Polymeric Micelles in Ocular Drug Delivery: Rationale, Strategies and Challenges

I. Pepić,* J. Lovrić, and J. Filipović-Grčić
Department of Pharmaceutics, University of Zagreb,
Faculty of Pharmacy and Biochemistry, Zagreb, Croatia

Original scientific paper
Received: May 14, 2012
Accepted: July 30, 2012

Polymeric micelles that can deliver drug to intended sites of the eye have attracted much scientific attention recently. The aim here is to review the aqueous-based formulation of drug loaded polymeric micelles that hold significant promise for ophthalmic drug delivery. These innovative nanosystems can provide the biopharmaceutical advantages of higher permeation and enhancement of residence time at ocular surface for better drug absorption through ocular barriers. Mucoadhesive properties of biopolymers forming micelle enhance their contact time and minimize their elimination from the absorbing surface, consequently increasing the bioavailability of the drug. Their physicochemical characteristics are also important with respect to the industrial production and patient compliance. Drug loaded polymeric micelles can be fabricated by simple and cost effective techniques with improved physical stability which fulfils the requirements for industrial acceptance. Innovative polymeric micelle formulations allow their easy application in the form of eye drops without blurring of vision and discomfort, thus achieving patient compliance requirements.

Key words:
Polymeric micelles, ocular barriers, ocular delivery, ocular bioavailability, size, surface charge, *in vitro* stability, eye biofate, ocular permeability, safety

Introduction

Ocular drug delivery is faced with significant challenges due to dynamic, tissue and ocular-blood barriers. In terms of drug delivery, the eye can be considered to have four target sites: (*i*) the pre-ocular structures of the front of the eye (e.g. conjunctiva, eyelids); (*ii*) the cornea; (*iii*) the anterior and posterior chamber and associated tissues; and (*iv*) posterior eye segment (e.g. retina, vitreous cavity).[1]

The administration of the drug to the front of the eye is typical for the anterior segment therapies (pre-ocular, corneal and anterior/posterior regions). The tear dynamics creates a lively environment of the front of the eye. The solution drainage rate constant from the pre-corneal area is 1.45 min^{-1}. The rate of drug loss from the eye surface can be 500 to 700 times greater than the rate of drug absorption into the anterior chamber, and consequently, less than 5% of the applied dose reaches the intraocular tissues.[2,3] The rate of aqueous humor turnover is estimated to be 1.0% to 1.5% of the anterior chamber volume per minute (i.e. total turnover 1.5–2 h). The drug can reach the anterior chamber via trans-corneal permeation from tears or via blood-aqueous barrier from the systemic circulation. Distribution volume of drugs in anterior chamber is in the range of 150–3000 μl. The drug clearance rates generally are in range of 1–30 μl min^{-1}.[2]

Cornea is the major route of anterior drug absorption. There are three corneal layers, namely epithelium, stroma, and endothelium, and all of them have a distinct role in trans-corneal drug permeability. The corneal epithelium is the major limiting barrier in trans-corneal drug absorption. The drug permeation across corneal epithelium is determined by passive diffusion, facilitated diffusion or active transport. The passive permeation of lipophilic drugs occurs via the transcellular pathway, while the passive permeation of hydrophilic drugs occurs via the tight junctions regulated paracellular pathway. In general, drug permeation across the corneal epithelium is 10^{-7}–10^{-5} cm s^{-1}. In contrast to the epithelium, the hydrophilic stroma presents a barrier to lipophilic substances, which move along the transcellular route through the epithelium. Finally, the endothelium is a leaky monolayer that is easily permeated and participates only marginally in the corneal barrier function. Clinically used drugs are generally small and lipophilic. Thus, the trans-corneal route of drug absorption is currently dominating, although the trans-corneal rate constants are relatively low ($1–5 \times 10^{-3}$ min^{-1}).[4–7]

The surface area of human conjunctiva is about 17-fold larger than human corneal surface area. Conjunctival epithelium is composed of 2–3 cell layers with tight junctions that present the barrier to the passive permeation of hydrophilic molecules

*Correspondence author: Ivan Pepić, PhD, E-mail: ipepic@pharma.hr

found at the apical surface of these cells. However, human conjunctiva is between two- and 30-fold more permeable to drugs than the cornea.[8] The conjunctival epithelium has 2 times larger paracellular pores and 16 times higher paracellular pore density than the cornea. The total paracellular space in the conjunctiva was estimated to be 230 times greater than that in the cornea.[9]

The drug absorption through the conjunctival-scleral pathway has gained increasing attention recently, due to the permeability of conjunctiva to the hydrophilic and large molecules. This pathway can be of high importance for the intraocular delivery of new biotech-drugs such as proteins, peptides and nucleic acid based therapeutics.[5] Trans-scleral route may be suitable for delivery of biotech-drugs to the retina and vitreous, if appropriate delivery systems are developed.[10] Permeability through the sclera is considered to be comparable to that of the corneal stroma, i.e. hydrophilic drugs may diffuse through the scleral matrix pores more easily than lipophilic drugs. The permeability of drug molecules across the sclera is inversely proportional to the molecular radius as well as lipophilicity of drug molecules.[7] The charge of the drug molecule also affects its permeability across the sclera. Positively charged molecules exhibit poor permeability presumably due to their binding to the negatively charged scleral matrix. [8,11]

The blood-ocular barriers limit the effectiveness of the drug delivery to the posterior segment from the systemic blood circulation. The blood-aqueous barrier is located in the anterior segment of the eye and limits the entry of drugs from the blood into the aqueous humor. The blood-retinal barrier (BRB) restricts the transport of drugs from blood into the retina i.e. inward movement following periocular or systemic drug administration and outward movement after intravitreal drug administration.[7,12] The drug permeation from the vitreous humor across the retina is restricted by retinal layered structures.[13] The common method for posterior drug delivery is ocular injection. Intravitreal injection can provide adequate drug concentrations in the posterior segment of the eye, but with the highest risk of ocular complications. The risk is lower with periocular (e.g. sub-tenon) drug administration. Owing to the invasive nature of the injection, it is important to design drug formulations to maintain the therapeutic drug concentration over prolonged periods and minimize the number of injections.[10,14–16]

Nano-ophthalmology, the application of nanomedicine to ocular diagnosis and treatment, has the potential to transform clinical ophthalmology by enhancing the efficacy of eye therapy for a wide spectrum of innovative formulation of either new or existing ocular drugs. Ocular drug delivery nanosystems have been widely explored to bypass or transport the drug across various ocular barriers, as well as to sustain drug levels at the target site of the eye. Design of the innovative ocular drug delivery nanosystems and development of non-invasive techniques for their ocular applications may considerably improve the field of ocular drug delivery. Advances in the nano-based ocular dosage forms are expected to provide new tools for the treatment of the various anterior and posterior eye segment diseases. A number of nanocarriers intended to bypass and/or transport the drugs to the anterior and posterior segment of the eye have been investigated, namely: nanoparticles,[2,17–19] nanosuspensions,[20,21] solid lipid nanoparticles,[22] nanostructured lipid carriers,[23] liposomes,[24] niosomes,[25] cubosomes,[26] microemulsions,[27,28] dendrimers.[29] Yet, the potential of polymeric micelles intended for the ophthalmic application is less explored in comparison to their application in cancer therapy.[30,31] To date, the greatest contributions in this area have been made by Gupta et al.,[32] by the Kataoka group,[33] Liaw group,[34,35] and our group.[36,37] Several of these examples are listed in Table 1.

Ocular drug delivery nanosystems can be prepared as aqueous-based ophthalmic colloidal dispersions, which allow their application in the form of eye drops or eye injections. Eye drops are preferred ocular form used by patients, due to their ease in usage and low interference with vision. However, incorporation of drugs into specially designed nanocarriers would lead to the improved biopharmaceutical properties (e.g. prolonged retention at the site of application, modified drug release). These systems offer additional industrial advantages, especially relatively simple and inexpensive sterile production i.e. the possibility of sterile filtration due to the small particle size (usually less than 100 nm).[17,48–50] However, the majority of these systems are not on the pharmaceutical market due to unresolved problems related to the cost, bulk manufacturing (e.g., the ability to scale up the production), patient compliance (e.g., vision interference, discomfort) and approval by regulatory authorities.

The innovative nano-based ocular dosage forms should maintain the advantages of the conventional ocular therapeutics (i.e., patient compliance and industrial acceptance) while enhancing biopharmaceutical properties (i.e., enhanced ocular bioavailability).[17,48,51] The challenge is to provide a system with improved ocular drug bioavailability and prolonged duration of activity, but still with a minimum risk of ocular complications. These systems can potentially increase patient compliance by reducing the dosing frequency and invasive treatment.

Table 1

Nanosystem	Active compound	Size / surface charge	Observations	References
MPEG-hexPLA polymeric micelles	CsA physically entrapped into polymeric micelles; CsA-loading: 4.9 mg ml^{-1}	d_h = 54 nm	CsA-loaded micelles overcome corneal barriers delivering therapeutics CsA concentration in ocular tissues for an effective action of the drug.	38,39
Vitamin E TPGS/octoxynol 40 mixed polymeric micelles	DEX physically entrapped into mixed micelle hydrophobic core at a concentration 0.01–1 % (*w*/*v*) improving DEX solubility by up to about 10 fold	d_h 10–20 nm	DEX-loaded mixed micelles after topical application at the front of the eye efficiently accumulate into targeted retinal tissue providing DEX therapeutic concentrations in retinal pigment epithelia.	40
F 127/chitosan polymeric micelles	DEX physically entrapped into micelles by direct dissolution method; drug-loading ratio: 0.48–0.56%	d_h 25.4–28.9 nm ζ +9.3-+17.6 mV	2.4-fold increase in bioavailability to a single instillation of DEX-loaded micelle after a fourfold lower dose in comparison with the marked DEX eye drops.	37
F 68 polymeric micelles	Plasmid (pCMV–bcl-x_L–eGFP) encoding functional anti-apoptotic gene (bcl-x_L) physically entrapped into micelles by complex formation	d_h = 47.6 nm ζ = – 1.3 mV	Eye drop of pCMV–bcl-x_L–eGFP-loaded micelles reduced corneal apoptosis following epithelial debridement.	41
Poly(hydroxyethyl-aspartamide)-polyethylene glycol--hexadecylamine (PHEA-PEG-C_{16}) micelles	DEX	d_h 19–23 nm	Drug loaded micelles provide improved both the drug permeation across cell-based epithelial models as well as the drug *in vivo* bioavailability.	42
F 68 polymeric micelles	Plasmids (pCMV-Lac Z, pK12-Lac Z and pKera3.2-Lac Z) containing the Lac Z gene physically entrapped into micelles by complex formation	d_h 150–200 nm	Cornea epithelium- and stroma-specific gene expression could be achieved using cornea-specific promoters of keratin 12 and keratocan genes, and the gene was delivered with PM formulation through non-invasive, eye drop in mice and rabbits. The transfection mechanism of plasmid-PM may involve endocytosis and particle size dependent paracellular transport.	35
CS-modified CH self-aggregated micelles radiolabeled by ^{99m}Tc	CsA physically entrapped into micelles by dialysis method; drug-loading ratio: 6.2%	d_h < 230 nm	Prolonged residence time of CsA-loaded CS-CH micelles applied topically onto the eye.	43
PEG-PLL or PEG-PAA micelles	Ionic dendrimer type porphyrin derivate physically entrapped into PIC micelles	d_h 10–100 nm	Improved ocular photodynamic therapy after intravenous application of photosensitive drug-loaded PIC micelles intended for treatment posterior eye diseases.	33,44
F 127 polymeric micelles	PLC physically entrapped into micelles by direct dissolution method; entrapment efficiency: 1.9%	d_h 18.7–30.3 nm	Enhanced miotic response to a single instillation of PLC-loaded micelle formulations in comparison with the marked PLC eye drops.	36
TY/CR/P 85 or CR/P 85 mixed polymeric micelles	TR physically entrapped into micelles at a concentration 1 % (*w*/*v*)		TR-loaded micelle formulation improved bioavailability to a small but statistically significant extent in comparison with the marked TR eye drops.	45
Polyoxyl 40 stearate polymeric micelles	CsA physically entrapped into micelles at a concentration 0.1 % (*w*/*v*)	d_h = 200 nm	The micelle formulation improves both the CsA trans-corneal permeation and the CsA distribution into eye tissues (cornea, conjunctiva, lacrimal gland).	46
F 68 polymeric micelles	plasmid DNA with lacZ gene physically entrapped into micelles by complex formation	d_h = 155 nm ζ = – 4.4 mV	Enhanced gene expression in the iris, sclera, conjunctiva, and lateral muscle of rabbit eyes and also in the intraocular tissues of mice after topical application.	34
F 127 polymeric micelles	IND physically entrapped into micelles at a concentration 0.1 % (*w*/*v*)		Improved bioavailability and faster onset time of IND-loaded micelle formulation in comparison with the marketed IND eye drops.	47
polymeric micelles of copolymer of NIPAAM, VP and AA having cross--linkage with MBA	KT physically entrapped into micelles; entrapment efficiency: 80%	$d_h \approx$ 35 nm	Corneal penetration of KT from micelles is much higher compared to aqueous suspension of drug. The formulation also shows much higher anti-inflammatory activity for longer duration compared to that of aqueous suspension of drug.	32

Poly(hydroxyethylaspartamide) (PHEA); polyethylene glycol (PEG); PEG-*b*-poly(L-lysine) (PEG-PLL); PEG-*b*-poly(aspartic acid) (PEG-PAA); hexadecylamine (C_{16}); methoxy poly(ethylene) glycol-hexylsubstituted poly(lactides) (MPEG-hexPLA) copolymers; D-alpha tocopheryl polyethylene glycol 1000 succinate (vitamin E TPGS), PEG-40 octyl phenyl ether (Octoxynol-40); cholesterol (CS); chitosan (CH); Tyloxapol® (TY); Cremophor EL (CR); Pluronic® P 85 (P 85); Pluronic® F 68 (F 68); Pluronic® F 127 (F 127); *N*-isopropylacrylamide (NIPAAM); vinyl pyrrolidone (VP); acrylic acid (AA); *N*,*N*-methylene bis-acrylamide (MBA); cyclosporine A (CsA); dexamethasone (DEX); indomethacin (IND); pilocarpine (PCL); tropicamide (TR); ketorolac (KT); polyion complex (PIC); hydrodynamic diameter (d_h); zeta-potential (ζ).

Physicochemical considerations

Polymeric micelles are spontaneously formed in aqueous media via the self-assembly of amphiphilic block copolymers into nano-sized particles at or above the critical micelle concentration (cmc). During the micellization process, the hydrophobic blocks associate to form the core region, whereas the hydrophilic segments form hydrophilic shell of micelles. Amphiphilic block copolymers can be tailored to have unique properties with respect to delivery requirements, such as to prolong the stability of micelles in the eye fluids, to enhance residence time at the absorption membrane, and to modify the drug release profiles. The shell is responsible for micelle stabilization and in particular circumstances interactions with absorption membranes. The most commonly used shell-forming polymer is poly(ethylene oxide) (PEO) or poly(ethylene glycol) (PEG). PEO has unique solution properties, including minimal interfacial free energy with water, high aqueous solubility, high mobility, and large exclusion volume. PEO stabilizes the interface between the bulk aqueous phase and the hydrophobic core of micelles by steric repulsive inter-particle forces. From biopharmaceutical viewpoint, PEO has low toxicity and immunogenicity, minimize protein adsorption to micelle surfaces and improve the micelles biocompatibility.[52] The use of other hydrophilic polymers as shell-forming blocks has been reported for their bioadhesive or thermoresponsive properties.

Biodegradable polyesters such as poly(lactic acid) (PLA), poly(ε-caprolactone) (PCL), and poly(glycolic acid) (PGA) are most commonly used polymers to encapsulate small lipophilic drugs. Yet, the polyion-complex (PIC) micelles are formed by electrostatic interaction of charged polymer blocks, such as poly(ethyleneimine) (PEI), poly(aspartic acid) and poly(L-lysine) (PLL), and nucleic acid-based therapeutics (e.g., plasmid DNA, siRNA) or oppositely charged protein drugs.

The drugs may be loaded in the micelle core by chemical, physical, or electrostatic means. However, the physical procedures of encapsulation are preferred. Commonly used physical methods include direct dissolution, dialysis, oil-in-water emulsion, various modifications of film-methods, and complexation. Depending on the method, drug loading may occur during or after micelle self-assembly.[52,53] The dialysis method consists in bringing the active components and copolymer from an organic water-miscible solvent in which they are both soluble (e.g. ethanol, dimethylsulfoxide, dimethylacetamide, tetrahydrofuran) to a solvent that is selective only for the hydrophilic part of the copolymer (i.e. different aqueous based solvent).[53] The size and polydispersity as well as the weight fraction or yield of polymeric micelles obtained may vary depending on the organic solvent employed.[54] However, to ensure the complete removal of the organic solvent used, the dialysis has to be extended over several days, which is quite inappropriate for industry. The oil-in-water emulsion method consists in preparing an aqueous solution of the copolymer to which the solution of drug in the water-immiscible volatile solvent (e.g. chloroform) is added in order to prepare an oil in water emulsion followed by evaporation of volatile solvent.[53] This method should be avoided when preparing the micelles intended for ocular delivery because it is almost impossible to completely remove the toxic chlorinated solvents during the evaporation process. The film-method was subjected to various modifications but usually include following steps: the addition of copolymer and organic drug solution to an empty vial, the evaporation of organic solvent and the formation of copolymer/drug matrix film on the vial walls. The micelles are formed during the rehydration of the matrix film by the addition of the aqueous solvent.[53,54] This method is suitable for the preparation of micelle for ocular delivery because the organic solvent can be practically completely removed. This method may also result in the significant increase in the drug loading. Finally, the drug-loaded micelles can be prepared by the simple equilibration of the drug and polymeric micelles in aqueous media. Even though, direct dissolution method may not result in the high levels of drug loading, it should be emphasized that this method is the simplest method of micelle preparation with the great potential for scale-up through industry. Furthermore, the usage of toxic solvent is avoided in this method. By the principles of simplicity and safety, this method should be preferred for the preparation of micelles envisioned for ophthalmic delivery. Yet, combing the hydrophobic effect between block copolymers and drugs with other interactions such as hydrogen bonding, electrostatic interaction and dipole-dipole interaction should enhance the drug loading capacity of polymer micelles. PIC micelles, however, are prepared by simple mixing of aqueous solutions of block copolymer containing neutral block and ionic block (e.g. PEG-*b*-polycationic copolymers), and aqueous solution of pDNA, siRNA or negatively charged proteins.[55] The hydrophobic micelle core is formed by electrostatic interaction of polycationic portion of copolymer and oppositely charged biomolecules. Due to the highly dense hydrophilic shell surrounding the PIC core, the PIC micelles exhibit excellent stability in aqueous media and high tolerability against nuclease or protease degradation of encapsulated biomolecules.[56] However, further modification of PIC micelles will be necessary to retain their stabil-

ity under harsh *in vivo* conditions in the eye, and to achieve the delivery of the cargo in the right cellular space.

The release of drug from polymeric micelles depends on the rate of diffusion of the drug from the micelles, micelle stability and the rate of biodegradation of the copolymer. If the micelle is stable and the rate of biodegradation of copolymer is slow, the release rate will be mostly influenced by the strength of the interaction between the drug and the core-forming block.[54] The drug release rate is particularly important for ophthalmic delivery. It is crucial to establish the right balance between the drug release rate from the micelles and their drainage from the various eye compartments. The drug release profile should be tailored depending on the dynamics of eye compartments in which the drug is applied. More precisely, if the site of drug action is the tissue associated with aqueous humor and the drug-loaded polymeric micelles are not absorbed across cornea after topical drop-wise application, the drug needs to be released from the micelles and the free drug needs to be absorbed in the great extent across the cornea in the process that is faster than the drug-loaded micelle and/or free drug drainage occurs. Contrary, if the drug-loaded polymeric micelles are applied by injection into vitreous humor, than there is no danger of washing out of micelles from the site of application because the vitreous humor is very static environment and the release profile of drugs should be set up to ensure effective dose of drug during the prolonged period of time and thus reduce the frequency of invasive application.

In the case of PIC micelles, it is of crucial importance that the release of the encapsulated biotech-drugs does not occur before the internalization of intact micelles because the biotech-drugs cannot cross the plasma membrane themselves. This points out the importance of the stability of PIC micelles in the tears if applied as eye drops and the stability in the extracellular matrix in ocular tissues if injected intraocularly.

Polymeric micelles usually range in size between 10 and 100 nm. This range is even smaller than the size of other new self-assembled delivery system such as dexamethasone-loaded cubosomes ranging in sizes from about 214 to about 226 nm.[26] The sizes of polymeric micelles are similar to the sizes of natural nanocarriers such as serum lipoproteins and viral particles. Different physicochemical and biopharmaceutical properties of the polymeric micelles are related to their size. With respect to the patient convenience, it is important that the particle size for ophthalmic applications is within the nano range because with larger sizes a scratching feeling of foreign body sensation might occur. An aqueous-based micelle nanodispersions are similar to conventional eye drops because they have drug-loaded particles in colloidal form in a liquid vehicle similar to eye drops. These nanodispersions can be conveniently administered to the ocular surface like aqueous solution but with lower frequency of dose. The stability of polymeric micelles, both physical and biological, is closely associated with their colloidal size range as well as their common narrow size distributions. Drug loading efficiency as well as its release profile might be dependent on size and shape of micelle nanocarriers. The size of polymeric micelles can largely influence on their eye biofate. It plays an important role in the ability of polymeric micelles to interact with eye surfaces, in particular, with the mucosal epithelia. Furthermore, the size of externally applied micelles could influence the absorption or permeation through the ocular barriers. The size plays a crucial role in determining the efficiency of endocytosis, and also in determining the mechanism of internalization of nanosystems in the cell (e.g. clathrin mediated or caveolin-mediated endocytosis).[57,58] Moreover, the size could play a decisive role for the paracellular transport of polymeric micelles through the hydrophilic pores of tight junctions.

Besides the size, the surface charge of polymeric micelles is also important considering their physicochemical and biological properties. Polymeric micelles can be stabilized either by electrostatic stabilization or by steric stabilization or by a combination of both. The micelle surface charge determined by the zeta-potential values allows the formation of the stable micelle nanodispersion. Furthermore, surface charge of polymeric micelles determines their interactions with eye surfaces and/or cell membranes. In particular, different types of electrostatic interactions (e.g. ionic interaction or hydrogen bonds) with the negatively charged sialic acid residues of mucin, usually depending on the environmental pH and/or ionic strength, define polymeric micelles retention on the absorbing surface. For comparison, positively charged liposomes seem to be preferentially captured at the negatively charged corneal surface compared to neutral and negatively charged liposomes.[17]

Even though the polymer micelle is the physically self-assembled dynamic structure, their physical stability in aqueous solutions is enhanced in comparison to low-molecular-weight surfactants. The physical stability of polymeric micelles includes their thermodynamic and kinetic stability. The micelles formed from amphiphilic block copolymers are more thermodynamically stable than those formed from low-molecular-weight surfactants. Increased thermodynamic stability is related to low cmc values of polymeric micelles allowing

them to be less prone to disassembly at low concentrations than standard surfactants.[52,59] For example, the cmc value of polyoxyethylated nonionic surfactants in general is about two orders of magnitude lower than the corresponding anionics with the same alkyl chain length.[60] Polymeric micelles also possess noteworthy kinetic stability, meaning that the dissociation of polymeric micelle structures into unimers is a slow process, even when the system is subjected to dilution below cmc.[52,59] Moreover, it is proposed that loading of water insoluble drugs into micelle hydrophobic cores can further stabilize polymer micelles.[61] Slow dissociation upon dilution allows polymeric micelles to retain their integrity and drug content in eye fluids for some time giving them a chance to reach the target site before dissociation into unimers. However, the interactions of polymeric micelles with components of eye fluids (e.g. proteins, mucin, glycosaminoglycans) and cells need to be evaluated in terms of micelle stability and drug release.

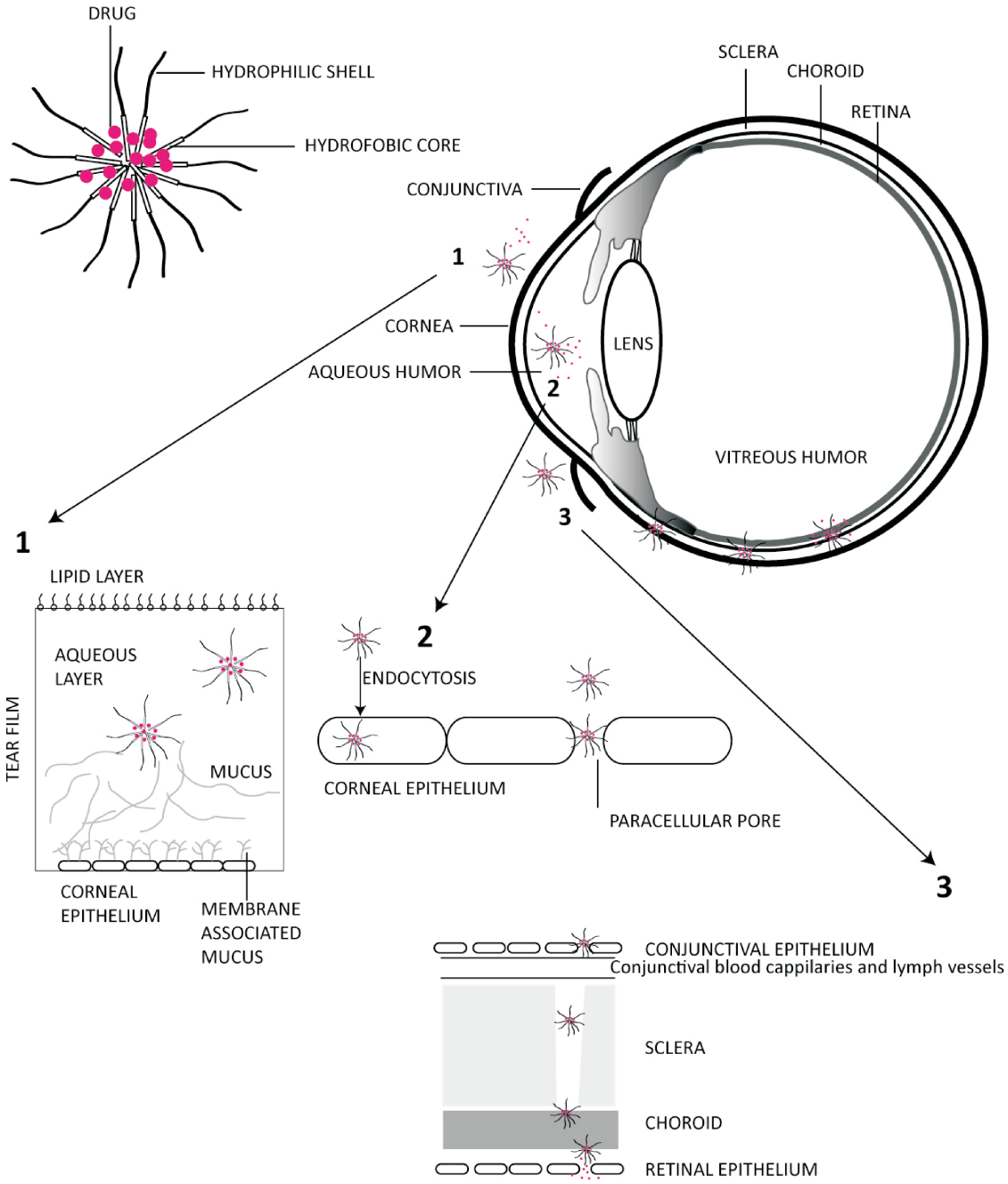
The polymeric micelles *in vivo* are confronted with numerous eye fluid components affecting their stability. The influence of these components should be discriminated within *in vitro* stability studies. Thus far, the *in vitro* stability of ocular drug delivery nanosystems including polymeric micelles, and their efficiency in the delivery of encapsulated drugs in the ocular tissues was studied in the artificial eye fluids with relatively simple composition. The artificial tear fluid (pH 7.4, ionic strength 0.188) commonly contains different salts (i.e. sodium chloride, sodium bicarbonate, calcium chloride) dissolved in water[18], while the human tears, in addition to being aqueous solution of electrolytes, are rather complex mixture of different proteins (e.g. lysozyme, lactoferrin, lipocalin, secretory IgA), lipids, and mucin. The importance of stability studies in complex media is highlighted by the finding that the PEG-*b*-PCL micelles are stable in simple media such as phosphate-buffered saline but disassembled to varying extents with increasing chemical complexity of media and addition of serum.[62]

The design strategies for *in vitro* micelle stability should pay special attention on the physicochemical properties, composition and dynamics of the eye fluids. The tears are isotonic (308 mOsmol/kg), low viscous non-Newtonian pseudoplastic fluid (1–10 mPa s) with pH values in range 7.3–7.7.[3,63] The tear film is composed primarily of mucus whose primary component is mucin, a high-molecular-mass glycoprotein that is negatively charged at physiological pH. The concentration of mucin in human tears is 100 μg ml^{-1}. Proteins in biological fluids can compromise the stability of polymeric micelles by adsorption to the micelle surface leading to their disintegration, and consequent decrease in the transport across the ocular barriers. For example, negatively charged mucin may destabilize the PIC micelles by the counter polyelectrolyte reaction.

Biopharmaceutical considerations

Drug-loaded polymeric micelles should be carefully designed in order to deliver the drug at the site of its action. If the site of drug action is cornea or conjuctiva, polymeric micelles should be retained at the ocular surface long enough to ensure sustained drug release as well as more efficient drug permeation in the front eye surface tissue (Figure 1, case 1). At the same time, the adsorption of intact drug-loaded micelles may be hypothesized providing sustained drug release and prolonged therapeutic activity in the corneal tissue. When dealing with the drug whose site of action is aqueous humor associated tissues, then the drug-loaded polymeric micelles must have potential to be adsorbed across the cornea, releasing the drug in aqueous chamber (Figure 1, case 2). In the case of the diseases affecting the posterior eye segment, and in order to evade the invasive ocular injections thereby improving patient compliance, polymeric micelles should reach the posterior of the eye most probably by trans-scleral route around conjunctiva, through the sclera, choroid and finally retina (Figure 1, case 3).[40] However, after topical application, polymeric micelles will encounter different challenges that they have to overcome in order to successfully deliver the drug to the site of action.

Since the volume of human tears in the pre-corneal area is only 7 μl, there will be no significant dilution of micelle formulation upon the application. However, the micelles will be exposed to the dynamic and complex eye environment, and they can be immediately washed away from the eye surface. The tear flow is in the range of 0.5 to 2.2 μl min^{-1} and the contact time with the ocular absorbing surfaces (cornea or conjunctiva) is typically less than 2 min. The eye surface is covered by a thin fluid layer, the so-called precorneal tear film with the thickness of 3–10 μm. The human tear film is composed of three distinct layers: the thin lipid layer, the aqueous layer and the mucus layer.[64,65] The secreted mucin in tears forms a hydrophilic layer that covers ocular surface and thereby creates the negative charge of both corneal and conjunctival surface. In order to prolong the residence time at the ocular surface, polymeric micelles should make an intimate contact with the mucus layer. Pre-ocular retention of polymeric micelles can be achieved by the presence of the positive charge in the hydrophilic micelle shell that will en-



F i g . 1 – *Fate of drug-loaded polymeric micelles after topical ocular application*

hance the interactions with negatively charged mucins at the corneal surface.[66] Coating of polymeric micelles by mucoadhesive polymers like chitosan improves bioavailability of the encapsulated drug either by prolonging the residence time of micelles at the mucus site due to ionic interaction between the positively charged amino groups of chitosan and the negatively charged sialic acid residues in mucus, or by the strong effect of chitosan as permeation enhancer, or by a combination of both.[37] Without bioadhesion and/or permeation enhancer, the micelles would be eliminated from the pre-corneal site almost as quickly as aqueous solution.

The precorneal tear film serves as permeability barrier that could discriminate molecules or particles by surface properties. Namely, molecules or particles that engage in strong binding interactions with the mucus hydrogel become trapped in the hydrogel matrix independent of their size and thereby the hydrogel matrix modulates their diffusion behaviour (so-called interaction-filtering mechanism).[67] However, the polymeric micelles must be mobile and flexible enough to interdiffuse into the mucus and penetrate to a sufficient depth. Yet, the stability of the micelles on the negatively charged mucin surfaces can be compromised.

There are several possible scenarios predicting the pre-corneal drug fate regarding the polymeric micelle relaxation time and the process of drug partitioning. If the relaxation time of drug-loaded polymeric micelles is short and/or the process of drug partitioning in and out of micelles is fast, than the free drug is absorbed into the eye and instantly new free drug is released from micelles according to a shift in the equilibrium between free drug and drug in micelle.[60] However, this productive absorption process takes place as long as drug-loaded polymeric micelles are close to absorbing surface. Once the micelles are removed from the productive absorption area, the non-productive (systemic) absorption takes place. Thus, the ocular drug absorption rates from a micelle and a solution system are comparable if the effects of surfactants on the physical properties of the delivery systems and the integrity of ocular epithelium are negligible. In contrast, if we are dealing with the drug-loaded polymeric micelles having the longer relaxation time and/or the slower process of drug partitioning in and out of micelles, it is possible to achieve micelle depot at the absorbing surface with prolonged drug release and therapeutic activity. To achieve sustained drug release and prolonged therapeutic activity, the micelles need to be retained in the pre-ocular area after topical administration, and the consequent release of the drug from the micelles at an appropriate rate.

Polymeric micelles have potential to increase drug permeability via transcellular and/or paracellular pathway. The effects of amphiphilic copolymers (unimers) on the transcellular permeability are well established. Unimers dissociated from micelles may perturb the plasma membrane, resulting in acceleration of drug permeation through cell membranes.[61] More precisely, these copolymers can be incorporated into the lipid bilayer, forming polar defects, which change the physical properties of the cell membranes. When the lipid bilayer is saturated, mixed micelles begin to form, resulting in the removal of phospholipids from the cell membranes and hence leading to the increased transcellular permeability.[60] In addition, intact polymer micelles can also enter cells possibly by endocytosis[68], but the mechanisms explaining endocytosis of polymer micelles have not been fully clarified. Moreover, the trans-corneal absorption of particles smaller than 100 nm has been also reported.[17]

Tight junctions regulate paracellular movement of hydrophilic drugs across epithelia. The dimensions of the hydrophilic paracellular pores lie between 3–5 nm, suggesting that solutes with a molecular radius exceeding 1.5 nm will be excluded from this uptake route under normal conditions. Permeation enhancers could improve corneal barrier restrictions. Polycationic polymers such as chitosan and poly-L-arginine are reported to increase the trans-epithelial absorption by dissociation of tight junction assemblies, which restrict the paracellular permeation in ocular epithelia without producing significant epithelial damage.[66] Taking into account the flexibility of the micelle structure, as well as permeation enhancer induced opening of the tight junctions and enlargement of the paracellular pore dimensions, it is possible to envision the transport of intact micelle through the opened hydrophilic paracellular pores.[17]

Moreover, the majority of polyoxyethylated nonionic surfactants are P-glycoprotein (P-gp) efflux pump inhibitors that can increase drug absorption, resulting in a higher bioavailability. The mechanism of P-gp inhibition by polyoxyethylated nonionic surfactants has not yet been completely understood. The use of the nonionic surfactant systems as a means of delivering poorly absorbed drugs has been recognized as a strategy to overcome P-gp efflux activity and improve drug efficacy (e.g. dexamethasone, timolol).[60]

The approaches to improve the *in vivo* stability of polymeric micelles include one or combination of following strategies: stimuli sensitivity, targeting moiety, and cross-linking strategy. Stimuli-sensitive polymer micelles can actively respond to environmental signals, provoking changes of their physical stability. A representative example is the pH-sensitive polymer micelle, which becomes destabilized and releases drugs depending on the environmental pH. For example, Gupta et al.[32] prepared the polymeric micelles of N-isopropylacrylamide copolymer (NIPAAM), vinyl pyrrolidone (VP) and acrylic acid (AA) having cross-linkage with N,N-methylene bis-acrylamide (MBA) loaded ketorolac. The temperature and pH dependent release of drug in aqueous buffer (pH 7.2) from the polymeric micelles at 25°C were 20 and 60% after 2 and 8 h, respectively. *In vitro* corneal permeation studies through excised rabbit cornea indicated two fold increases in ocular availability with no corneal damage compared to an aqueous suspension containing same amount of drug as in micelles. The formulation showed significant inhibition of lid closure up to 3 h and PMN migration up to 5 h compared to the suspension containing non-entrapped drug, which did not show any significant effect. Corneal permeation of ketorolac and anti-inflammatory activity from nanoparticles were much higher compared to aqueous suspension of drug of equivalent concentration. This could be attributed to the small size (< 50 nm) of the polymeric micelles as well as their mucoadhesiveness. Also, the ligand-conjugated polymer micelle (e.g. folate, antibodies, growth factors, or homing peptides) increased the

probability of cellular uptake of polymer micelles. Those micelles are designed for intracellular delivery of anticancer drugs. However, this approach can be an important rationale to develop ligand-conjugated polymer micelle for ophthalmic delivery because eye cells and tissues expressed numerous receptors. Chemically or electrostatically cross-linked polymer micelles prevent micelle *in vivo* disintegration. However, cross-linking between polymer chains may result in another problem of controlled drug release owing to the lack of a biodegradation mechanism.[61]

Furthermore, the polymer micelle formulations have potential for injection applications into different eye compartments allowing micelles to be confronted with various compositions of the eye fluids (aqueous or vitreous humor). The hydrogel forming vitreous humor is diffusion barrier that restricts drug permeation to the retina. Interaction-filtering mechanism might also apply to the vitreous humor hydrogel that molecules have to permeate to reach the retinal cells. The major components of vitreous humor are collagens and anionic glycosaminoglycans including hyaluronic acid, chondroitin sulphate and heparan sulphate. The permeability properties of the vitreous humor are also selective – some antibiotics can penetrate the vitreous humor whereas others are blocked. Similarly, the diffusion of some small dyes is delayed in the hydrogel, whereas others, although similar in size, are able to diffuse freely. This suggests that, also in the ocular hydrogel, interaction-filtering strategies contribute to the microscopic regulation of diffusion.[67] However, systematic studies on the permeability properties of the vitreous humor are still lacking.

The understanding of the behaviour of polymeric micelles and their fate in real time in the eye is necessitated. To clarify the biodistribution of drug-loaded polymer micelles, their structural integrity and fate in the eye should be visualized.[69] The biodistribution data can represent the real location of the polymer micelles only during the time within the polymeric micelles are stabile *in vivo*. A novel fluorogenic-based approach to micelle integrity assessment may be useful to monitor the micelle stability and biodistribution *in vivo*.[62]

Eye biofate and safety considerations

The unique characteristics and high sensitivity of the eye tissues impose distinctive safety requirements and great restrictions on the selection of the components that can be used in the topical ocular preparations. A special consideration should be given to the ability of the polymeric surfactants to affect the integrity of epithelial surfaces potentially causing irritation and inflammatory changes (burning, stinging, corneal opacity, conjunctival redness or chemosis, and discharge). Polymeric surfactants have potential to induce cytotoxic side effects to the eye which are dependent on the type and chemical structure of surfactants to which the eye is exposed.[60]

The surfactants applied to the eye can be absorbed through the non-productive routes (conjunctiva, nasal mucosa, lacrimal drainage system, pharynx, gastrointestinal tract, skin at the cheek and lips) and enter the systemic circulation.[60] In most cases, the molecular weight of polymeric micelles is in the order of 10^6 g mol^{-1}. Possessing a much larger molecular weight than that for critical filtration in the kidney, polymeric micelles can evade renal filtration.[52] Although polymeric micelles are able to persist for several hours after dilution below the cmc, upon entering the systemic circulation they will fall apart at some point through biodegradation or through simple dilution into unimers. The tissue distribution of these surfactants is relatively low due to a moderate to high hydrophilicity and/or possible strong binding with plasma proteins. The dissociated individual surfactant molecules are below the renal molecular weight threshold and will be eliminated mainly by renal excretion with only a small portion removed by the biliary secretion. Yet, the clearance path of polymeric surfactants through the kidneys or by other means of excretion opens the potential for micelles that are very stable in plasma to alter the blood disposition and pharmacokinetics of biologically active compounds which may affect the toxicity profiles of the ophthalmic drugs applied topically as eye drops.[60]

Drug-loaded polymeric micelles applied topically if able to permeate into the eye across the cornea would firstly enter the aqueous humor and then be distributed to the surrounding tissues. The aqueous humor in the anterior and the posterior chambers flow in opposite directions and would hinder the passage of the micelles from the aqueous humor to the lens and vitreous humor, thus making this unfavourable pathway for the posterior of the eye. The absorbed drug and polymeric surfactant in the anterior eye tissues would be mainly eliminated via aqueous humor turnover and venous blood flow in the anterior uvea. Yet, topically applied drug-loaded polymeric micelles have potential to reach the posterior of the eye by the unconventional pathway of going around the eye, i.e. trans-scleral route around conjunctiva, through the sclera, choroid and finally retina. Permeation of micelles through the trans-scleral route would be enabled by their small size and hydrophilic surface properties. Polymeric micelles would probably travel through the hydrophilic pores of the sclera, which range from about

30 nm to about 300 nm in size. Moreover, it was suggested that the hydrophilic chains in the micelle corona could partially evade wash-out by the conjunctival/choroidal blood vessels and lymphatics allowing the micelles to reach retina.[40] The absorbed drug and polymeric surfactant from the posterior eye tissues would be eliminated via posterior (choroidal blood flow) or, less likely, via the anterior route by diffusion through the vitreous to posterior chamber and elimination via aqueous turnover and uveal blood flow.[7]

Polymeric surfactants are known to be safer than low-molecular-weight surfactants.[70] Polymeric nonionic surfactants are relatively harmless to the eye in comparison to the cationic, anionic, or amphoteric counterparts. They are generally less toxic, less haemolytic, and less irritating to the eye tissues, and tend to maintain near physiological pH values when in solution. Polyoxyethylated nonionic surfactants have found widespread applications in ophthalmics among the nonionics. Some polyoxyethylated nonionic surfactants have a long history of being safe in ophthalmic use. Polysorbate 80 was reported to be non-irritating to the rabbit eye up to a concentration of 10% and has been used in a number of marketed eye drop solutions. Tyloxapol and Pluronics are also used as formulation components of several commercial topical ocular products and have proven to be safe for use.[60] Pluronic block copolymers are practically safe, particularly those with a high content of PEO. Core-forming blocks such as polyesters and poly(L-amino acids) undergo hydrolysis and/or enzymatic degradation in biological environment, producing biocompatible monomers allowing renal elimination. Since the body effectively deals with these monomers, there is very minimal systemic toxicity associated by using polyesters and poly(L-amino acids) for drug delivery.[71] For ocular delivery biodegradable polymers are preferable and in most cases required.

The surface or interfacial tension that polyethoxylated nonionic surfactants produce in the concentration they form aggregates is usually higher compared to that of ionics, making the nonionics less destructive on cell membranes and thus less irritating and toxic.[60] The polyoxyethylated nonionic surfactants are safe as permeation promoters. Their apparent safety at the tested concentrations was confirmed by their inability to increase the corneal hydration level beyond the normal value, and by lack of irritant effect *in vivo*, as evidenced by a Draize test.[60]

Industrial considerations

The major factors that must be taken into account while formulating aqueous based polymeric micelle nanodispersion include required drug therapeutic concentration, ocular safety and toxicity of polymeric micelles, compatibility of polymeric micelles with other formulation ingredients as well as packaging components, the effect of pH and ionic strength on stability and/or solubility of drug-loaded micelles, choice of formulation preservative, ocular formulation comfort and its ease of manufacturing.[48] The aqueous based nanodispersions of polymeric micelles can be easily manufactured by the direct dissolution method. After the simple mixing of the drug and polymeric micelles in aqueous media and sterilization via sterile filtration, this sterile micelle nanodispersion may further be mixed with previously sterilized solutions of additional components such as viscosity enhancing, lubricating, bioadhesive, buffering, tonicity, chelating, antioxidant and preservative agents. Then the batch is brought to final volume with additional sterile water. For comparison, the manufacture of sterile nanoparticles is more complicated as compared to polymeric micelles including more and much complicated manufacturing steps (e.g. milling, homogenization, emulsion techniques).

The major benefit associated with nano-sized dimensions of polymeric micelles is related to their sterilization processes in pharmaceutical productions. Polymeric micelles are simply and inexpensively sterilized by filtration using typical sterilization filters with 0.45 μm or 0.22 μm pores owing to a fact that polymeric micelles are essentially free of micro-sized particles contamination. In contrast, other typical pharmaceutical nano-sized carrier systems (e.g., nanoparticles, liposomes) need a removal of contaminated micron-sized particles.[70]

The stability of ophthalmic dosage forms determines the shelf life and expiration dating of the product.[48] It has been already explained that polymeric micelles possess high structural stability provided by the entanglement of copolymer chains in the inner core. Thus, polymeric micelles can be fabricated by simple techniques with better physical stability in comparison to other nanocarrier systems.

Among the possible difficulties related with the stability of ophthalmic formulations containing polymeric micelles is the oxidative damage of active pharmaceutical ingredients due to the presence of residual level of peroxides in the polymeric surfactants used for the micelle preparation and/or the susceptibility of polymeric surfactant to autoxidation. This problem is highlighted in the formulations containing high excipient-to-drug ratios. Furthermore, nonionic polymeric surfactant can negatively influence on the effectiveness of preservatives present in the formulation. The free preservative can be depleted below its biological effective

level due to the interaction with polymeric surfactants. In the development of ophthalmic formulation containing polymeric micelles a careful evaluation of interactions of polymeric surfactants and other formulation ingredients should therefore be performed in order to attain required stability of polymeric micelles, the shelf-life of the formulation, and the performance of the product.

Nonionic polymeric surfactants influence on physicochemical characteristics of the formulation, in particular the dynamic surface tension, viscosity or other rheological behaviour, which needs to be taken into consideration during the innovative ophthalmic product development. For example, it has been shown that the lower is the dynamic surface tension of the solution; the lower is the weight of drops delivered. The change in viscosity or other rheological properties including yield stress, storage/loss modulus, and thixotropy as a result of addition of nonionic surfactants to eye drops may not significantly affect the drop volume/weight within the usual concentration range of surfactants used.[60]

Regulatory considerations related to new ophthalmic delivery systems are very complex issues including preclinical and clinical testing and filing appropriate applications. The differences between global medical regulatory authorities create an additional obstacle for industry aiming to lunch the innovative ophthalmic products in various world markets.[48]

Patient considerations

To treat the diseases of the anterior part of the eye, drugs are commonly applied in the form of eye drops to the eye surface. Due to the physiological and anatomical constraints, only a small fraction of drug is ocularly adsorbed, and to achieve pharmacological effect the high frequency of dosing is requested. Nanodispersion of polymeric micelles in a suitable ophthalmic vehicle has the advantage of application in liquid form just like eye drop solutions while maintaining the drug activity at the site of action. Bioadhesive properties of polymeric micelles minimize the drainage from the ocular surface by interacting with the mucin present in the ocular surface resulting in the enhancement of the contact time and the increase of the drug bioavailability. Thus, the frequency of dosing can be significantly reduced. Moreover, the discomfort associated with the application of viscous or sticky preparations that also prolong the residence time on the eye surface, but lead to a blurring of vision, can be avoided. The additional advantage of polymeric micelles is permeation enhancement of the encapsulated drug through ocular barriers. Thus, properly formulated drug-loaded polymeric micelles may provide ease of application with lower frequency of dosing, giving to the ophthalmic products containing polymeric micelles the advantage of being patient friendly.

The drug from topically applied drops hardly reaches the posterior tissues such as choroid and retina. Thus there is a real problem for the drug to be efficiently delivered to the posterior segment of the eye. For the treatment of the diseases affecting posterior segment of the eye, ophthalmic formulation containing polymeric micelles can be administered by two routes, topical and injection [17]. Injectable route is an invasive method for administration of therapeutic agents to ocular tissues, however due to the sustained release of the drug from the polymeric micelles, the frequency of injections could be significantly decreased. The further achievements in the design of polymeric micelles that may bypass or transport the drug across various ocular barriers and sustain its levels at the target site of the eye will significantly improve ocular drug delivery, and thus the risk of ocular complications associated with ocular injections could be omitted.[40]

Concluding remarks

Polymeric micelles have the potential to target ocular tissues at high therapeutic value offering several favourable biological properties, such as biodegradability, biocompatibility and mucoadhesiveness, which fulfil the requirements for ophthalmic application. Physicochemical characteristics such as size, surface charge, morphology, physical state of the encapsulated drug, drug release properties and stability of the nanosystems are of particular importance for topical ocular application. Polymeric micelles dispersed in aqueous solutions can be stabilized either by electrostatic stabilization or by a combination of both. Adequate surface charge of polymeric micelles can be achieved to produce a stable colloidal dispersion. Furthermore, surface charge of topically applied polymeric micelles determines their performance at the absorbing surfaces, e.g. their interactions with the cell membranes as well as the glycoproteins of the eye tissue and/or fluids (e.g. cornea, conjunctiva, tears, vitreous humor) forming a depot with prolonged release of the loaded drug. Once the drug is released from the polymeric micelles, drug molecule size, charge, lipophilicity, solubility in the eye fluid and metabolic stability determine its *in vivo* fate in the eye (e.g. trans-corneal, trans-conjunctival/scleral absorption, metabolic degradation, loss through the tear drainage). Particle size of topically applied colloidal carriers influences absorption or permeation through the ocular barriers. For example, the nanoparticles of 100 nm are able to permeate across the

corneal barrier. Surface modification or coating by biocompatible hydrophilic polymers improves uptake of polymeric micelles and enhances their stability.

ACKNOWLEDGEMENTS

This study was supported by Grant 006-0061117-1244 of the Ministry of Science, Education and Sports of the Republic of Croatia.

References

1. *Davies, N.M.*, Clin Exp Pharmacol Physiol **27** (2000) 558.
2. *Zhang, W., Prausnitz, M.R., Edwards, A.*, J Control Release **99** (2004) 241.
3. *Tiffany, J.M.*, Eye (Lond) **17** (2003) 923.
4. *Reichl, S., Kolln, C., Hahne, M., Verstraelen, J.*, Expert Opin Drug Metab Toxicol **7** (2011) 559.
5. *Urtti, A.*, Adv Drug Deliv Rev **58** (2006) 1131.
6. *Mannermaa, E., Vellonen, K.S., Urtti, A.*, Adv Drug Deliv Rev **58** (2006) 1136.
7. *Kuno, N., Fujii, S.*, Polymers **3** (2011) 193.
8. *Barar J., Asadi M., Mortazavi-Tabatabaei S.A, Omidi, Y.*, I. Ophtalmic Vis. Res. **4** (2009) 238.
9. *Hamalainen, K.M., Kananen, K., Auriola, S., Kontturi, K., Urtti, A.*, I. Ophthalmol Vis Sci **38** (1997) 627.
10. *Del Amo, E.M., Urtti, A.*, Drug Discov Today **13** (2008) 135.
11. *Gaudana, R., Ananthula, H.K., Parenky, A., Mitra, A.K.*, AAPS J **12** (2010) 348.
12. Anand-Apte, B., Hollyfield, J.G., in *Encyclopedia of the Eye*, edited by Dartt, D.A. (Elsevier Ltd, 2010).
13. *Candiello, J., Balasubramani, M., Schreiber, E.M., Cole, G.J., Mayer, U., Halfter, W., Lin, H.*, FEBS J **274** (2007) 2897.
14. *Yasukawa, T., Ogura, Y., Sakurai, E., Tabata, Y., Kimura, H.*, Adv Drug Deliv Rev **57** (2005) 2033.
15. *Yasukawa, T., Ogura, Y., Kimura, H., Sakurai, E., Tabata, Y.*, Expert Opin Drug Deliv **3** (2006) 261.
16. *Hsu, J.*, Curr Opin Ophthalmol **18** (2007) 235.
17. *Nagarwal, R.C., Kant, S., Singh, P.N., Maiti, P., Pandit, J.K.*, J Control Release **136** (2009) 2.
18. *Motwani, S.K., Chopra, S., Talegaonkar, S., Kohli, K., Ahmad, F.J., Khar, R.K.*, Eur J Pharm Biopharm **68** (2008) 513.
19. *Diebold, Y., Jarrin, M., Saez, V., Carvalho, E.L., Orea, M., Calonge, M., Seijo, B., Alonso, M.J.*, Biomaterials **28** (2007) 1553.
20. *Agnihotri, S.M., Vavia, P.R.*, Nanomedicine **5** (2009) 90.
21. *Kassem, M.A., Abdel Rahman, A.A., Ghorab, M.M., Ahmed, M.B., Khalil, R.M.*, Int J Pharm **340** (2007) 126.
22. *Attama, A.A., Reichl, S., Muller-Goymann, C.C.*, Int J Pharm **355** (2008) 307.
23. *Li, X., Nie, S.F., Kong, J., Li, N., Ju, C.Y., Pan, W.S.*, Int J Pharm **363** (2008) 177.
24. *Mishra, G.P., Bagui, M., Tamboli, V., Mitra, A.K.*, J Drug Deliv **2011** (2011) 1.
25. *Aggarwal, D., Pal, D., Mitra, A.K., Kaur, I.P.*, Int J Pharm **338** (2007) 21.
26. *Gan, L., Han, S., Shen, J., Zhu, J., Zhu, C., Zhang, X., Gan, Y.*, Int J Pharm **396** (2010) 179.
27. *Gan, L., Gan, Y., Zhu, C., Zhang, X., Zhu, J.*, Int J Pharm **365** (2009) 143.
28. *Fialho, S.L., da Silva-Cunha, A.*, Clin Experiment Ophthalmol **32** (2004) 626.
29. *Vandamme, T.F., Brobeck, L.*, J Control Release **102** (2005) 23.
30. *Yokoyama, M.*, Expert Opin Drug Deliv **7** (2012) 145.
31. *Gong, J., Chen, M., Zheng, Y., Wang, S., Wang, Y.*, J Control Release (2012)
32. *Gupta, A.K., Madan, S., Majumdar, D.K., Maitra, A.*, Int J Pharm **209** (2000) 1.
33. *Kataoka, K., Tamaki, Y., Harada, A., Tasaka, F.*, USA Patent No. US 2006/0110356 A1 (2006).
34. *Liaw, J., Chang, S.F., Hsiao, F.C.*, Gene Ther **8** (2001) 999.
35. *Tong, Y.C., Chang, S.F., Liu, C.Y., Kao, W.W., Huang, C.H., Liaw, J.*, J Gene Med **9** (2007) 956.
36. *Pepić, I., Jalšenjak, N., Jalšenjak, I.*, Int J Pharm **272** (2004) 57.
37. *Pepić, I., Hafner, A., Lovrić, J., Pirkić, B., Filipović-Grčić, J.*, J Pharm Sci **99** (2010) 4317.
38. *Di Tommaso, C., Torriglia, A., Furrer, P., Behar-Cohen, F., Gurny, R., Moller, M.*, Int J Pharm **416** (2011) 515.
39. *Di Tommaso, C., Bourges, J.L., Valamanesh, F., Trubitsyn, G., Torriglia, A., Jeanny, J.C., Behar-Cohen, F., Gurny, R., Moller, M.*, Eur J Pharm Biopharm **81** (2012) 257.
40. *Mitra, A.K., Velagaleti, P.R., Grau, U.M.*, USA Patent No. US 2010/0310642 A1 (2010).
41. *Tong, Y.C., Chang, S.F., Kao, W.W., Liu, C.Y., Liaw, J.*, J Control Release **147** (2010) 76.
42. *Civiale, C., Licciardi, M., Cavallaro, G., Giammona, G., Mazzone, M.G.*, Int J Pharm **378** (2009) 177.
43. *Yuan, X.B., Li, H., Yuan, Y.B.*, Carbohydrate Polymers **65** (2006) 337.
44. *Christie, J.G., Kompella, U.B.*, Drug Discov Today **13** (2008) 124.
45. *Carmignani, C., Rossi, S., Saettone, M.F., Burgalassi, S.*, Drug Dev Ind Pharm **28** (2002) 101.
46. *Kuwano, M., Ibuki, H., Morikawa, N., Ota, A., Kawashima, Y.*, Pharm Res **19** (2002) 108.
47. *Chetoni, P., Panichi, L., Burgalassi, S., Benelli, U., Saettone, M.F.*, J Ocul Pharmacol Ther **16** (2000) 363.
48. *Ali, Y., Lehmussaari, K.*, Adv Drug Deliv Rev **58** (2006) 1258.
49. *Sahoo, S.K., Dilnawaz, F., Krishnakumar, S.*, Drug Discov Today **13** (2008) 144.
50. Sánchez, A., Alonso, M.J., in *Nanoparticulates as drug carriers*, edited by Torchilin, V. (World Scientific-Imperial College Press, London, 2006).
51. *Lang, J.C.*, Adv Drug Deliv Rev **16** (1995) 39.
52. *Adams, M.L., Lavasanifar, A., Kwon, G.S.*, J Pharm Sci **92** (2003) 1343.
53. *Jones, M., Leroux, J.*, Eur J Pharm Biopharm **48** (1999) 101.
54. *Allen, C., Maysinger, D., Eisenberg, A.*, Colloids Surf. B. **16** (1999) 3.
55. *Nishiyama, N., Kataoka, K.*, Pharmacol Ther **112** (2006) 630.
56. *Itaka, K., Yamauchi, K., Harada, A., Nakamura, K., Kawaguchi, H., Kataoka, K.*, Biomaterials **24** (2003) 4495.
57. *Jiang, W., Kim, B.Y., Rutka, J.T., Chan, W.C.*, Nat Nanotechnol **3** (2008) 145.

58. *Rejman, J., Oberle, V., Zuhorn, I.S., Hoekstra, D.*, Biochem J **377** (2004) 159.
59. *Lavasanifar, A., Samuel, J., Kwon, G.S.*, Adv Drug Deliv Rev **54** (2002) 169.
60. *Jiao, J.*, Adv Drug Deliv Rev **60** (2008) 1663.
61. *Kim, S., Shi, Y., Kim, J.Y., Park, K., Cheng, J.X.*, Expert Opin Drug Deliv **7** (2010) 49.
62. *Savic, R., Azzam, T., Eisenberg, A., Maysinger, D.*, Langmuir **22** (2006) 3570.
63. *Kaur, I.P., Smitha, R.*, Drug Dev Ind Pharm **28** (2002) 353.
64. *Bron, A.J., Tiffany, J.M., Gouveia, S.M., Yokoi, N., Voon, L.W.*, Exp Eye Res **78** (2004) 347.
65. *Gipson, I.K.*, Exp Eye Res **78** (2004) 379.
66. *de la Fuente, M., Ravina, M., Paolicelli, P., Sanchez, A., Seijo, B., Alonso, M.J.*, Adv Drug Deliv Rev **62** (2010) 100.
67. *Lieleg, O., Ribbeck, K.*, Trends Cell Biol **21** (2011) 543.
68. *Savic, R., Luo, L., Eisenberg, A., Maysinger, D.*, Science **300** (2003) 615.
69. *Maysinger, D., Lovric, J., Eisenberg, A., Savic, R.*, Eur J Pharm Biopharm **65** (2007) 270.
70. *Yokoyama, M.*, J Exp Clin Med **3** (2011) 151.
71. *Kumari, A., Yadav, S.K., Yadav, S.C.*, Colloids Surf. B. **75** (2010) 1.