboxylate-modified Fluospheres were administered subconjunctivally at 10 times lower dose (40 μg) in the same volume of injection as above and their retention at the site of administration was evaluated immediately and at 60 days post administration.

Effect of surface properties on retention of particles in the periocular tissue

Carboxylate-, sulfate- and amine-modified 200 nm Fluospheres were administered subconjunctivally and the retention of these particles was evaluated at the end of day 1. The carboxylate-modified Fluospheres, with a high density of pendant carboxyl groups on the surface, are negatively charged and relatively hydrophilic. Amine-modified microspheres are prepared by further modification of carboxylate-modified microspheres and these particles are positively charged. The sulfate-modified microspheres have sulfate surface groups and have a hydrophobic surface that can adsorb proteins (more information on the surface properties of these and other particles is available at http://www.probes.com/media/pis/mp05001.pdf). To evaluate the effect of surface properties on the retention of particulate systems at the site of administration, the three types of 200 nm particles were administered subconjunctivally and the retention of these particulate systems and ocular tissue levels were analysed at the end of day 1. In addition, the retention of smaller 20 nm particles was investigated using carboxylate-modified (negatively charged, hydrophilic) and aldehyde-sulfate-modified (neutral, hydrophobic) particles for a period of 1 day. Amine-modified 20 nm particles and sulfate- or aldehyde-sulfate-modified 2 μm particles were not available from a commercial source for comparative purposes.

Tissue isolation and the quantification of intact particles

The ocular tissues, including the sclera, retina, vitreous, lens and cornea, were isolated from the frozen eye. Briefly, the frozen eye was sliced along the corneo-scleral junction or limbus region to separate posterior and anterior segments. The vitreous was collected and the retina was peeled off from the sclera. The cornea and lens were isolated from the anterior segment. The sclera was used without scraping off the choroid. The tissues were homogenized in 1 mL of PBS and incubated for 3 h after adding 1 mL of 2% Triton-X solution. The fluorescence intensity was measured at the appropriate excitation and emission wavelengths. The concentrations were calculated using standards processed similarly.

The periocular tissues, including the conjunctiva remaining after the isolation of the above tissues, were analysed for particle levels at the site of administration. For analysis, the tissue was minced and homogenized and incubated for 3 h in 1 mL of 2% Triton-X. The incubation mixture was ultrasonicated at 1.5 watts for 3–5 min and the tissue and the supernatant were separated. The supernatant was diluted appropriately and the fluorescence was measured using a Shimadzu RF 5000 spectroflorometer at the appropriate excitation and emission wavelengths.

The method detection limit (MDL) and method quantification limit (MQL)

MDL and MQL of the particulate systems (Fluospheres) were determined using a previously reported method (Smith 1999; Corley 2003). After verifying that a linear relationship exists between concentration and instrument response, the instrument detection limit (IDL) and instrument quantification limit (IQL) were determined as follows using a standard curve of the particles in PBS + 1% Triton-X. The root mean square error (RMSE) was calculated from the difference in the concentration predicted using the regression line and the actual concentration of the particles used. The IDL was defined as $3 \times$ RMSE/slope and the IQL was defined as $10 \times$ RMSE/slope. The method detection limit (MDL) and method quantification limit (MQL) were then estimated by fortifying the ocular tissues with the particles at the IQL. Ocular and periocular tissues were removed from five eyes and the tissues ($n = 5$) were fortified with particles at IQL. The tissues were homogenized in 1 mL of PBS and the particles were extracted and quantified using the method described above. The method detection limit (MDL) and method quantification limit (MQL) were calculated from the standard deviation (SD) at the IQL by using the formulae $MDL = t_{99(n-1)} \times SD$ at IQL and $MQL = 3 \times MDL$, where $t_{99(n-1)}$ is the one tailed t statistic for $n - 1$ observations at the 99% confidence level.

The standard curve in the tissues obtained by spiking particles 0.2–1 μg mL^{-1} onto each of the ocular tissues, including the sclera-choroid, retina, vitreous, lens and the cornea, was compared with a standard curve in PBS and the correlation was determined.

Tissue histology

For histology at 60 days post administration of the Fluospheres, the eyes and the periocular tissue were isolated and immediately transferred to a 10% neutral buffered formalin solution. After 1 day the eyes or the

Table 1 The properties of microparticles and nanoparticles used in this study

Particle type	Mean particle size (nm) provided by supplier	Mean particle size (nm) measured using dynamic light scattering[a]	Zeta-potential[a] (mV)	Excitation λ/emission λ (nm)
Carboxylate modified	20	25.5 ± 2.1	n.d.	505/515
Aldehyde sulfate modified	20	37 ± 0.8	n.d.	505/515
Carboxylate modified	200	205.3 ± 4.1	-24.5 ± 7	580/605
Sulfate modified	200	238.2 ± 5.7	-41.14 ± 7.18	505/515
Amine modified	200	285.6 ± 5.8	5.8 ± 1.4	580/605
Carboxylate modified	2000	1510.4 ± 4.8	-56.9 ± 0.6	505/515

[a]The size and zeta-potential are expressed as mean ± s.d. for 5 measurements. n.d., not determined due to instrument limitation.

periocular tissue were dehydrated, embedded in paraffin and 10-μm-thick sections were cut. The sections were stained with hematoxylin and eosin and viewed with a Zeiss Axioscope 40 microscope. Images were taken with a Hitachi HV-D25 digital camera using the EPIX- XCAP-Lite software V2.2 for windows.

Data analysis

All in-vivo data are expressed as mean ± s.d. for n = 3 or 4. Particle retention comparisons for the three particle sizes (20 nm, 200 nm and 2 μm) and three different modifications (carboxylate, amine and sulfate) of 200 nm particles were performed by non-parametric Kruskal–Wallis analysis of variance. The comparison for the two types of 20 nm particles was done by the Mann–Whitney *U*-test. The results were considered statistically significant at $P < 0.05$. Statistical analysis was performed using SPSS 11.5 for windows (SPSS Inc, Chicago, IL).

Results

Particle size and zeta-potential of Fluospheres

The various particles used in this study and their properties are summarized in Table 1. The particle sizes obtained from the supplier were, in general, comparable with those obtained by dynamic light scattering. All the particles, except the amine-modified Fluospheres, had a negative zeta-potential. The positively charged amine groups in the amine-modified Fluospheres were responsible for the positive zeta-potential.

Limit of detection and quantification of Fluospheres in ocular tissues with the fluorescence spectrophotometry

The limits of detection and quantification were first evaluated in the extraction buffer (PBS with 1% Triton-X). The limits were then measured using various ocular tissue matrices (Table 2). The limits of detection and quantification for the particles were similar to those in the buffer for retina, vitreous and cornea. The limits were higher for the sclera and the lens probably due to some interference from the tissues.

Table 2 Detection and quantification limits of Fluospheres in various ocular and periocular tissues

Matrix	Method detection limit (ng mL^{-1})[a]			Method quantification limit (ng mL^{-1})[a]		
	20 nm	200 nm	2 μm	20 nm	200 nm	2 μm
Triton-X	32	45	31	109	135	93
Cornea	38	60	58	114	180	174
Sclera-choroid	64	100	97	192	300	291
Retina	35	52	54	105	156	162
Vitreous	30	50	46	90	150	138
Lens	127	204	190	381	612	570
Periocular tissue	38	46	59	114	138	177

[a]The units reflect the detection and quantification limits of the particles in the ocular tissues when ocular tissue from one eye is extracted for the particles using 1 mL of the extraction buffer (PBS + 1% Triton-X). That is, the numbers stated in the table in nanograms is the amount required in each tissue for detection/quantification.

Linearity of standard curves of particles in the ocular tissues

The standard curves of the particles (20, 200 and 2 μm) with various surface modifications were linear in all the ocular tissues, including the sclera-choroid, retina, vitreous, lens and the cornea. When compared with the standard curve in PBS, a correlation coefficient of > 0.99 was observed with all the above-mentioned tissues except the sclera-choroid. The correlation coefficient was > 0.98 for the sclera-choroid.

Subconjunctival disposition of particles

Ocular tissue distribution of particles following subconjunctival administration

There was negligible penetration of particles of any size (20, 200 or 200 nm) with the carboxylate-modified Fluospheres for a period of 60 days. With 20 nm nanoparticles, significant levels could be detected only in the

sclera-choroid (n = 4) with the amount being 25.5 ± 6.3 μg (g of tissue)$^{-1}$ one day post-administration. At all other time points (7 and 60 days post administration) for 20 nm particles and at all time points for the 200 nm and 2 μm particles, levels in all the ocular tissues were below detection limits. All the particles were undetectable in the plasma at the various sampling times. In addition, neither the nano- nor the microparticles could be detected in the contralateral eye at any interval.

Retention at the site of administration

Quantification of particles remaining at the site of administration provided interesting results. For the 2 μm, 200 nm and 20 nm particles, 77.2 ± 7.7, 79.3 ± 7.2 and 82.8 ± 2.8% of the administered dose, respectively, could be quantified at the site immediately after dosing. Microparticle (2 μm) and 200 nm particle levels at the site of administration did not differ at the various intervals up to 60 days (Figure 1). With the 20 nm nanoparticles, there was a rapid decline in the particle fraction retained at the site of administration, with the levels being about 15 ± 2.8% and 8.1 ± 3.5%, respectively, on days 1 and 7. These nanoparticles were not detectable at the site of administration on day 60.

Influence of dose on particle retention and ocular tissue distribution

At the lower dose, the nanoparticles (20 nm) could not be detected in the periocular tissues at the end of 60 days (Figure 2). Microparticle (2 μm) levels at the end of 60 days were not statistically different from the levels at time zero ($P > 0.05$). Neither nano- nor microparticles could be detected in any of the intraocular tissues at 60 days post-administration of the lower dose. There was no significant difference in particle retention with the two doses tested at either of the time points.

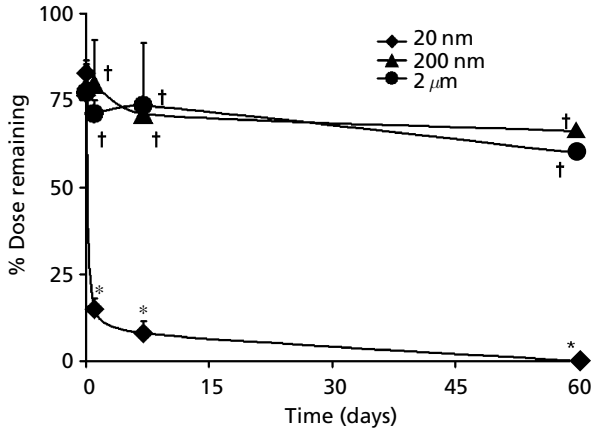

Figure 1 Retention of microparticles and nanoparticles at the site of administration following subconjunctival injection in rats. Following administration of a 400-μg dose of 2 μm, 200 nm and 20 nm particles, the percentage dose remaining at the site of administration was determined up to 2 months post dosing. The data are expressed as mean ± s.d., n = 4. †$P < 0.05$, compared with 20 nm nanoparticles; *$P < 0.05$, compared with the particle fraction remaining at time 0.

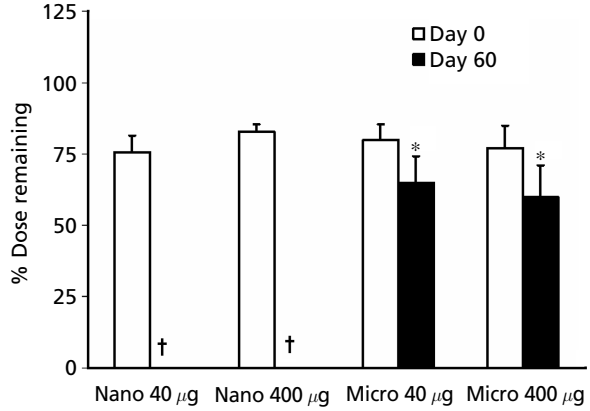

Figure 2 Effect of dose on the retention of particles after subconjunctival administration in rats. Following administration of 40 or 400 μg doses of 2 μm (Micro) and 20 nm (Nano) Fluospheres, the percent dose remaining at the site of administration was determined immediately and at 60 days post-dosing. The data are expressed as mean ± s.d., n = 4. *$P < 0.05$, compared with nanoparticles; †$P < 0.05$, compared with the particle fraction remaining at time 0.

Influence of surface properties on the retention and ocular tissue distribution of 200 nm and 20 nm particles

With all three systems, the retention of the 200 nm particles at the site of administration was similar on day 0 and day 1 (Table 3A). Also, none of these particles were detectable in the sclera-choroid, retina, vitreous, lens and cornea. The retention of these particles at the site of administration did not significantly differ from that of the 2 μm particles. The retention of the 20 nm particles with aldehyde sulfate modification (hydrophobic) was significantly greater than the retention of the 20 nm carboxylate-modified particles (hydrophilic) (Table 3B). However the measured size was 26 nm and 37 nm for the carboxylate- and aldehyde-sulfate modified particles, respectively (Table 1).

Tissue histology

No adverse effects or signs of inflammation were observed in any of the intraocular tissues 60 days after the administration of the Fluospheres. There was no atrophy in the retina or any other ocular tissue. There were a few inflammatory cells seen in the periocular tissue with the 200 nm and 2 μm particles but not with the 20 nm Fluospheres at 60 days post administration. No such changes were seen in the contralateral periocular tissue in any of the groups (Figure 3).

Discussion

There is ample evidence in the literature to support the hypothesis that therapeutic concentrations of drug can be achieved in the retina following periocular administration (Geroski & Edelhauser 2001). In our previous investigations, we have observed that the retinal availability of celecoxib is several-fold higher following subconjunctival

Table 3A Influence of particle surface modification on the retention of 200 nm nanoparticles at the site of administration[a]

Particle type	Day 0	Day 1
	% Remaining	% Remaining
Carboxylate modified	79.98 ± 34.28	70.45 ± 10.64
Sulfate modified	79.27 ± 7.22	79.87 ± 12.57
Amine modified	67.77 ± 6.15	68.89 ± 16.83

Table 3B Influence of particle surface modifications on the retention of 20 nm nanoparticles at the site of administration[a]

Particle type	Day 0	Day 1
	% Remaining	% Remaining
Carboxylate modified	82.82 ± 2.70	15.03 ± 2.89
Aldehyde sulfate modified	78.48 ± 14.28	37.18 ± 17.05†

[a]Following subconjunctival administration of a 400-μg dose of 20 or 200 nm Fluospheres with various surface modifications, the percentage dose remaining at the site of administration was determined immediately and at 1 day post dosing. The data are expressed as mean ± s.d., n = 4. †$P = 0.034$, compared with 20 nm carboxylate-modified particles at day 1.

administration than after systemic administration (Ayalasomayajula & Kompella 2004). We have also demonstrated that low-molecular-weight (< 500) drugs can be transported across the sclera and delivered to the intraocular tissues (Cheruvu et al 2003a, b). Even macromolecules have been shown to reach the retina following subconjunctival administration (Ambati et al 2000a). However, it is not known whether particles given by the subconjunctival route could gain access to the retina.

Effective control over the delivery of drugs in-vivo using drug delivery systems depends not only on the design of the system but also on its disposition in-vivo. Thus, understanding of the in-vivo disposition of delivery systems, such as microparticles and nanoparticles, is essential. Information on the disposition of particulate systems given by the intravenous and the intravitreal route is available (Sjoholm & Edman 1979; Edman & Sjoholm 1983; Laakso et al 1986; Martini 1992). However, there are no reports describing the disposition of such systems following periocular administration. To investigate the influence of particle size on their disposition in the absence of confounding polymer degradation factors, we have assessed non-degradable particles of varying sizes in this study.

The fate of subconjunctivally administered particles can include, firstly, entry into the intraocular tissues via the sclera, secondly, entry into the systemic circulation through the local intraocular circulation or following uptake by the conjunctival or episcleral blood vessels and, thirdly, retention at the site of administration until degradation or clearance by the lymphatic system. The fate will likely depend on the particle size, polymer and surface properties and degradation rates. Intravitreally administered nanoparticles have been shown to diffuse in the vitreous, with some degree of penetration into the retina and preferential localization in the retinal pigment epithelium (Bourges et al 2003). However, the authors utilized biodegradable nanoparticles entrapping

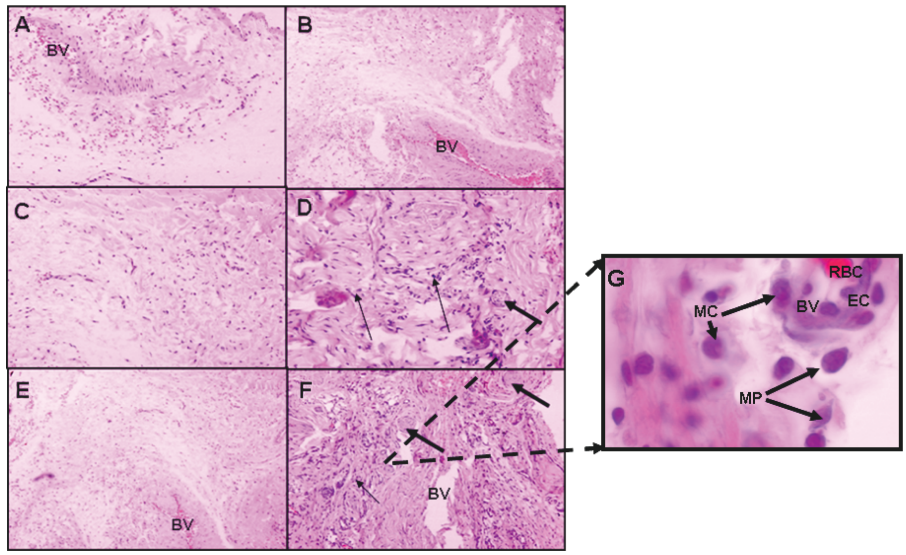

Figure 3 Representative histopathologic sections of the periocular tissue of rats 60 days post administration of the Fluospheres, showing contralateral and ipsilateral periocular tissues for 20 nm Fluospheres (A and B, respectively), contralateral and ipsilateral periocular tissues for 200 nm Fluospheres (C and D, respectively) and contralateral and ipsilateral periocular tissues for 2 μm Fluospheres (E and F, respectively). Larger arrows indicate nerve endings and smaller arrows indicate inflammatory cells. BV, blood vessel. Total magnifications 100×. G. Higher magnification (1000×) micrograph of F, showing presence of inflammatory cells. EC, endothelial cell; BV, blood vessel; RBC, red blood cell; MP, macrophage; MC, monocyte.

a fluorescent dye and a clear conclusion as to whether the released dye or the particles migrated could not be made. To overcome such limitations and to understand the role of particle size and surface properties on retention in subconjunctival tissue, non-biodegradable fluorescent polystyrene particles were chosen for this study. The fluorescent label does not leach from these particles.

We did not observe significant levels of nano- or microparticles in the retina, vitreous, lens or cornea. Only with 20 nm nanoparticles, significant quantifiable levels of particles could be detected in the sclera-choroid. The sclera is a collagenous tissue with very few scattered cells. It can be considered as a porous membrane containing water-filled channels. The principle mechanism of penetration into the sclera could thus be passive diffusion. Active transport is unlikely due to the absence of cellular barriers in the tissue. Thus, if delivering the entire particle to the retina is the goal, the subconjunctival route of administration is not a useful approach and other approaches, such as systemic, sub-retinal or intravitreal injection should be investigated.

With insignificant entry into the eye, particles must either be retained at the site of administration or be cleared by other means, including entry into the systemic and lymphatic circulations. Since 200 nm and 2 μm particles could be almost completely accounted for their dose at the site of administration for up to two months, these particles did not undergo significant clearance by the above means. On the other hand, 20 nm particles were cleared rapidly from the site of administration. This clearance might be due to removal by the conjunctival, episcleral or other periocular circulatory systems and entry into the circulation. The conjunctiva, in contrast to the sclera, is a cellular tissue. The mechanism of entry of the particles into the conjunctiva probably involves active transport processes. In-vitro studies with conjunctival cells have demonstrated that uptake of poly lactide-co-glycolide (PLGA) nanoparticles (size range 100–10 000 nm) reduces with increasing size and occurs mostly by adsorptive endocytosis (Qaddoumi et al 2004). Conjunctival uptake was also reduced by lowering the temperature to 4°C from 37°C, and by various cellular energy depletors, suggesting the involvement of an energy-dependent process in the uptake of these particles (Qaddoumi et al 2004). Such a mechanism of clearance via the conjunctiva cannot be ruled out for 20 nm particles in our study. Another possible mechanism for rapid escape of the 20 nm particles could be rapid bulk flow escape through capillary fenestrations in the episcleral, conjunctival or periocular blood vessels. Capillary fenestrations are gaps in the capillaries, which range in size from 30 to 100 nm depending on the tissue (Williams et al 1989). The smallest nanoparticles tested in this study are more likely to enter blood vessels. Once in the circulation, the particles exhibit short half-lives (Simon et al 1995) due to clearance by the organs of the reticuloendothelial system (Moghimi et al 1991). Another possible explanation for the loss of 20 nm particles is the more rapid leakage of these particles along the needle track into the precorneal area. At the intervals tested, particles of all sizes were undetectable in plasma following subconjunctival administration, indicating either poor absorption of these particles into the systemic circulation or their rapid distribution and clearance from the systemic circulation. This may also be because the particle levels in plasma were below detection limits.

The retention of particles in the periocular tissue was not dose dependent, indicating the absence of any saturable processes in the dose range tested. Also, particle surface properties did not influence the retention of 200 nm particles, possibly because these particles were above the cut-off size for entry into the circulation and intraocular tissues across the sclera. With the 20 nm particles, the mean particle retention was 86% greater with the hydrophobic particles (aldehyde sulfate modified) as compared with the hydrophilic ones (carboxylate modified), suggesting greater retention with hydrophobic particulate systems. However, the mean particle size determined by dynamic light scattering was higher for the aldehyde-sulfate-modified particles as compared with the carboxylate-modified particles. This measured size difference could partially contribute to the greater retention of the aldehyde-sulfate-modified particles.

Another important finding of this study is that particles of diameters 200 nm and above are almost completely retained in the periocular tissue for at least 60 days. This observation suggests the potential use of particulate systems of 200 nm and above for sustained drug release in periocular spaces for transscleral drug delivery to the retina. If biodegradable microparticles are injected subconjunctivally, they will likely be retained in the periocular spaces until they reach a much smaller dimension that is suitable for clearance. We have previously demonstrated the ability of subconjunctival drug containing microparticles in sustaining retinal drug delivery and alleviating biochemical changes associated with diabetic retinopathy (Kompella et al 2003; Ayalasomayajula & Kompella 2005). The previously observed inability of subconjunctivally administered biodegradable budesonide-poly(lactide) nanoparticles (345 nm) to sustain retinal budesonide delivery for durations as long as budesonide–PLA microparticles (3.6 μm) might not be due to the differences in retention of these systems, but most likely due to the differences in drug release behaviour of the nano- and microparticles. The nanoparticles exhibited a higher burst followed by much lower release rates compared with the microparticles (Kompella et al 2003).

We observed inflammatory cells in histological sections taken 60 days post administration with the 200 nm and 2 μm particles but not with the 20 nm particles. This could be because the 200 nm and 2 μm particles were retained for 60 days, whereas the 20 nm particles were cleared much earlier. The inflammatory cells might participate in the degradation (in case of biodegradable polymeric particles) and clearance (for both biodegradable and non-biodegradable polymeric particles) of the particles. The role of inflammatory cells in the clearance of the particles has been demonstrated in several pulmonary studies (Zhang et al 2000). Also, the presence of inflammatory cells in periocular tissues has been demonstrated 10 days post administration of poly-lactic-acid (PLA) microparticles containing a protein kinase C (PKC) inhibitor (Saishin et al 2003). The major pathways for the clearance of 20 nm particles might

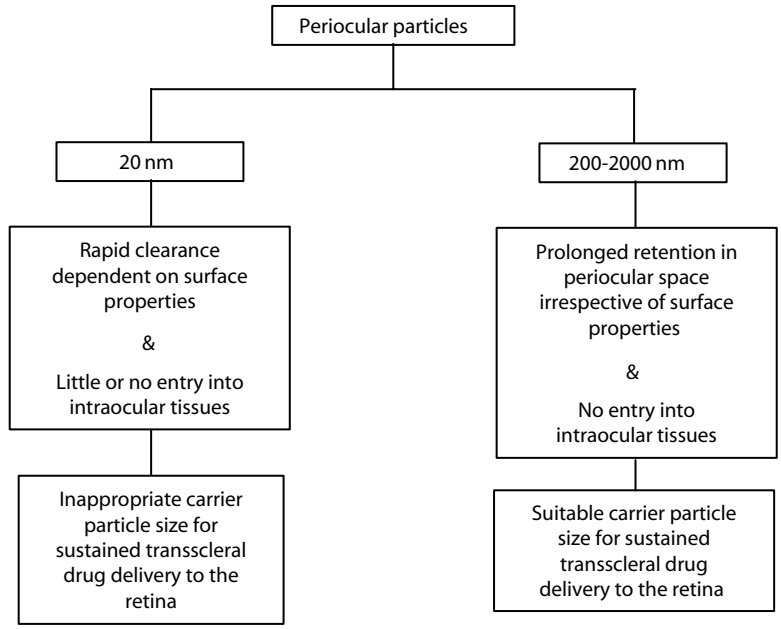

Figure 4 Disposition of periocularly administered nano- and micro-particles and their suitability for sustained transscleral drug delivery to the retina.

be entry into the systemic or lymphatic circulation, whereas with the larger particles (200 nm and above), the clearance would take place mainly by retention and degradation at the site of administration (if the particles are biodegradable) until they reach a size sufficiently small enough for entry into the systemic or lymphatic circulation.

Conclusions

In summary (Figure 4), particulate systems at and above 200 nm are retained in the periocular tissue for at least 2 months. The retention of 200 nm particles is not affected by surface charge or hydrophobicity, probably because this size is above the cut-off size for clearance from periocular tissue. Thus, particulate systems of size above 200 nm formulated using biodegradable polymers encapsulating drugs will likely be retained in periocular tissue for prolonged periods and can be used for sustained retinal drug delivery via the transscleral pathway. Small nanoparticles (20 nm) rapidly disappear from the periocular tissue but do not gain access to the retina. For these smaller particles, the surface properties are more critical in determining their disposition. The periocular route of administration does not allow significant delivery of intact particles to the retina. If delivery of intact particulate systems to the retina is the goal, other routes of administration, such as intravenous, intravitreal and sub-retinal, should be investigated.

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