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Research paper

Novel Cyclosporin A formulations using MPEG–hexyl-substituted polylactide micelles: A suitability study

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ABSTRACT

The immunosuppressive agent Cyclosporin A (CsA) has very poor solubility in water and, in consequence, non-aqueous formulations have been developed for its intravenous administration to treat patients with transplant rejection. In this article, aqueous micelle solutions of novel amphiphilic copolymers based on methoxy-poly(ethylene glycol) (MPEG) and hexyl-substituted poly(lactides) (hexPLA) were studied for possible incorporation and formulation of CsA, and for their biocompatibility towards novel pharmaceutical applications. Above the critical micellar concentration (CMC), MPEG–hexPLA block-copolymers self-assemble into unimodal micelles with diameters of around 30 nm, either unloaded or drug-loaded. The best shelf-life stability of these formulations was observed when stored at 4 °C with a drug loss inferior to 7% after 1 year. The polymer and micelle toxicities were evaluated *in vitro* for three different cell lines and *in vivo* using the chick embryo chorioallantoic membrane (CAM) model. The hemolytic property was assessed using human blood samples. As the studies revealed, MPEG–hexPLAs are non-toxic and do not show hemolysis; the same was found for the comparable MPEG–PLAs, both as unimers below their CMC and as polymeric micelles up to copolymer concentrations of 20 mg/mL. At this concentration, CsA was efficiently incorporated into MPEG–hexPLA micelles up to 6 mg/mL, which corresponds to a 500-fold increase of its water solubility. The current recommended clinical concentration administered per infusion (0.5–2.5 mg/mL) can be easily achieved and requires four times less copolymer than with the often-used Cremophor®EL surfactant. In this regard, MPEG–hexPLA micelle formulations can be an applicable formulation in transplant rejection treatments as an injectable CsA carrier system.

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1. Introduction

To deliver drugs to the right pathological site, at the right time, in the right dose and without altering surrounding tissues is one of the important aims of drug delivery systems. For this purpose, nanocarriers, such as nanoparticles [1,2], liposomes [3] and micelles [4–6], have been studied in pharmaceutical research. Due to their small size of less than 1 μm, nanosized vehicles can improve the therapeutic and pharmacological properties of drugs by escaping the typical response of the immune system and therefore reaching and accumulating into pathological sites, such as tumors, due to the enhanced permeability and retention effect (EPR) [7]. For other pathological sites, the nanosize of micelles allows for better internalization in cells and increases the therapeutic effect of the drug compared to larger carriers [8–10].

Polymeric micelles of amphiphilic copolymers, which are composed of hydrophobic and hydrophilic blocks, are characterized by self-assembled core–shell structures in aqueous media. For pharmaceutical use, polymeric micelles with such a structure are of interest due to their ability to incorporate and protect hydrophobic drugs within their hydrophobic core, while the hydrophilic corona stabilizes and solubilizes the micelles in the aqueous environment. Usually the hydrophilic block is formed by the water-soluble, biocompatible and non-immunogenic poly(ethylene glycol) (PEG), which is known for its ability to reduce opsonization and recognition by the mononuclear phagocytic system (MPS). This consequently provides the long circulation properties of, for example, pegylated nanoparticles [11]. The hydrophobic block can be composed of various polymers, which must be non-toxic and biocompatible for pharmaceutical applications, i.e., polyethers [12], polyamino acids [13], or polyesters [14]. Amongst the hydrolysable polyesters, polycaprolactone (PCL) and polylactides (PLA) are the polymers of highest interest due to their known biodegradability, biocompatibility and their FDA status of "Generally Regarded as Safe" (GRAS). Due to its faster degradation time, PLA is often preferred to PCL in medical applications. However, PLA has limited applicability and performance as a carrier of hydrophobic drugs; the ability of micelles to solubilize drugs within their core depends on the compatibility of the core-forming block and the drug itself

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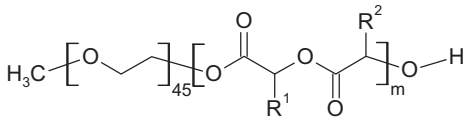
[15–17]. Therefore, novel hydrophobic and biocompatible polymers based on the biodegradable PLA backbone were developed by Trimaille et al., who synthesized different alkyl substituted polylactides by ring opening polymerization (ROP) [18]. Due to their interesting physico-chemical properties, viscous and injectable hexyl-substituted polylactides (hexPLA) were investigated for pharmaceutical applications [19]. HexPLA polymers of the two different structures, that is to say the mono- and di-hexyl-substituted polylactides (replacement of every second methyl group and all methyl groups by hexyl groups, respectively along the polymer backbone), are named here as monohexPLA and dihexPLA, respectively (Scheme 1). The presence of hexyl groups results in the highest hydrophobicity for dihexPLA, followed by monohexPLA, and the smallest for PLA. When hexPLAs are copolymerized with methoxy-poly(ethylene glycol) (MPEG), amphiphilic copolymers that self-assemble in aqueous media into polymeric micelles are formed and they incorporate hydrophobic drugs like griseofulvin [20] or the photosensitizer meso-tetra(p-hydroxyphenyl)porphine (THPP) [21] much more efficiently than comparable "standard" MPEG–PLA micelles. In this work, the very hydrophobic drug Cyclosporin A (CsA), which has a log *P* of 8.2, was formulated for the first time with MPEG–hexPLA micelles. We also investigated their biocompatibilities to determine the feasibility of these novel drug nanocarriers as conceivable pharmaceutical formulations. The neutral, cyclic, undecapeptide CsA is a potent immunosuppressive agent used to reduce graft rejection after organ transplantation [22,23]. With its rigid structure and its poor water solubility (0.012 mg/mL, determined experimentally at 25 °C), CsA must be solubilized in non-aqueous media with surfactants like Cremophor®EL, as is done for the Sandimmune® formulation (Novartis Pharma). Here, MPEG–hexPLA micelles can facilitate an aqueous formulation for the solubilization and delivery of CsA for intravenous applications. Due to this newly developed drug carrier system, the biocompatibility issues of the MPEG–hexPLA micelles needed to be addressed. Therefore, we first characterized both unloaded and CsA-loaded micelle formulations and studied their drug incorporations and shelf-life stabilities. Secondly, the biocompatibilities of the unloaded micelles and their unimers (possible compounds from disassembled micelles below their CMC that can occur under the dilution conditions in intravenous applications) were studied. These studies included (a) the *in vitro* toxicity on three different cell lines, which differed in their mammalian origins (murine or human) and their biological origins (intestinal or cancerous), (b) the *in vivo* toxicity using the chick embryo chorioallantoic membrane (CAM) model and (c) the hemolytic property using human blood. The obtained favorable and encouraging results are presented here.

2. Materials and methods

2.1. Materials

Triton® X-100 was purchased from AppliChem (Gatersleben, Germany). Tetrahydrofuran (THF) was supplied by SDS (Toulouse, France) and distilled over sodium to make it anhydrous when needed. Methoxy-poly(ethylene glycol) with a molecular weight of 2000 g/mol ($MPEG_{2000\ g/mol}$) was a gift from BASF (Ludwigshafen, Germany). D,L-lactide, tin(II) 2-ethylhexanoate ($Sn(Oct)_2$) and acetone p.a. were purchased from Purac Biochem (Gorinchem, The Netherlands), Aldrich (Buchs, Switzerland) and Fluka (Buchs, Switzerland), respectively, and used as received. The monomers, mono-hexyl-substituted lactide (monohexLA) and di-hexyl-substituted lactide (dihexLA) were synthesized as described previously (Trimaille et al., 2004). Cremophor®EL was supplied by BASF and Sandimmune® (Novartis Pharma, Bern, CH) was purchased from a local pharmacy. Cyclosporine A was supplied by Fluka (Buchs, Switzerland).



$R^1, R^2 = CH_3$: $MPEG_{2000g/mol}$-$PLA_{3000g/mol}$ for m=21
$R^1=C_6H_{13}, R^2=CH_3$: $MPEG_{2000g/mol}$-monohexPLA$_{3000g/mol}$ for m=14
$R^1, R^2 =C_6H_{13}$: $MPEG_{2000g/mol}$-dihexPLA$_{3000g/mol}$ for m=11

Scheme 1. Structure of MPEG–hexPLA and MPEG–PLA block-copolymers.

2.2. Synthesis and characterization of MPEG–(hex)PLA copolymers

Please note that in the following text, "MPEG–(hex)PLA" refers to both MPEG–PLA and MPEG–hexPLA polymers.

MPEG–hexPLA and MPEG–PLA copolymers of 5000 g/mol were synthesized by ring opening polymerization (ROP). Briefly, 3.0 g of the corresponding lactide monomer was polymerized in bulk at 100 °C with the required amount of the initiator $MPEG_{2000\ g/mol}$ to reach the targeted molecular weight. The catalyst $Sn(Oct)_2$ was added in an equimolar ratio to the initiator. The copolymerization reactions were stopped after 1.5 h by adding 5 mL of THF (1% water) into the reaction mixture. After the removal of THF by evaporation, the resulting copolymers were precipitated dropwise into 120 mL of cold methanol, then filtrated and dried under vacuum. Finally, they were analyzed by ^{1}H NMR (Brüker, 300 MHz) to detect any residual organic solvent traces.

The copolymers were characterized to determine their molecular weight (M_n) and polydispersity index (P.I.) using gel permeation chromatography (GPC). The GPC setup was composed of a Waters system with Waters Styragel HR1-3 columns and a Waters 410 differential refractometer (Waters, Milford, USA). The analysis was carried out using polystyrene (PS) of different molecular weights as calibration standards (PSS, Mainz, Germany).

2.3. Preparation and size characterization of unloaded MPEG–(hex)PLA micelles

A MPEG–(hex)PLA copolymer solution was dissolved at 100 mg/mL in acetone (2 mL) and was added dropwise under sonication into 4 mL of isotonic saline solution. The organic solvent was slowly removed by evaporation at 15 mbar. Final micelle concentrations were adjusted to 50 $mg_{copolymer}$/mL by adding isotonic saline solution. Afterwards, MPEG–(hex)PLA micelles were analyzed for their size. The hydrodynamic- (Z_{av}) and number-weighted (d_n) diameters were measured using dynamic light scattering (DLS) with a Zetasizer HS 3000 (Malvern, Worcestershire, UK). The analyses were performed at an angle of 90° at 25 °C. For each sample, the mean diameters were obtained after three runs of ten measurements.

2.4. Incorporation of CsA into MPEG–hexPLA micelles

The preparation and incorporation of CsA into MPEG–(hex)PLA micelles followed the similar co-solvent evaporation method as described for unloaded micelles. Here, CsA and 20 mg MPEG–(hex)PLA were dissolved in acetone (2 mL), and the final micelle concentrations were adjusted to 5 $mg_{copolymer}$/mL by adding ultra-pure water or isotonic saline solution. When needed, the isotonicity of micelles solution prepared in water was obtained by addition of the corresponding amounts of sodium chloride. The solutions were left to equilibrate overnight and then centrifuged at 9500*g* for 15 min to remove non-incorporated, non-soluble

CsA. For drug-loading determination, the supernatant was diluted in acetonitrile at a ratio 1:10 to break up the micelles and to release the CsA for quantitative analysis.

For more highly concentrated CsA loadings in MPEG–dihexPLA micelles, the copolymer concentration was increased up to 20 mg/mL with a targeted drug loading set at 400 $mg_{CsA}/g_{copolymer}$. To ensure complete micelle destruction before CsA quantification, a 1:100 dilution in acetonitrile was made.

The CsA concentration was quantified by HPLC using a C-18 column (250 mm × 4.6 mm) heated at 65 °C. The flow rate was 1.2 mL/min and the mobile phase was a mixture of acetonitrile/water (75:25) with a pH of 3.1 after the addition of phosphoric acid. Samples of 20 μL were injected with a running time fixed at 6 min. The CsA peak was detected by UV at λ = 210 nm and appeared at 4.1 min. The CsA calibration standards from 0.15 to 200 μg/mL were prepared, and the resulting calibration curves were obtained with a regression coefficient superior to 0.99. All samples were measured in triplicates.

2.5. Preparation of Cremophor®EL micelles

Cremophor®EL micelles were prepared by adding 400 mg of the surfactant into 4 mL isotonic saline solution with stirring to reach a final concentration of 100 mg/mL. They have been characterized by size as previously mentioned for unloaded MPEG–(hex)PLA micelles.

2.6. Cell culture

A Caco-2 cell line, a human epithelial colorectal adenocarcinoma cell line was maintained in Dulbecco's Modified Eagle's medium (DMEM) (Gibco Life Technologies, Carlsbad, USA) supplemented with 10% fetal bovine serum (FBS) (Brunschwig, Amsterdam, The Netherlands), 1% of non-essential amino acids and 1% of penicillin and streptomycin [24]. The cells were grown for 2 weeks to allow for monolayer formation and cell differentiation [25].

A NuTu-19 cell line, a poorly differentiated Fischer 344 rat-derivative epithelial ovarian cancer cell line [26], was kindly provided by Dr. A. Major (Geneva University Hospital, Geneva, Switzerland). The cells were cultured in DMEM culture medium supplemented with 10% FBS and 100 U/mL penicillin–streptomycin (Gibco Life Technologies).

A SKOV-3 (HTB-77) cell line, a human ovarian carcinoma cell line, was purchased from American Tissue Culture Collection (ATCC, Manassas, USA). The cells were grown in Roswell Park Memorial Institute medium (RPMI 1640) (Gibco Life Technologies) and supplemented with 10% FBS, 1% l-glutamine and 1% penicillin/streptomycin.

All cell lines were cultured at 37 °C in a humidified atmosphere containing 5% CO_2.

2.7. In vitro toxicity

The *in vitro* toxicity of unloaded micelles was determined by standard MTT tests on Caco-2, NuTu-19 and SKOV-3 cells. Briefly, the cells were seeded in a 96-well plate at a density of 1.5×10^4 cells per well in 100 μL of culture medium and incubated in a humidified atmosphere with 5% CO_2 at 37 °C for 24 h. After removal of the culture media and washing with PBS, the cells were incubated with 100 μL MPEG–(hex)PLA and Cremophor®EL micelle solutions or isotonic saline solution. After 24-h incubation, 50 μL of a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution of 1 mg/mL in PBS was added to each well, and the plate was incubated for 3 h to allow the soluble yellow MTT to be reduced into the dark-blue, insoluble formazan crystals by the metabolically active cells. Afterwards, the formazan crystals were dissolved by the addition of 200 μL dimethyl sulfoxide (DMSO) in the incubator at 37 °C for 1 h. The UV absorbance of individual wells was measured at 595 nm with a microplate reader (Model 550, Bio-RAD, Hercules, USA).

Formulation concentrations below the CMC (0.01 $mg_{copolymer}$/mL) were studied to determine the toxicity of MPEG–(hex)PLA as unimers, whereas concentrations above the CMC yielded the toxicity of MPEG–(hex)PLA as micellar structures. Different copolymer concentrations were obtained by dilution in culture media from the most concentrated MPEG–(hex)PLA formulation (50 $mg_{copolymer}$/mL). The same procedure was carried out for Cremophor®EL solutions, which have a CMC of 0.9 mg/mL. The culture media and a 0.5% Triton® X-100 solution in 1 N NaOH were used as positive (100% survival) and negative (0% survival) controls, respectively.

The cell viability was determined by the following formula:

$$\text{Cell viability } (\%) = \frac{A_{\text{sample}} - A_{0\%}}{A_{100\%} - A_{0\%}} \times 100$$

where A_{sample}, $A_{0\%}$ and $A_{100\%}$ are the respective absorbances of the sample, the negative and positive control.

2.8. In vivo toxicity by the CAM model

The *in vivo* toxicity of the MPEG–(hex)PLA micelles was assessed by using the chick embryo chorioallantoic membrane (CAM) model adapted from Lange et al. [27]. The egg incubation procedure followed the one described by Vargas et al. [28]. Briefly, fertilized hen eggs, kindly provided by the Animalerie Universitaire of the University of Geneva (Geneva, Switzerland), were placed with the narrow apex down into an incubator Savimat MG 200 at 37 °C with a relative humidity of 65%. The eggs were rotated twice a day until the embryo development day 3 (EDD3). On EDD4, a 3-mm hole was drilled into the eggshell and the narrow apex was covered by an adhesive tape. The eggs were incubated in a static mode until EDD12. After the removal of the tape, a bigger hole of 2–3 cm was drilled, thus allowing the visualization of the CAM vasculature. Portions of 20 μL of unloaded MPEG–(hex)PLA formulations were injected via the main vessel. The eggs were returned to the incubator and the survival rate was evaluated at 24-h post-injection.

The unloaded MPEG–(hex)PLA micelles at 0.0001, 5, 10 and 50 $mg_{copolymer}$/mL from five chick egg embryos were investigated. The highest concentrated micelle solution of 50 $mg_{copolymer}$/mL was used as prepared and the other three were obtained by dilution with isotonic saline solution. The isotonic saline solution itself was used as the 100% survival control.

2.9. Hemolysis test

Blood samples were freshly collected from healthy human volunteers in acid citrate dextrose-coated (ACD) tubes at the Blood Transfusion Center (Geneva University Hospital, Geneva, Switzerland). The formulation samples were incubated with blood at a 3:1 sample/blood ratio at 37 °C for 24 h. After centrifugation at 770*g* for 10 min, the supernatant was collected in a 96-well plate and the release of hemoglobin was measured by UV absorbance at 575 nm with four points of measurement per well using a microplate reader (Safire, Tecan, Männedorf, Switzerland).

Various concentrations (below and above the CMC) of unloaded MPEG–(hex)PLA, CsA-loaded MPEG–dihexPLA micelles and Sandimmune® were tested. Samples of different concentrations were prepared by dilution of the highest copolymer or surfactant concentration with isotonic saline solution. Both the concentrated CsA-loaded MPEG–dihexPLA formulations and the concentrated Sandimmune® were diluted in order to obtain final

CsA concentrations of 0.5 mg/mL and of 2.5 mg/mL. An isotonic saline solution and 1% Triton® X-100 solution were tested as the 0% lysis control and 100% lysis control, respectively.

The percentage of hemolysis was calculated as follows:

$$\text{Hemolysis } (\%) = \frac{A_{\text{sample}} - A_{0\%}}{A_{100\%} - A_{0\%}} \times 100$$

where A_{sample}, $A_{0\%}$ and $A_{100\%}$ represent the absorbances of the sample, the negative and positive control, respectively.

2.10. Shelf-life stability

The size stability of unloaded and CsA-loaded micelles in ultra-pure water was investigated at 25 °C over 1 year. The shelf-life of CsA-loaded micelles in isotonic saline solution in terms of drug content and size was studied at three different temperatures (4 °C, 25 °C and 37 °C) over 3 months and 1 year.

The micelle size was determined by DLS at multiple detection angles with a goniometer ALV/CGS-5 (ALV-GmbH, Langen, Germany) and a power of 0.2 W. Briefly, 100 μL of unloaded or CsA-loaded MPEG–(hex)PLA micelle formulations was diluted in 2 mL of isotonic saline solution or ultra-pure water in a clean, clear and capped tube resulting in a copolymer concentration of 0.24 mg/mL. After initial size measurements, the samples were stored at their respective storage conditions. At the desired time points, they were allowed to equilibrate to room temperature for 2 h before being analyzed directly in the sample tubes.

A similar procedure was applied for the formulation stability tests. After the desired storage time, the CsA-loaded MPEG–(hex)PLA formulation samples were allowed to equilibrate to room temperature and then centrifuged at 9500*g* for 15 min to remove any non-entrapped CsA. The CsA drug content in micelles was quantified by the HPLC method described above.

3. Results

3.1. Synthesis of MPEG–(hex)PLA copolymers

The MPEG–monohexPLA and MPEG–dihexPLA copolymers were prepared by ring opening polymerization (ROP) in bulk using $Sn(Oct)_2$ as the catalyst and $MPEG_{2000\ g/mol}$ as the initiator. These molecular structures are presented in Scheme 1. The MPEG–PLA was synthesized by the same method and was used as the "reference". Copolymers with molecular weights of 5000 g/mol (±1 monomer unit) were obtained with low polydispersity indices (P.I.) smaller than to 1.1 (Table 1). The MPEG–hexPLAs of the same molecular weight have half and double number of hydrophobic hexyl side groups, respectively, thus permitting us to investigate the influence of hydrophobicity on the micellization, stability, drug loading and toxicity of their resulting micelles.

3.2. Unloaded MPEG–hexPLA micelles

Unloaded MPEG–hexPLA micelles were prepared in isotonic saline solution by the co-solvent evaporation method and had the number-weighted (d_n) sizes between 18 and 27 nm with a population of 99–100% (Table 1). The values obtained for the hydrodynamic diameter (Z_{av}) showed sizes of 70–90 nm with a polydispersity between 0.22 and 0.35. This higher polydispersity is related to a minor population of some larger micelles, which could be filtered off.

Table 1
Characteristics of synthesized MPEG–(hex)PLA copolymers and their corresponding unloaded micelles prepared in isotonic saline solution.

Copolymer	Mw[a] (g/mol)	P.I.[a]	Micelle size			
			d_n (nm)	[%]d_n	Z_{av} (nm)	P.I.
MPEG–PLA	5100	1.07	18	100.0	75	0.35
MPEG–monohexPLA	5000	1.03	26	99.1	70	0.22
MPEG–dihexPLA	5300	1.05	27	99.6	87	0.31

[a] Determined by GPC using PS standards.

3.3. CsA incorporation in MPEG–hexPLA micelles

Formulations with a copolymer concentration of 5 mg/mL and different targeted drug loadings from 10 to 1000 $mg_{CsA}/g_{copolymer}$ were prepared. The amount of incorporated CsA was assessed by HPLC after micelle disruption by the addition of acetonitrile. The results are summarized in Fig. 1, where the incorporation efficiency of 100% and 80% is drawn in plain and dashed lines, respectively. As seen in the figure, the CsA loading increased with the targeted loadings until a plateau at 80 $mg_{CsA}/g_{copolymer}$ for MPEG–PLA, 230 $mg_{CsA}/g_{copolymer}$ for MPEG–monohexPLA and 300 $mg_{CsA}/g_{copolymer}$ for MPEG–dihexPLA copolymer was reached. At the latter angular points, loadings with efficiencies of 80% and higher could be achieved. For MPEG–monohexPLA micelles, the highest and most efficient drug loading of 230 $mg_{CsA}/g_{copolymer}$ was obtained from the targeted loading of 300 mg/g, with an incorporation efficiency of 77%. For the same target loading, MPEG–dihexPLA incorporated 265 $mg_{CsA}/g_{copolymer}$ with an efficiency of 88%. For the higher targeted loading of 500 mg/g, the highest drug loading of 320 mg/g for a MPEG–hexPLA formulation could be achieved, still with an efficiency of 65%. Any non-incorporated drug could be removed by centrifugation of the formulation.

Micelle sizes, Z_{av} and d_n of CsA-loaded MPEG–(hex)PLA micelles were determined by DLS at a detection angle of 90°. The results for different drug loadings are presented in Fig. 2, where the sizes are given on the primary *Y*-axis and the [%]d_n on the secondary axis. The MPEG–(hex)PLA micelles had a number-weighted diameter between 16 and 30 nm for all tested formulations. The sizes were identical to the unloaded micelle solutions (Table 1). It is notable that the average hydrodynamic diameter Z_{av} increased with the incorporation amount of CsA in MPEG–hexPLA micelles, which was due to the presence of a small number of larger micelles in contrast to unloaded micelle formulations. Still, 98–99% of all the micelles had sizes d_n smaller than 30 nm (Fig. 2). For the formulations envisioned for intravenous application, isotonic saline solutions were prepared by first generating MPEG–hexPLA polymeric micelles in water and then adjusting the solution isotonicity with the addition of NaCl. The average hydrodynamic diameter, $Z_{av,}$ increased with the isotonicity change in the medium (Table 2), whereas the diameter d_n remained at 25 nm. The CsA solubility in polymeric micelles was found to be a little bit lower with 1.4 mg/mL, when compared to 1.5 mg/mL in water. The more

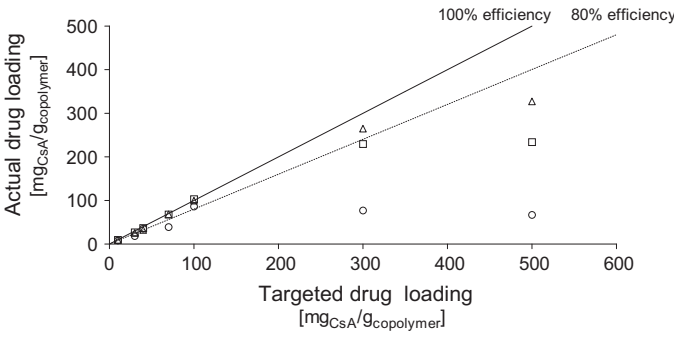


Fig. 1. Incorporation of CsA in MPEG–PLA (○), MPEG–monohexPLA (□) and MPEG–dihexPLA (△) micelles in function of the targeted drug loading.

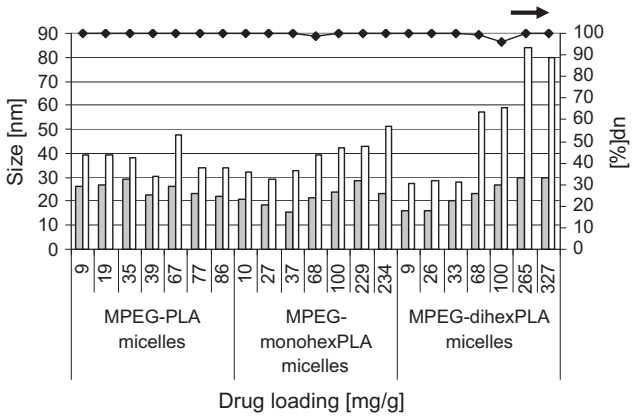


Fig. 2. Size characteristics of CsA-loaded PEG-(hex)PLA micelles in dependence of the drug loading with the hydrodynamic diameter Z_{av} (□), the number-weighted diameter d_n (■) and the percentage of micelles with a given d_n ([%]d_n (●)).

hydrophobic MPEG–dihexPLA micelles incorporated higher amounts of CsA than MPEG–monohexPLA and MPEG–PLA-based formulations. More highly concentrated micelle solutions of 6 mg/mL could be prepared by increasing the MPEG–dihexPLA copolymer concentration in the medium while keeping the same drug to copolymer ratio.

3.4. In vitro toxicity of MPEG–hexPLA solutions

To evaluate the *in vitro* toxicity of the unloaded MPEG–(hex)PLA micelles, three different cell lines were tested: human intestinal epithelial Caco-2 cells, murine NuTu-19 and human SKOV-3 ovarian cancer cells. Various copolymer concentrations were investigated, both below the CMC for studying the toxicity of MPEG–(hex)PLA as unimers and above the CMC for studying the micelle solutions. The isotonic saline solution and Cremophor®EL solutions were tested for comparison (Fig. 3). Triton X-100 and culture media were used as 0% survival and 100% survival controls, respectively.

After 24-h incubation at 37 °C on Caco-2 cells (Fig. 3a), MPEG–(hex)PLA polymeric micelles were non-toxic with a cell survival above 80% for copolymer concentrations ranging from 0.0001 to 25 $mg_{copolymer}$/mL, whereas Cremophor®EL micelles showed no toxicity only for solutions with low concentrations (<1 mg/mL). Negative cell survivals were observed for higher concentrations, which can be explained by a washing-out of the cells from the test plates. On NuTu-19 cells, MPEG–(hex)PLA micelles were non-toxic for concentrations up to a 10 $mg_{copolymer}$/mL for MPEG–dihexPLA and MPEG–PLA solutions, and up to 1 $mg_{copolymer}$/mL for the comparable MPEG–monohexPLA solutions (Fig. 3b). Cremophor®EL micelles showed no toxicity for low concentrations (<0.5 mg/mL). On the human SKOV-3 ovarian cancer cells, the three MPEG–(hex)PLA micelle solutions were non-toxic up to concentrations of 10 $mg_{copolymer}$/mL, whereas Cremophor®EL micelles started to show a toxic effect for a concentration of 1 mg/mL (Fig. 3c). At higher concentrations, MPEG–hexPLA micelles showed a slightly better cell viability than compared to MPEG–PLA micelles.

3.5. In vivo toxicity of MPEG–hexPLA micelle solutions

In vivo toxicity of MPEG–(hex)PLA micelles was evaluated using the CAM model. The chick embryo survival was determined 24 h after the intravascular injection of 20 μL micelle solution for four different copolymer concentrations of 0.0001, 5, 10 and 50 mg/mL. All tested concentrations of MPEG–(hex)PLA formulations were non-toxic, since 100% of the tested chick embryos were still alive at this crucial time point.

3.6. Hemolysis test of MPEG–hexPLA micelle solutions

The hemolytic property of MPEG–(hex)PLA solutions was assessed on fresh human blood after incubation at 37 °C for 24 h. The method was adapted from a hemolysis test protocol described earlier by Mottu et al. [29]. The percentage of hemolysis was determined for various copolymer concentrations (Table 3). When compared to the control of an isotonic saline solution, the unloaded MPEG–(hex)PLA micelle solutions showed less than 1% of red blood cell lysis up to a copolymer concentration of 20 $mg_{copolymer}$/mL. Likewise, under much diluted conditions that were below the CMC, the non-hemolytic activity of MPEG–(hex)PLA unimers was proven (Table 3a). For the highest concentration of 50 $mg_{copolymer}$/mL, which is much higher than the formulation concentration that is considered to be practical, a hemolysis of 2.0% for MPEG–dihexPLA, 9.2% for MPEG–monohexPLA and 9.7% for MPEG–PLA was observed. The incorporation of CsA into MPEG–dihexPLA micelles did not induce any red blood cell lysis (Table 3b). A formulation of CsA in MPEG–dihexPLA (20 mg/mL) with a CsA concentration of 6 mg/mL affected less than 1% lysis (Entry 1). Also, the formulations with lower concentrations that are similar to those used in current clinical CsA doses (Entries 2 and 3) do not show hemolysis.

3.7. Shelf-life stability

The sizes and possible size changes of MPEG–hexPLA micelles were monitored using DLS at different detection angles over a period of 1 year. At small detection angles of 30–40°, large particles scatter more light than small particles, whereas at larger detection angles of 50–140°, mainly the small particles are detectable. Therefore, it is important to measure micelle sizes at the full range of detection angles in order to observe a possible micelle enlargement or aggregation with time. The results obtained for MPEG–hexPLA micelles prepared in ultra-pure water and stored at 25 °C for 1 year are given in Fig. 4a. Directly after preparation, the unloaded MPEG–hexPLA micelles had an average hydrodynamic diameter (observation between 50° and 140°) of around 30 nm for MPEG–monohexPLA and of around 40 nm for MPEG–dihexPLA micelles, respectively. Only a few micelles larger than 50 nm were visible at the small angles of 30–40°. After 1 year of storage, the size curves of the initial and stored solutions were similar, except at the low detection angle of 30°, where some larger micelles in the

Table 2
Characteristics of CsA-loaded MPEG–dihexPLA micelles with different copolymer concentrations in different aqueous media.

MPEG–dihexPLA concentration (mg/mL)	Aqueous medium	CsA loading ($mg_{CsA}/g_{copolymer}$)	CsA concentration ($mg_{CsA}/mL_{micelles}$)	Size d_n (nm)	[%]d_n	Size Z_{av} (nm)	P.I.
5	Water	307 ± 1	1.51 ± 0.01	26	100.0	40	0.24
5	Isoton. Saline Sol.	286 ± 11	1.43 ± 0.06	25	99.7	83	0.50
10	Water	318 ± 4	3.23 ± 0.05	26	100.0	40	0.24
20	Water	295 ± 5	5.97 ± 0.11	27	99.7	41	0.28

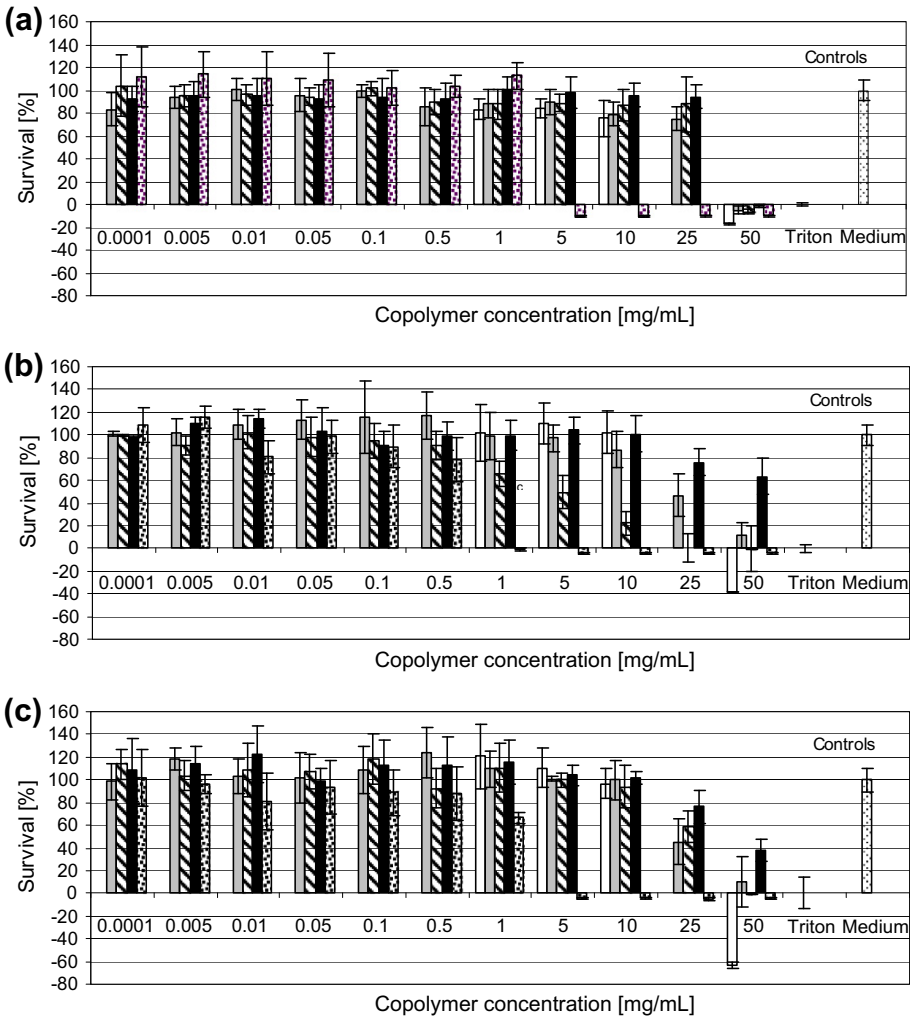


Fig. 3. In vitro toxicity on (a) Caco-2, (b) Nutu-19 and (c) SKOV-3 cells of MPEG–PLA (▒), MPEG–monohexPLA (▒), MPEG–dihexPLA (■), Cremophor®EL (▒) and NaCl 0.9% (□) for different concentrations. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 3
Hemolytic activity of (a) unloaded and (b) CsA-loaded MPEG–hexPLA micelles of different copolymer concentrations after 24-h incubation with human blood.

(a)

Copolymer concentration (mg/mL)	% Lysis		
	MPEG–dihexPLA	MPEG–monohexPLA	MPEG–PLA
50	2.0 ± 0.9	9.2 ± 3.6	9.7 ± 2.3
0.01–20	<1	<1	<1
0.0001–0.005 [a]	<1	<1	<1

(b)

Cremophor®EL				MPEG–dihexPLA	
Entry	CsA concentration (mg/mL)	Surfactant concentration (mg/mL)	% Lysis	Copolymer concentration (mg/mL)	% Lysis
1	6	78	<1	20	<1
2	2.5	32.5	<1	8.3	<1
3	0.5	6.5	<1	1.7	<1
4	–	<0.09[a]	<1	<0.008[a]	<1

[a] Concentrations below CMC.

stored solutions were observable. For all other detection angles, size changes of only 10 nm for MPEG–dihexPLA and of 17 nm for MPEG–monohexPLA were observed. The formation of larger micelles at small angles was also found for CsA-loaded micelles (Fig. 4b). This was more pronounced for MPEG–monohexPLA micelles, for which a substantial increase of the diameter occurred at detection angles between 30° and 60°, showing the existence of 1–2% of larger micelles in the stored solutions. For all other detection angles, the difference in diameter values after 1 year at 25 °C varied in the same range as for the unloaded micelles. The results from intermediate time points (data not shown) revealed that micelle size enlargement or aggregation started only after five months for CsA-loaded MPEG–monohexPLA and MPEG–PLA micelles.

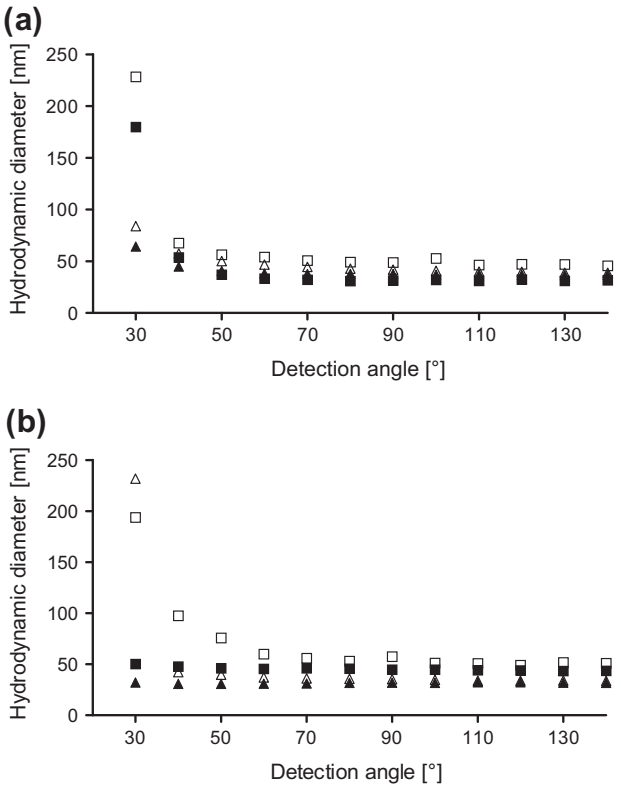


Fig. 4. Hydrodynamic diameters Z_{av} at different detection angles of (a) unloaded and (b) CsA-loaded MPEG–monohexPLA (■,□) and MPEG–dihexPLA (▲,△) micelles in ultra-pure water directly after preparation (filled symbols) and after 1 year (unfilled symbols) at 25 °C.

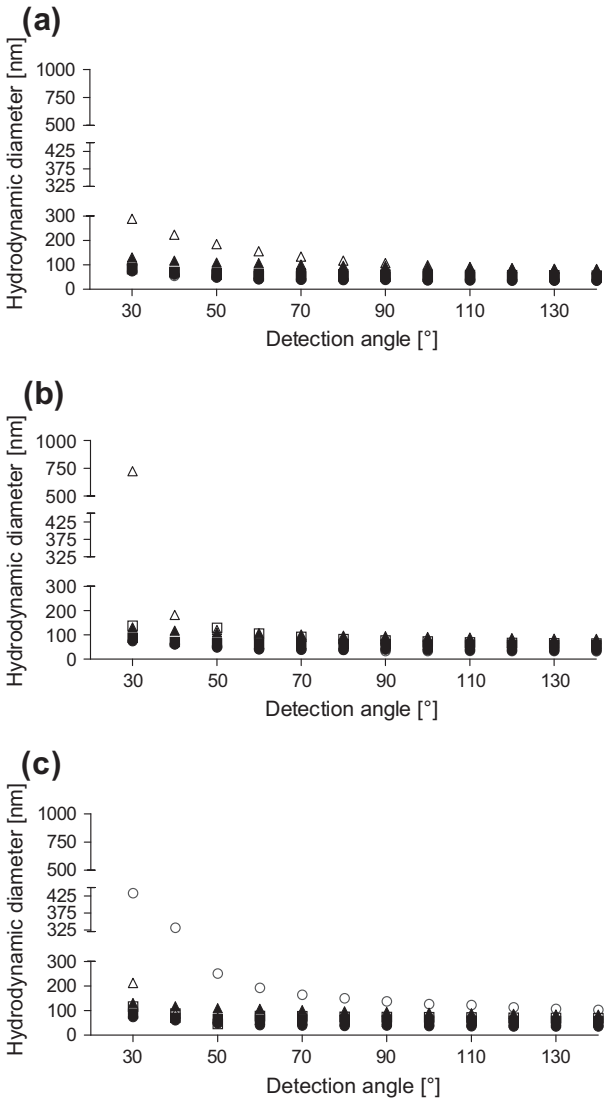


Fig. 5. Hydrodynamic diameters Z_{av} at different detection angles of CsA-loaded MPEG–PLA (●,○), MPEG–monohexPLA (■,□) and MPEG–dihexPLA (▲,△) polymeric micelles in isotonic saline solution directly after preparation (filled symbols) and after 3 months (unfilled symbols) at (a) 4 °C, (b) 25 °C and (c) 37 °C.

MPEG–(hex)PLA micelle formulations in isotonic saline solution were assessed for their shelf-life stability, including a size and drug content evaluation. Fig. 5 shows the micelle hydrodynamic diameters of the three micelle solutions at (a) 4 °C, (b) 25 °C and (c) 37 °C. Here, the formation of larger micelles was visible at detection angles between 30° and 60° after three months of storage time. The size change in these formulations was influenced by the storage temperature. Larger micelles were observed at 37 °C (200–450 nm) than at 25 °C (100–750 nm) and at 4 °C (150–220 nm). The drug content of CsA-loaded MPEG–(hex)PLA formulations in isotonic saline solution at the same storage temperatures was followed up over time (Fig. 6). The CsA content in micelles remained most stable at 4 °C, with a drug loss of less than 7% for all tested formulations after 1 year. At 25 °C, the drug loss increased to 26%, 82% and 100% for MPEG–dihexPLA, monohexPLA and MPEG–PLA micelles, respectively. At 37 °C, the entire incorporated amount of CsA was released after three months in MPEG–PLA micelles, after seven months in MPEG–monohexPLA and 13 months in MPEG–dihexPLA micelles.

4. Discussion

Polymeric micelles based on MPEG–hexPLA are characterized by an increased hydrophobicity of the micelle core in comparison with the standard MPEG–PLA. It has been demonstrated that this enables higher drug loadings of poorly water-soluble drugs [21]. In this paper, we investigated the biocompatibility of these novel micelles towards formulations of the poorly water-soluble drug Cyclosporin (CsA) for intravenous applications.

The novel copolymers, MPEG–monohexPLA and MPEG–dihexPLA, and the "reference" standard MPEG–PLA were synthesized in a controlled manner by ROP with a defined molecular weight of 5000 g/mol (±1 monomer unit), thus allowing a good comparison of the influence of the introduced hexyl substituents along the PLA backbone. The increase of hydrophobic interactions between copolymer chains favors micellization at lower critical micellar concentrations (CMC). Indeed, the CMCs of MPEG–hexPLA micelles decrease with the increase of hexyl groups in the core-forming block, leading to a value of 1.0036×10^{-6} M and 1.7×10^{-6} M for MPEG–dihexPLA and monohexPLA compared to a value of 2.0×10^{-6} M for MPEG–PLA micelles [19]. These values in the micro molar range are in agreement with data reported in the literature [30,4]. Compared to MPEG–PLA and to other typical surfactants, which have CMCs with 10–1000 times higher concentrations [5], the low CMC of MPEG–hexPLA micelles facilitates better stability upon dilution, which can improve these formulations for envisioned intravenous applications.

Moreover, MPEG–hexPLA micelles are very small and truly nanocarriers. Around 99% of MPEG–hexPLA micelles had number-weighted diameters (d_n) between 18 and 30 nm, confirming a unimodal size distribution in the unloaded (Table 1) or CsA-loaded

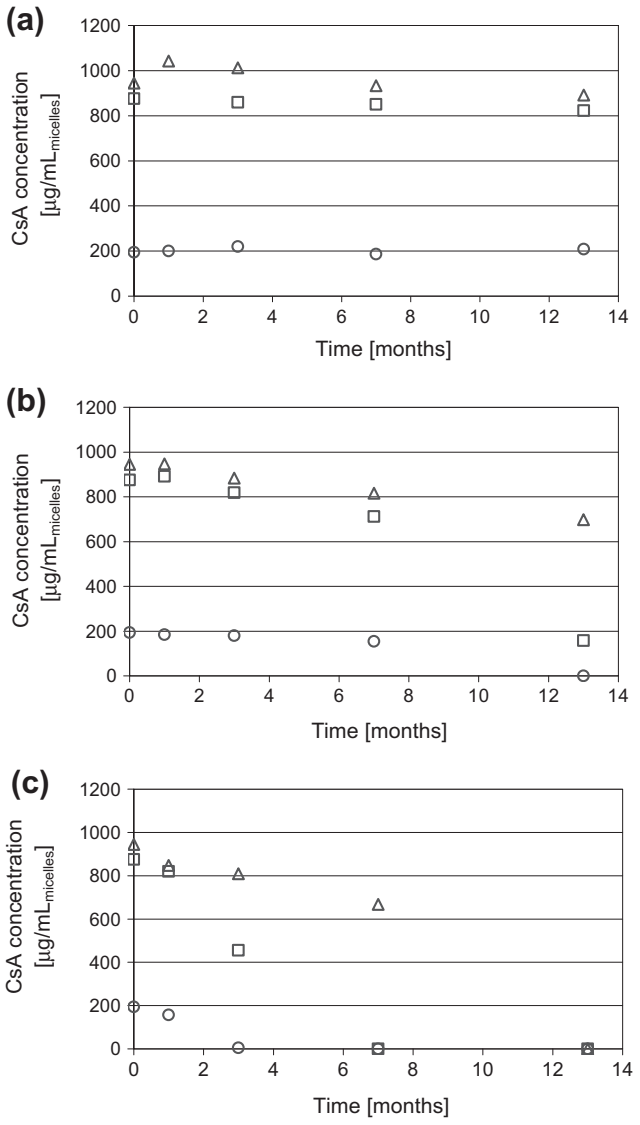


Fig. 6. Formulation stability of CsA incorporated in MPEG–PLA (○), MPEG–monohexPLA (□) and MPEG–dihexPLA (△) micelles in isotonic saline solution (a) at 4 °C, (b) 25 °C and (c) 37 °C ($n = 3$, standard deviation for most time points smaller than symbols).

state (Fig. 2). The incorporation of CsA into the micelles did not influence the micelle size, d_n. As a nanosized drug delivery system, MPEG–hexPLA micelles should have the same advantages as described for other polymeric micelles, in particular the ability to escape the mononuclear phagocyte system (MPS) uptake and renal clearance, allowing a long circulation in the body, which should result in a higher probability of reaching the target [30].

The potential of MPEG–hexPLA micelles to solubilize the hydrophobic drug CsA was herein demonstrated in comparison with MPEG–PLA micelles and the Cremophor®EL surfactant. Comparing the obtained CsA loadings for MPEG–hexPLA with the corresponding MPEG–PLA formulations, it becomes obvious that the increased hydrophobicity of the new hexPLA-based micelles improve the loading and the solubility capacity. In aqueous MPEG–hexPLA formulations, CsA was solubilized very efficiently up to the concentration of 1.5 mg/mL with 5.0 mg/mL MPEG–dihexPLA excipient (Table 2). Increasing the concentration of the MPEG–hexPLA copolymer while keeping the drug/copolymer ratio constant leads to higher concentrated formulations. At comparable polymer concentrations of 10 mg/mL, MPEG–hexPLA could incorporate twice the amount of CsA as MPEG–polycaprolactone ($MPEG_{5000\ g/mol}$–$PCL_{13,000\ g/mol}$) micelles (~1.3 mg/mL) as reported by Aliabadi et al. [31]. Formulations with 20 mg/mL MPEG–hexPLA facilitated a CsA concentration of 6 mg/mL, corresponding to an increase in water solubility of CsA by a factor of 500. For comparable CsA concentrations, MPEG–dihexPLA formulations would require four times less excipient than a currently marketed formulation with Cremophor®EL as the surfactant.

Toxicity studies carried out on three different cell lines showed that MPEG–hexPLA micelles had a lower toxicity than formulations with Cremophor®EL micelles on human ovarian cancer SKOV-3 cells. On the two other cell lines, Cremophor®EL formulations at higher concentrations than 0.5 mg/mL washed out cells from the test plates, and thus cell viabilities could not be determined (Fig. 3). This was not the case for MPEG–hexPLA micelle formulations, for which non-toxicity could be found for concentrations at least up to 10 mg/mL.

For possible intravenous pharmaceutical applications, isotonic micelle solutions of MPEG–hexPLA micelles were prepared first in water and then adjusted by the addition of NaCl crystals before use. By this procedure, CsA formulations with a concentration of 1.4 mg/mL could be more efficiently prepared and the loss of drug observed in other procedures could be significantly reduced. For example, by direct preparation in isotonic saline solution, MPEG–hexPLA micelles could solubilize around 1 mg/mL CsA, which corresponds to an incorporation of 48% (results not shown) as opposed to 73% when prepared in water and adjusted afterwards to isotonicity. This was also observed for other copolymers like $MPEG_{5000\ g/mol}$–$PCL_{13,000\ g/mol}$ micelles, which incorporated CsA with 28% or 37% efficiency when prepared in isotonic solution, whereas in water a 37% or 64% efficiency could be achieved, depending on the initial applied drug loading [32]. The choice of the medium is not only of importance for the drug-loading efficiency, but it is also an important factor regarding the stability of the micelle formulations. In isotonic saline solutions, the formation of larger micelles was observable after three months, whereas in water, no size increase could be detected for MPEG–dihexPLA formulations after 1 year of storage (Fig. 4). A micelle size enlargement after addition of salt has also been observed by Jain et al. and has been explained by the dehydratation effects on the PEG units of the micelle shell [33]. For the investigated formulations, the increase in micelle diameter was dependent on the storage temperature and the copolymer. A storage temperature of 4 °C showed the best formulation shelf-life stability, whereas in the isotonic saline solution only a very few larger MPEG–hexPLA micelles were observed after three months (Fig. 5), still with diameters below 200 nm, which is the maximum size for particles with long-circulating properties [34]. To avoid their potential consequences, larger micelles or aggregates could be simply filtered off with common sterile filters after extended storage times. Regarding the CsA concentrations in the MPEG–hexPLA formulations, the CsA amounts remained very constant over 1 year when stored at 4 °C, with a final drug loss of only 7% (Fig. 6). In contrast, at 25 °C and 37 °C, the initial drug content in the micelles decreased more rapidly. At 37 °C, a complete CsA release was observed at earlier time points; the MPEG–hexPLA micelles showed a higher stability than the MPEG–PLA micelles. A recent paper of Nottelet et al. have also demonstrated the superior stability of MPEG–hexPLA micelles in PBS modeling intravenous conditions (pH 7.4 at 37 °C) compared to MPEG–PLA micelles [35]. These results could be associated with the degradation of the polymers under these conditions. Previous studies on hexPLA polymers have shown that, despite a similar degradation profile, the homopolymer PLA had a faster molecular weight decrease and weight loss than hexPLA in PBS at pH 7.4 after 50 days [19]. Thus, the slower polymer degradation and the lower CMC increase the excipient stability in the formulations and

stabilize the solubilized CsA drug content. Nevertheless, the data obtained here for the higher temperatures indicate that the carrier system has the properties of a certain shelf-life stability next to its ultimate degradation and drug release, when applied in the human body.

The biocompatibility aspects of both MPEG–hexPLA unimers and micelles were investigated by studying their toxicity *in vitro* and *in vivo* and by assessing their hemolytic activity on red blood cells. Practically, the biocompatibility of unimers was carried out by modeling the dilution effect after intravenous application and thus the disassembly of micelles. Unloaded MPEG–hexPLA micelles were tested on three cell lines with different mammalian origins and from different sources: human epithelial intestinal cells (Caco-2), murine (NuTu-19) and human ovarian cancer cells (SKOV-3). Non-toxicity was found for MPEG–dihexPLA unimers and for their polymeric micelles at least up to 10 $mg_{copolymer}$/mL on all tested cell lines and was comparable to the results obtained for MPEG–PLA copolymers as controls (Fig. 3). In addition to the MTT tests, the *in vivo* toxicity of MPEG–hexPLA micelles was studied on the CAM model. This model is an alternative to mammalian models and has been proven helpful for testing intravenous formulations due to its well-developed vasculature network [36]. Injection volumes of 20 μL into the vasculature of the chick embryos were found to be well-tolerated [28] and were therefore chosen for the toxicity studies of the MPEG–hexPLA micelle solutions. The survival rate of chick embryos was assessed 24 h after injection, since longer times usually give identical results. The CAM model results showed neither a toxicity for MPEG–hexPLA unimers nor for the MPEG–hexPLA micelle formulations of the three tested concentrations. All chick embryos survived the injection of the formulations with the novel excipient. It has to be pointed out that MPEG–hexPLA micelles were non-toxic up to a concentration of 50 $mg_{copolymer}$/mL, the same as was found for the control MPEG–PLA micelle solution, whose biocompatibility has been reported in the literature [37]. To add to these toxicity studies, the compatibility with human blood was assessed. The hemolytic property of MPEG–hexPLA unimers and unloaded micelles was investigated at different concentrations and showed no hemolytic activity (<1% lysis) up to a copolymer concentration of 20 mg/mL. A slight hemolytic activity was observed for the three different MPEG–(hex)PLA micelles for the "extreme" copolymer concentration of 50 $mg_{copolymer}$/mL, which is far above the concentrations possibly needed for an envisioned intravenous application. Also, under these conditions, the novel hexPLA-based micelles did not differ in their hemolytic activity from the standard PLA-based micelles.

Considering the practical application of CsA MPEG–hexPLA formulations, the corresponding drug concentrations of 0.5 mg/mL up to 2.5 mg/mL, as used in the current Cremophor®EL based products, were found to not induce hemolysis. The high drug-loading capacity of MPEG–hexPLA micelles allows a significant reduction of polymeric excipient for formulating the same amounts of CsA. This increases the potential intravenous use of such micelle formulations, which may improve the maximum tolerated dose (MTD) in the treatment. In a previous study, MPEG–PLA micelles could increase four times the MTD of paclitaxel in nude mice compared to the current treatment with Taxol®, which is also formulated with Cremophor®EL [38]. In addition, regarding the influence of the physical state of the hydrophobic core-forming block on the micelle stability, MPEG–hexPLA micelles are expected to retain the drug more efficiently than Cremophor®EL micelles [37,31].

5. Summary and conclusions

In this paper, we demonstrated the biocompatibility and non-toxicity of polymeric micelles based on MPEG–hexPLA. The amphiphilic MPEG–hexPLA copolymers self-assembled in aqueous media into polymeric micelles of 30 nm. The resulting micelle formulations show no toxicity and no hemolytic activity in MTT tests, the CAM model and on human blood tests. All results are comparably good or better than those obtained for the controls of standard MPEG–PLA. By increasing the MPEG–hexPLA copolymer concentration while keeping the drug:copolymer ratio constant, the immunosuppressive and hydrophobic drug Cyclosporin A could be solubilized and formulated with concentrations up to 6 mg/mL, which is equivalent to a 500-fold increase of the drug's water solubility. The current clinically used CsA concentration administrated intravenously with Sandimmune® can be prepared in a MPEG–hexPLA micelle formulation by a simple procedure and would need four times less copolymer than is used with the surfactant Cremophor®EL. MPEG–hexPLA micelles have the potential to be very interesting non-toxic injectable nanosized drug carriers for improved formulations of poorly water soluble drugs.

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