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Pseudoprotein-based nanoparticles show promise as carriers for ophthalmic drug delivery

Temur Kantaria[a], Tengiz Kantaria[a], Sophio Kobauri[a], Wenliang Zhang[b], Nicole Eter[b], Peter Heiduschka[c], Archil Kezeli[c], George Chichua[c], David Tugushi[a], Ramaz Katsarava*[a].

[a]Agricultural University of Georgia, Institute of Chemistry and Molecular Engineering, 240, David Aghmashenebeli Ave., Tbilisi, 0159, Georgia,

[b]University of Münster Medical School, Research laboratory of the Department of Ophthalmology, 15, Domagkstrasse, Münster, , D-48149 , Germany,

[c]Ivane Beritashvili Center of Experimental Biomedicine, Laboratory of Physiology of Vision, 14, Gotua Str., Tbilisi, 0160,Georgia.

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ABSTRACT

Drug delivery used to treat ocular disease still poses a challenge to modern ophthalmology. Well-established intravitreal injections imply discomfort to the patients and risk of ocular complications. Therefore, opportunities to deliver drugs by topical administration are investigated thoroughly. Despite its seemingly easy accessibility, the eye is well protected by efficient mechanisms that rapidly remove drugs after instillation on the eye surface. Hence, eye drops are less effective for the treatment of various diseases, which necessitates a risk-containing procedure of intravitreal injection. One of the rational ways to overcome the problem is the application of drug-loaded polymeric nanoparticles (NPs) that are able to penetrate through ocular barriers when administered topically. Pseudo-proteins (PPs) - amino acid-based biodegradable polymers are one of the most suitable materials for the design of drug delivering NPs. One of the most important features of such kind of nanovehicles is “disappearance” from the body after their function is fulfilled. We have prepared biodegradable NPs of various types by nanoprecipitation of the PEA-class of PP composed of L-leucine, 1,6-hexanediol and sebacic acid (8L6). The originally designed arginine-based cationic PEA and comb-like PEA containing lateral PEG-2000 chains along with 8L6 anchoring fragments in the backbones were used to construct positively charged and PEGylated NPs. The NPs were loaded with fluorescein diacetate (FDA) as a fluorescent probe to detect if the NP penetrated through the ocular barriers. A preliminary *in vivo* study on intraocular infiltration of the NPs has been done using wild-type C57BL/6 mice. After penetrating into the cellular lysosomes, FDA probes became visible due to the hydrolysis of the diacetate groups, thus allowing for the detection of the NPs as tiny fluorescent spots inside the tissues. One day after administration, fluorescent dots were found at various sites - always in the peripheral cornea and the sclera, and in different layers of the outer retina depending on the type of NPs used. Four days after administration, fluorescent dots were still visible in the peripheral cornea and the sclera with some of the NPs. These results show that the new type of NPs infiltrate the ocular tissues after topical administration and are taken up by the cells. This raises hope that the NPs may be useful carriers for ocular delivery of therapeutic agents.

Keywords: Biodegradable polymers, Pseudo-proteins, Nanoparticles, Biodegradable surfactant, PEGylation, Ocular penetration.

*Corresponding author: Ramaz Katsarava; E-mail address: r.katsarava@agruni.edu.ge

1. Introduction

Most of the diseases affecting the posterior segment of the eye are related with visual impairment and blindness. The effective treatment of these pathologies is one of the major challenges in drug delivery as most of them are chronic and multifactorial. Among them, aged related macular degeneration, diabetic retinopathies and glaucoma produce irreversible visual damage and blindness [1]. These

diseases are becoming more and more prevalent in the aging populations, and nowadays tens of millions of patients are affected worldwide. Depending on the disease, the medications should be delivered to the retinal cells, retinal pigment epithelium or choroid. Furthermore, therapeutic concentrations of the active substance in the intraocular target site have to be maintained during a long period of time.

Due to the ocular barriers, it is difficult to deliver effective drug concentrations to the posterior tissues of the eye using non-invasive routes such as topical or systemic administration [2]. It is well known that after topical administration only very low drug concentrations are reached in the retina and choroid [3]. This is due to the obstacles of drug penetration that include the short residence time of formulations on the ocular surface, the presence of tissue barriers (cornea, lens, conjunctiva, sclera), and flow mediated drug loss factors (conjunctival blood flow, aqueous humor flow) that limit the drug access to the retina and choroid. Although systemic administration is used to deliver some drugs to the eye (e.g., corticosteroids), this route is restricted by the systemic toxicity of the drugs and reduced access to the target site, mainly due to the blood-aqueous and blood-retinal barriers [2]. The most effective method of drug delivery to the back of the eye is through intraocular administrations, mainly intravitreal injections. However, intravitreal administration is an invasive mode of drug delivery and it is sometimes associated with adverse effects (endophthalmitis, hemorrhages, ocular hypertension, damage of lens or retinal detachment) and it requires frequent visits of the patients to clinics. For this reason, ophthalmic drug delivery is one of the most challenging endeavour facing the ocular pharmacologists. A major challenge is to get over the ocular barriers and reach the tissue target. Controlled drug delivery systems able to release and maintain effective active substance levels over long periods of time, would prolong the dosing interval to months [4,5]. Therefore, the intraocular administration of drugs using sustained/controlled drug delivering (SCD) beads looks more promising.

Microspheres (MSs) are emerging therapeutic tools for SCD as they can be administered as a conventional injection by periocular and intraocular routes. MSs made of biodegradable polymers (BPs) are by far more promising since they can be cleared from the site of administration over time. Ophthalmic drug delivery systems can be made with a variety of biodegradable materials such as polyesters (lactide and glycolide copolymers (PLGA), polycaprolactones, poly(β-hydroxybutyrates)), polyamides (including natural polymers such as collagen, gelatine and albumin), heteropolysaccharides (chitosan). Synthetic polymers have an advantage over the naturally occurring once since they reveal from low to zero immunogenicity.

The obvious advantages of biodegradable implants over the non-degradable devices in the clinical practice have promoted the interest in novel BPs adequate for intraocular drug delivery purposes. However, biodegradable injectable implants mostly made of poly-α-hydoxy acids - lactide/glycolide polyesters (PEs) and co-PEs, degrade and release drugs in non-zero order kinetics, which is inefficient for long-term sustained drug release [6,7]. The degradation rate of the aliphatic PEs cannot be tuned easily as most of these polymers are synthesized from single monomers. Attempts to tune the degradation rate often involves copolymerization or blending with other polymers that are not always successful [8]. Besides, the PEs release acidic products upon biodegradation that are considered to be toxic to some cells causing undesired phenotype modulations that limits their biomedical applications [9-13]. In addition, the acidic products transiently decrease vitreous humour to pH 7 and increases the risk of inflammation before being metabolized. It should also be noted that lactide/glycolide polymers show low compatibility with acid sensitive bio-pharmaceuticals [6].

More promising for sophisticated biomedical applications look BPs made of naturally occurring α-amino acids, fatty diols and dicarboxylic acids, so called pseudo-proteins (PPs) [14-16]. These relatively new family of BPs in most cases release low acidic products, and some of them show self-buffering property thus preventing significant pH drop during the degradation process; in other words, no local acidic environment causing inflammation is built up upon the biodegradation of the PPs. The PP of co-poly(ester amide) (**co-PEA**) class, originally developed by Katsarava et al. [17,18], was successfully used recently by the team of Royal DSM for developing micronized particles for intraocular delivery of dexamethasone (DEX) - microfibriles [6] and microsphere [7]. The co-PEA based micronized devices showed excellent biocompatibility when used for intraocular purposes [6,7]. The use of PP-based micronized particles is a progressive method for the delivery drugs into the ocular tissues, however, also needs, though less often, painful and undesirable intraocular injection.

To overcome the problems, nanotechnology involving drug-loaded polymeric nanoparticles (NPs), has been proposed as ophthalmic drug delivery systems that may control drug release and maintain therapeutic levels over a prolonged period of time. The use of NPs, designed in due manner, can provide high corneal penetration of drugs and exclude the need of painful injections. In addition, NPs were shown to adhere preferentially to inflamed precorneal tissues of the eye. This seems to be an effective targeting of drugs to inflamed parts of the eye and might be a promising application for nanoformulations [19].

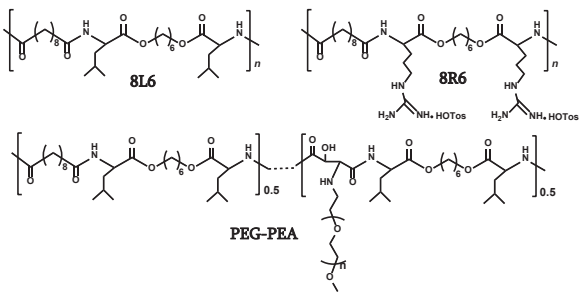
Various NPs-based ophthalmic drug delivery systems have been offered: liposomes, dendimers, β-cyclodextrine, various emulsions, and nanospheres [20]. Liposomes and emulsions liposomes are less popular because of their short shelf life, limited drug capacity, use of aggressive conditions for preparation and problems in sterilization [21]. More promising for ophthalmic drug delivery are NPs [20]. Various non-degradable and degradable polymers of both natural and synthetic origin were used in ophthalmic drug nanoformulations [22-24]. Synthetic BPs are more suitable for drug delivery purposes since they can be cleared from the body after their function is fulfilled. The most usable to date are PEs such as lactide/glycolide polymers/copolymers. The serious drawbacks of the PEs limiting their biomedical applications were discussed above (see Refs. [6-13]).

Pseudo-proteins (PPs) - a new family of amino acid based synthetic analogous of proteins, merits of which were discussed in brief above, look more promising for constructing the drug delivery NPs. Three basic classes of the PPs obtained are – poly(ester amide)s (PEAs), poly(ester urethane)s, and poly(ester urea)s [14-16].

For constructing nanosized drug delivery vehicles we have selected PP-PEAs. Namely, we used the *homo*-PEA labelled as 8L6 (Scheme 1), composed of sebacic acid (8), L-leucine (L) and 1,6-hexanediol (6), 8L6. The selected PEA 8L6 represents a fragment of the highly biocompatible co-PEA of complex structure used by Royal-DSM (DSM-PEA) as a drug delivery platform [6,7]. The DSM-PEA labelled as $[8L6]_{0.30}$-$[8LDAS]_{0.45}$-$[8K(Bn)]_{0.25}$, is made of amino acid based three monomers: bis-(L-leucine)-1,6-hexanediol diester, L6 (30 mol%), bis-(L-leucine)-1,4-dianhydrosorbitol diester, LDAS (45 mol%), and lysine benzyl-ester, KBn (25 mol%) originally designed previously [17,18] and obtained *via* solution active polycondensation (SAP). The selected PP-PEA 8L6 is by far more simple than DSM-PEA (that means its biodegradation products are more definite and predictable) and was obtained *via* very fast (lasts 15-20 min) interfacial polycondensation (IP), in contrast to SAP which lasts 16 h. The polymer was synthesized using one amino acid based monomer - L6, by its IP with sebacoyl chloride [25], which is a cheap and purchasable product. Hence, the PEA 8L6 is by far simple and cost-effective as compared with the DSM-PEA.

Previous systematic study of four PPs of various structures in terms of particles size, stability and cell compatibility showed the PP-PEA 8L6 was the best [26]. Along with 8L6 in the present study we used also cationic PP-PEA 8R6 [27] (Scheme 1) for imparting positive charge to the NPs by blending with neutral 8L6, and an originally designed PP-surfactant – PEG-attached co-PEA (PEG-PEA) [28] which represents at the same time NPs' PEGylating agent.

Positive surface charge (positive zeta-potential) is favourable for penetration of NPs through biological (ophthalmic) barriers such as cornea, lens, etc. It is known that a positive charge helps with the NPs adhesion to the surface of cells and stimulates penetration into the cells *via* endocytosis [29,30]. Surface PEGylation decreases the affinity plasma proteins (opsonins) for adsorption on NPs and in that way suppresses phagocytosis [31,32]. Along with the protection of the NPs from phagocytosis the PEGylation increases ocular drug bioavailability of NPs [33].



Scheme 1. *PP-PEAs used for fabricating NPs.*

In the present study we have prepared NPs of various types loaded with fluorescent probe - fluorescein diacetate (FDA) which is non-fluorescent and become fluorescent after penetrating into cells and enzymatic hydrolysis of ester groups [34]. A preliminary study of permeability of the obtained NPs through the ocular barriers was done using wild-type C57BL/6 mice.

2. Materials and Methods

2.1. *Materials*

Surfactant Tween 20, Sorbitanmonolaurate (MW 1,228), was purchased from Sigma-Aldrich (St. Louis, MO, USA). Methoxy-PEG-amine with average molecular weight 2,000 Da (mPEG-amine-2000) was purchased from Laysan Bio. Fluorescent probe – fluorescein diacetate was purchased from Santa Cruz Biotechnology. Organic solvent - Dimethyl sulfoxide (DMSO) was purchased from Carl Roth (Karlsruhe, Germany). All the chemicals were used as received. The dialysis bag (MWCO 25 kDa) was purchased from Spectrum Laboratories, Inc., Rancho Dominguez, CA, USA. The PP-PEAs, selected for the proposed study, were originally synthesized as reported previously: the leucine (**L**) based PP-PEA 8L6 *via* the Interfacial Polycondensation (IP) [25,35], and the arginine (**R**) based biodegradable cationic PP-PEA 8R6 - *via* Solution Active Polycondensation (SAP) [27]. The new functional PP - surfactant/PEGylating agent composed of amino acid **L** and containing lateral PEG chains, PEG-PEA, was synthesized by interaction of epoxy-co-PEA $[8L6]_{0.5}$-$[t$ES-L6$]_{0.5}$ with mPEG-amine-2000 as reported previously [28].

For the *in vivo* experiments, we used adult wild-type C57BL/6J mice. They were held in ventilated cages in our own animal facility at 12 hours/12 hours light/dark cycle, with standard food and drinking water *ad libitum*. The experiments were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and the EU directive 2010/63/EU and were approved by the local authorities (LANUV, Recklinghausen, Germany, file number 84-02.04.2018.A175).

2.2. *Characterization of polymers*

The number-average (M_n), and weight-average (M_w) molecular weights (MWs), and dispersity ($Đ$) of the polymers were determined using the GPC. The MWs of the PPs - 8L6 and $[8L6]_{0.5}$-$[t$ES-L6$]_{0.5}$ were determined on a machine of Waters Associates, Inc., Milford, MA, USA, equipped with Styragel columns in DMF: HR4, HR3, HR0.5 (all 7.8 mm × 300 mm), a high-pressure liquid chromatography pump (Waters 1525 Binary HPLC) and a Waters refractive index detector 2414 and UV-detector (Waters 2487 dual absorbance detector, λ = 240 nm). A solution of LiBr (0.1 M) in DMF was used as an eluent. Injected volume was 100 μL, the sample concentration 5.0 mg/mL, flow rate 1.0 mL/min and temperature 35 °C. The columns were calibrated with PMMA standards. The MW of the cationic polymer 8R6 was determined on a Shimadzu GPC machine, model LC-8A equipped with an Empower computer program (Waters), a PL HFIP gel column (Polymer Lab, Theale, Berkshire, UK) and a refractive index detector (Shimadzu RID-10A, Shimadzu Scientific Instruments, Columbia, MD, USA). The polymer 8R6 was dissolved in and eluted with HFIP containing CF_3COONa (0.05 M, to suppress polyelectrolyte effects). The injected volume was 100 μL, the sample concentration 2.0 mg/mL, and the flow rate 0.5 mL/min. The columns were calibrated with PMMA standards.

2.3. Preparation of various types of fluorescently-tagged NPs

Different types of fluorescently-tagged NPs were prepared according to the polymer deposition/solvent displacement (nanoprecipitation) method under the optimal conditions previously established for PPs - amino acid based biodegradable ester polymers [26].

Non PEGylated NPs (labelled as TK-1 and TK-5 NPs) were prepared as follows: 6.0 mg of PEA 8L6 (in case of TK-1 NPs) or the 70/30 mixture of PEAs 8L6/8R6 (i.e. 4.2 mg of PEA 8L6 and 1.8 mg of cationic PEA 8R6) (in case of TK-5 NPs) was dissolved in 1.0 mL of DMSO (organic phase) along with the 6.0 μg of fluorescein diacetate (i.e. 0.1 w% from the polymer weight) and dropwise added (dropping rate 12 drops/min) to 10.0 mL of water (inorganic phase) containing 50.0 mg of the surfactant Tween 20 (organic/water phases ratio 1:10 v/v) at a stirring rate of 700 rpm using a magnetic stirrer. All manipulations were done at room temperature.

The surface PEGylated NPs (samples TK-2 and TK-6 NPs) were prepared as follows: 6.0 mg of PEA 8L6 (in case of TK-2 NPs) or the 70/30 mixture of PEAs 8L6/8R6 (in case of TK-6 NPs) was dissolved in 1.0 mL of DMSO (organic phase) along with the 6.0 μg of fluorescein diacetate (i.e. 0.1 w% from the polymer weight) and dropwise added (dropping rate 12 drops/min) to 10.0 mL of water (inorganic phase) containing 25.0 mg of the surfactant Tween 20 and 25.0 mg of biodegradable surfactant PEG-PEA (organic/water phases ratio 1:10 v/v) at a stirring rate of 700 rpm using a magnetic stirrer.

Other two types of surface PEGylated NPs (sam-

ples TK-3 and TK-7 NPs) were prepared without using the surfactant Tween 20. Manipulations were done as follows: 6.0 mg of PEA 8L6 (in case of TK-3 NPs) or the 70/30 mixture of PEAs 8L6/8R6 (in case of TK-7 NPs) was dissolved in 1.0 mL of DMSO (organic phase) along with the 6.0 μg of fluorescein diacetate (i.e. 0.1 w% from the polymer weight) and dropwise added (dropping rate 12 drops/min) to 10.0 mL of water (inorganic phase) containing 50.0 mg of the biodegradable surfactant PEG-PEA (organic/water phases ratio 1:10 v/v) at a stirring rate of 700 rpm using a magnetic stirrer.

The surface and bulk PEGylated NPs (samples TK-4 and TK-8 NPs) were prepared using so called modified nanoprecipitation method [26]. Manipulations were done as follows: 6.0 mg of PEA 8L6 (in case of TK-4 NPs) or the 70/30 mixture of PEAs 8L6/8R6 (in case of TK-8 NPs) was dissolved in 1.0 mL of DMSO (organic phase) along with the 6.0 μg of fluorescein diacetate (i.e. 0.1 w% from the polymer weight). A half portion of the biodegradable surfactant PEG-PEA (25.0 mg) was also dissolved in the organic phase. Afterwards, the prepared organic phase was added dropwise (dropping rate 12 drops/min) to 10.0 mL of water (inorganic phase) containing another half of the surfactant PEG-PEA (25.0 mg) at a stirring rate of 700 rpm using a magnetic stirrer. Note, that in case of TK-4 and TK-8 NPs the surfactant PEG-PEA was equally distributed between organic and water phases.

In all cases, after adding the organic phase, the aqueous phase became turbid indicating formation of NPs. The suspensions of the NPs, obtained after the complete addition of the organic phase, were stirred for 10-15 min and then dialyzed against distilled water for 2 h using the dialysis bag with MWCO 25 kDa to remove the organic solvent and residual surfactant. After dialysis the volume of suspension was reduced to 10.0 mL by evaporating water on a rotary evaporator under reduced pressure. The obtained nanosuspensions were stored in a refrigerator at 4-5°C.

2.4. NPs' size, size distribution and zeta-potential

The obtained PEGylated NPs were characterized by size (Average Diameter - AD), size distribution (Polydispersity Index - PDI), and zeta-potential (ZP), which were determined by dynamic light scattering (DLS) using a particle size analyzer (Zetasizer Nano ZS, Malvern Instruments, Malvern, UK) at 25 °C. The AD and PDI are presented as an average of five measurements ± standard deviation (SD). The PDI < 0.04 corresponds to a narrow distribution, 0.04 ≤ PDI ≤ 0.16 − to a mean distribution, and PDI > 0.16 − to a wide distribution.

2.5. Application of NPs onto the murine eyes and analysis of results

3. 10 m Results and discussion

3.1. *Selection of the PP-PEAs*

The PP-PEA 8L6 composed of L-leucine (**L**), 1,6-hexanediol (**6**) and sebacic acid (**8**) was selected as a basic polymer for preparing the various types of NPs (non PEGylated and PEGylated). We have found this PP-PEA as an optimal for fabricating resorbable NPs in terms of storage and cell compatibility [26]. For imparting a positive charge to the NPs that enhances both their stability and cellular uptake [29,30], arginine-based cationic PP-PEA 8R6 was used. Among recently designed arginine-based PP-PEAs [27] 8R6 showed desirable hydrophobicity - it dissolved in water only upon heating to 60–70 °C and precipitated when cooled to r.t. We assumed it would be retained by the NPs, i.e. would not easily be washed out from the NPs in the water phase. Note, the new biodegradable PP-surfactant PEG-PEA that at the same time represents the PEGylating agent was selected for preparing both the surface and bulk PEGylated NPs. The selected PEG-PEA contains backbone fragments similar to the backbones of 8L6 and 8R6 that provides a high affinity between these polymers, that in turn, should provide a firm anchoring of the PEG-PEA with NPs made of the 8L6 or 8L6/8R6 blend. The structures of the selected PEAs are depicted in Scheme 1, their MWs determined earlier and reported in our previous work [28], are given in Table 1.

Table 1. *MW characteristics of the PEAs*

Polymer	M_w	M_n	$Đ$
8L6	76,100	44,200	1.72
8R6	17,500	7,200	2.43
PEG-PEA	36,800	28,400	2.58

3.2. Fabrication of various types of fluorescently-tagged NPs

As noted above in the section 2 the different types of fluorescently-tagged NPs have been prepared us-

ing the nanoprecipitation method. All eight types of the obtained NPs labelled as TK-1 NPs, TK-2 NPs, etc. (see Table 2) can be divided into two groups – negatively charged NPs (from TK-1 to TK-4 NPs) and positively charged NPs (from TK-5 to TK-8 NPs). The negatively charged NPs were prepared on the basis of PP-PEA 8L6 whereas the positively charged NPs were fabricated on the basis of mixture of PP-PEAs 8L6/8R6 (70/30 w%). We suppose that the negative charge (i.e. negative Zeta-Potential, ZP) of the NPs is caused by a partial hydrolysis of the ester links of the PEAs generating free carboxyl groups (carboxylate anions $–COO^-$). As regards the positive charge of the NPs, it is provided by guanidine groups of the cationic PP-PEA 8R6.

The results given in Table 2 show that the size (Average Diameter, AD) of the obtained NPs varies within 68.5 – 130.2 nm. AD of the negatively charged NPs (samples from TK-1 to TK-4 NPs) is smaller than the AD of the positively charged NPs (samples from TK-5 to TK-8 NPs) - the AD of negatively charged NPs varies from 68.5 to 97.6 nm whilst the AD of positively charged NPs varies from 115.2 to 130.2 nm. With regard to the particle size distribution as we can see from Table 2, obtained fluorescently-tagged NPs showed mean ($0.04 \leq PDI \leq 0.16$) to wide size distribution ($PDI > 0.16$).

3.3. Stability of the NPs

All types of the obtained fluorescently tagged NPs were studied for stability upon storage at low temperature. The NPs' AD and PDI were measured right after the fabrication and then the NPs' suspensions were stored refrigerated at 4-5 °C. After predetermined time (30, 60, and 90 days), the suspensions were thoroughly shaken and analysed for the AD and PDI.

The results, listed in Table 3, show that the fabricated NPs were highly stable – no substantial change of the AD and PDI, or aggregation is observed after 90 days of storage. Along with the standard surfactant Tween 20 the new biodegradable PEG-PEA provided good stabilization of the NPs as well.

Table 2. *The obtained NPs and their parameters*

Type of NPs	AD (nm) ± SD	PDI ± SD	ZP (mV) ± SD
TK-1 NPs (Non PEGylated)	68,5 ± 3,1	0,228 ± 0,006	-24,2 ± 1,7
TK-2 NPs (Surface PEGylated)	73,4 ± 7,2	0,200 ± 0,012	-19,0 ± 0,4
TK-3 NPs (Surface PEGylated)	70,1 ± 2,3	0,188 ± 0,002	-14,5 ± 1,2
TK-4 NPs (Surface and bulk PEGylated)	97,6 ± 2,6	0,112 ± 0,008	-14,7 ± 1,1
TK-5 NPs (Non PEGylated)	115,2 ± 3,8	0,117 ± 0,009	+23,2 ± 1,3
TK-6 NPs (Surface PEGylated)	118,3 ± 4,1	0,142 ± 0,013	+15,5 ± 1,1
TK-7 NPs (Surface PEGylated)	125,7 ± 4,3	0,221 ± 0,014	+6,9 ± 1,2
TK-8 NPs (Surface and bulk PEGylated)	130,2 ± 3,8	0,143 ± 0,011	+7,5 ± 0,4

Table 3. *The stability of the prepared NPs upon storage at 4-5°C*

Type of NPs	Time			
	Freshly prepared	After 30 days	After 60 days	After 90 days
AD (nm) ± SD	[PDI ± SD]			
TK-1 NPs	68.5 ± 3.1 [0.228 ± 0.006]	69.1 ± 2.3 [0.221 ± 0.012]	72.3 ± 4.8 [0.218 ± 0.007]	71.3 ± 2.4 [0.219 ± 0.013]
TK-2 NPs	73.4 ± 7.2 [0.200 ± 0.012]	75.1 ± 2.3 [0.199 ± 0.009]	74.0 ± 2.1 [0.203 ± 0.011]	74.8 ± 1.2 [0.200 ± 0.012]

TK-3 NPs	70.1 ± 2.3 [0.188 ± 0.002]	72.2 ± 1.3 [0.181 ± 0.006]	70.4 ± 1.9 [0.179 ± 0.005]	71.8 ± 2.3 [0.178 ± 0.009]
TK-4 NPs	97.6 ± 2.6 [0.112 ± 0.008]	99.2 ± 3.2 [0.119 ± 0.006]	95.8 ± 3.4 [0.129 ± 0.012]	98.3 ± 2.8 [0.121 ± 0.011]
TK-5 NPs	115.2 ± 3.8 [0.117 ± 0.009]	113.6 ± 3.4 [0.119 ± 0.007]	118.2 ± 2.7 [0.122± 0.008]	116.1 ± 3.6 [0.124 ± 0.010]
TK-6 NPs	118.3 ± 4.1 [0.142 ± 0.013]	110.5 ± 1.9 [0.139 ± 0.012]	115.6 ± 2.1 [0.141± 0.011]	108.3 ± 1.2 [0.149 ± 0.013]
TK-7 NPs	125.7 ± 4.3 [0.221 ± 0.014]	118.4 ± 5.1 [0.229 ± 0.012]	121.9 ± 3.8 [0.219 ± 0.009]	119.2 ± 4.1 [0.218 ± 0.006]
TK-8 NPs	130.2 ± 3.8 [0.143 ± 0.011]	131.4 ± 3.1 [0.151 ± 0.016]	128.7 ± 4.2 [0.140 ± 0.009]	129.3± 4.4 [0.136 ± 0.010]

3.4. Penetration of the NPs into murine eyes

After giving drops of the NPs onto ocular surface of the mice, penetration of NPs into the eyes was checked by inspecting fluorescence at 488 nm in the frozen sections. As fluorescence becomes visible only after cleavage of the ester bond in the FDA molecule, only those NPs can be visualized that were taken up and digested by cells in the ocular tissues. We checked the different parts of the eye for fluorescent dots, which would indicate phagocytosed NPs. Results of a semi-quantitative evaluation of the extent of appearance of fluorescent dots are shown in Table 4.

We first inspected the cornea of the treated eyes, as localization of NPs would be anticipated there in the first instance. Fluorescence microscopy images of sections of the cornea are shown in Fig. 1. Besides a faint autofluorescence of the ocular tissue, some scattered bright dots are visible in the cornea (arrowheads in Fig. 1). Such dots do not appear in sections of eyes without NPs. These dots are located in the corneal epithelium and also in the stroma. Notably, they can be found only in the vicinity of the limbus between the cornea and the sclera and not in the central parts of the cornea.

Table 4. *The obtained NPs and their parameters*

Frozen section	Marginal Cornea	Central Cornea	Lens	OS/IS	Nearby OPL	Sclera
TK-1 NPs	++	-	-	+	++++	++
TK-2 NPs	+	-	-	+	-	+
TK-3 NPs	+	-	-	+	+	+
TK-4 NPs	+	-	-	++++	-	+
TK-5 NPs	++	-	-	+	+	+
TK-6 NPs	+	-	-	+++	?	+
TK-7 NPs	+	-	-	+	+	++
TK-8 NPs	+	-	-	+++	-	+

Symbols: + …++++ NPs are present to a different degree; - no NPs are visible; ? NPs not clearly visible.

Fluorescent dots were found also deeper in the eye, in the retina. Again, the tissue shows a faint autofluorescence, except for the photoreceptor inner segments and outer segments that show a higher autofluorescence due to rhodopsin, which is present there. Whereas all eight kinds of NPs were found to a similar extent in the cornea, there are clear differences between the types of NPs regarding their presence in the retina. Most fluorescent dots were seen when the NP TK4 were used, and such dots were also seen if TK6 or TK8 were applied (arrowheads in Fig. 2). Some more fluorescent dots were visible in the outer plexiform layer and, in a few cases, in the inner retina.

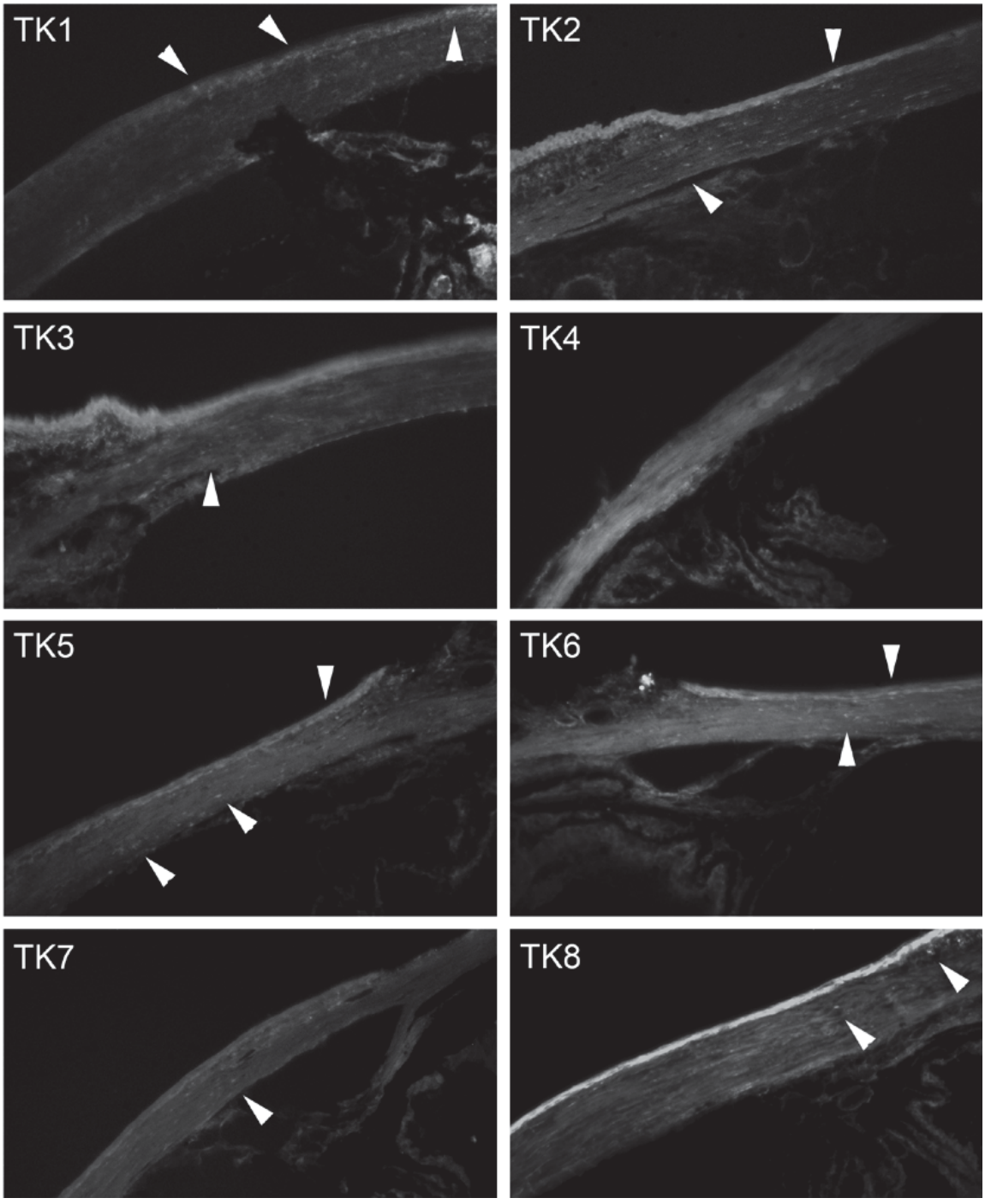


Fig. 1. *Fluorescence images of the cornea nearby the limbus in frozen sections of murine eyes after topical administration of NPs as indicated. Arrowheads point to fluorescent dots. Scale bar: 100 µm.*

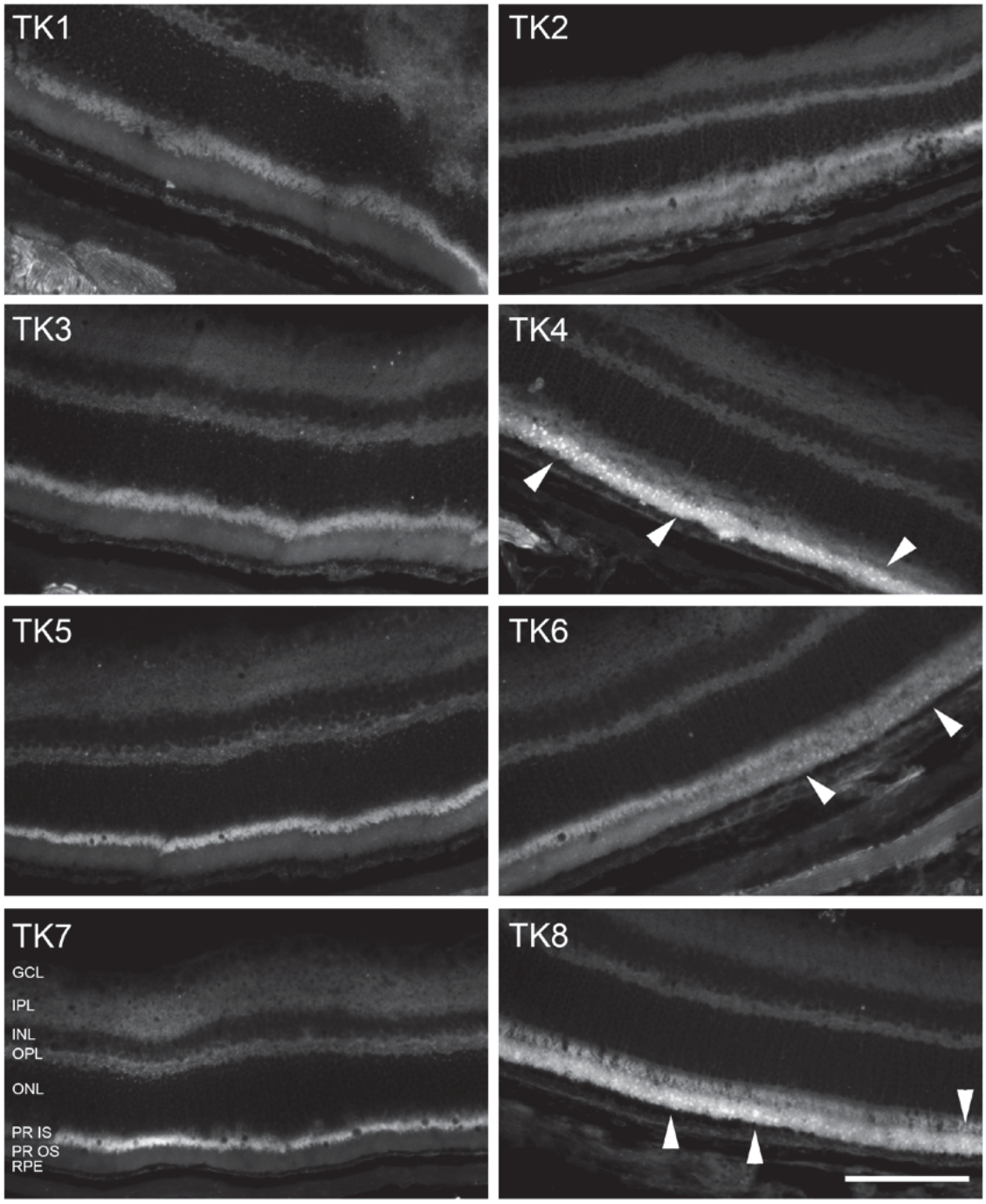


Fig. 2. *Fluorescence images of the retina in frozen sections of murine eyes after topical administration of NPs as indicated. Arrowheads point to fluorescent dots. Scale bar: 100 µm. Layers of the retina in a histological section: GCL – ganglion cell layer; IPL – inner plexiform layer; INL – inner nuclear layer; OPL – outer plexiform layer; ONL – outer nuclear layer; PR IS – photoreceptor inner segment; PR OS – photoreceptor outer segment; RPE – retinal pigment epithelium.*

4. Conclusion

Eight different types of PP-based NPs –negatively/positively charged and PEGylated/non-PEGylated ones, loaded with fluorescein diacetate as a fluorescent probe were successfully obtained. All the NPs were studied for the penetration through the ocular barriers using adult wild-type C57BL/6J mice. The fluorescent dots were found in the sclera after topical application of the NPs. Whereas this appears to be intelligible, the appearance of such dots within the eye, in the retina, was surprising.

At the moment, we cannot explain mechanisms by which the NPs may enter the eyes. Further studies will be performed using different fluorescent dyes to be able to trace the administered NPs within the eyes. The present results raise the hope that the used NPs can be suitable in the future to deliver ocular drugs to the diseased eye.

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