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RESEARCH PAPER

Drug Loading of Polymeric Micelles

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ABSTRACT

Purpose To gain mechanistic insights into drug loading and lyophilization of polymeric micelles.

Methods PEGylated poly-4-(vinylpyridine) micelles were loaded with dexamethasone. Three different methods were applied and compared: O/W emulsion, direct dialysis, cosolvent evaporation. Micellar dispersions with the highest drug load were lyophilized with varying lyoprotectors: sucrose, trehalose, maltose, a polyvinylpyrrolidine derivative, and β-cyclodextrin derivatives. For comparison, other PEGylated block copolymer micelles (PEGylated polylactic acid, polylactic acid-co-glycolic acid, polycaprolactone) were freeze-dried.

Results Drug loading via direct dialysis from acetone was a less effective loading method which led to dexamethasone loads <2% w/w. O/W emulsion technique from dichlormethane increased drug load up to ~13% w/w; optimized cosolvent evaporation increased load up to ~19% w/w. An important step for cosolvent evaporation was solubility screen of the drug prior to preparation. Loading was maintained upon lyophilization with β-cyclodextrins which proved to be versatile stabilizers for other block copolymer micelles.

Conclusion Careful solvent selection prior to cosolvent evaporation was a beneficial approach to load hydrophobic drugs into polymeric micelles. Moreover, β-cyclodextrins could be used as versatile lyoprotectors for these micelles.

KEY WORDS cosolvent evaporation · dexamethasone · direct dialysis · drug load · freeze-drying · HPβCD · lyophilization · O/W emulsion · polymeric micelles · polypseudorotaxane · SBEβCD · β-cyclodextrins

INTRODUCTION

High throughput screening (HTS) of drug candidates increased the number of hydrophobic new molecular entities in drug discovery (1,2). The amount of hydrophobic drugs among development compounds was estimated by Lipinski in 2002 to be around 40% (3). Recent investigations showed that the actual number of drug candidates which can be categorized in class II or IV in the Biopharmaceutics Classification System (BCS) (4) was 75% and higher. However, low aqueous solubility resulting from HTS remains a challenge in

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pharmaceutical industry leading to higher costs in development and drug attrition rates upon approval (5). Despite huge progress in advanced solubilization technologies, the options for hydrophobic compounds aiming on intravenous injection are still limited (5) and often bought dearly with toxic side effects of utilized excipients (e.g. Cremophor EL®/ethanol in Taxol® (6)). Therefore nanosized approaches to overcome solubility issues became more and more important over the last decades. However, encapsulation of therapeutically relevant drug doses are a basic requirement for reasonable treatment and has been regarded as one of the major drawbacks of nanoparticulate delivery systems (7).

Among nanoparticles a very promising technology with high solubilization potential is based on polymeric micelles. However, preparation of such micellar systems with high drug loads remains still challenging (8,9). A number of different drug loading procedures were elaborated on the basis of trial and error experiments and lack of a more systematic approach. The major reason is the limited knowledge of the underlying mechanisms leading to sufficiently high drug loadings. The organic solvent by which the drug loading process is initiated plays a key role in all preparation methods: According to the solvent properties (water-miscibility) an appropriate candidate is usually selected which finally defines the processing steps to follow. In this context the following drug loading methods have commonly been used: 1) O/W emulsion techniques (10), 2) W/O/W emulsion techniques (11,12), 3) direct dialysis (13), 4) cosolvent evaporation techniques (14) and 5) loading via lyophilization (15). Among those procedures especially 1), 3) and 4) are highly compatible with hydrophobic drugs whereas 2) focuses on the encapsulation of more hydrophilic compounds and 5) has limitations concerning polymer water solubility (15). In our study we selected the methods 1), 3) and 4) for hydrophobic drugs and prepared micelles by encapsulating the model drug dexamethasone. With its aqueous solubility of 80 μg/mL (16) it is a typical hydrophobic model compound which is in addition highly interesting for parenteral application. For the encapsulation study the PEGylated poly-(4-vinylpyridine) was selected that was known to form stable micelles in water (17).

As micelles are fragile dynamic systems it is highly desirable to stabilize the particles after freeze-drying. It is known from the literature that besides classic lyoprotectants such as sucrose or trehalose, β-cyclodextrins are particularly suitable lyoprotection excipients (18,19). We thus became interested in the questions of which kind of stabilizing mechanisms cyclodextrins might have compared to classical polyols and whether or not they might be helpful as versatile protectors for PEGlyated block copolymer micelles.

The purpose of this study was to gain mechanistic insight into the micellar encapsulation process of hydrophobic drugs by applying different preparation techniques using various organic solvents. In addition, β-cyclodextrins were compared to standard lyoprotection agents for their stabilizing properties. Such mechanistic insights in both the loading procedure and lyoprotector stabilization are of immediate relevance for the development of novel preparation techniques for polymeric micelles.

MATERIALS AND METHODS

Materials

PEGylated poly-(4-vinylpyridine) (PEG-PVPy) [5-b-20], PEGylated poly-(lactic acid) (PEG-PDLLA) [5-b-23] and PEGylated poly-(ε-caprolacton) (PEG-PCL) [5-b-32.5] were purchased from Polymer Source, Inc., Montreal, Canada. PEGylated poly-(lactic-co-glycolic acid) (PEG-PLGA) [5-b-28] (Resomer RGP d 50155) was delivered from Boehringer Ingelheim, Ingelheim, Germany. Dichlormethane (DCM), tetrahydrofurane (THF), acetonitrile (ACN), methanol, ethanol, dimethylsulfoxide (DMSO) were obtained from VWR, Darmstadt, Germany. Dexamethasone (Ph.Eur.) was delivered by Euro OTC Pharma from a local pharmacy store. Dulbeccos Saline PBS buffer concentrate was obtained from Sigma Aldrich, Rossdorf, Germany. Float-A-Lyzer G2 dialysis tubes (MWCO 8–10 kDa) and standard dialysis tubes (MWCO 6–8 kDa) were obtained from Spectrumlabs Inc., Breda, The Netherlands. For filtration of the micellar dispersions 0.2 μm Pall Acrodisc syringe filters with GHP membrane were used (Pall Life Sciences, Ann Arbor, MI, USA). Sucrose, trehalose, glucose, lactose, maltose and D-(-)-mannitol were obtained from VWR, Darmstadt, Germany. Kollidon 17PF was delivered from BASF, Ludwigshafen, Germany. Kleptose® (HPβCD) was obtained from Roquette, Frankfurt, Germany. Captisol® (SBEβCD) was delivered from Cydex, Lenexa, KS, USA. 2R-glass vials were purchased from Schott AG, Mainz, Germany. Water was of MilliQ grade.

Methods

Preparation of Drug-Loaded Micelles

All micelles were prepared with concentrations above their critical association concentration which were determined in a previous study (17).

Direct Dialysis from Acetone. 10 and 20 mg of block copolymer and 1 and 4 mg DXM, respectively, were dissolved in 1 mL acetone. This solution was filled into a dialysis bag and sealed. Dialysis was carried out over 24 h against 5 L water which was exchanged once after 4 h. The dispersion was filtered through a 0.2 μm membrane to remove drug

precipitates. Finally the mass of the resulting micellar dispersion was adjusted to 2 g by dilution with water.

O/W Emulsion Technique. Micelles without DXM were prepared as described above in the section "Direct Dialysis from Acetone" without drug. For drug loading, 2 mg DXM was dissolved in 1 mL DCM. This solution was injected under constant stirring to 2 mL aqueous micellar dispersion containing "empty" micelles. After stirring over night the resulting dispersion was filtered through a 0.2 μm filter membrane to remove drug precipitates and the mass was adjusted to 2 g by dilution with water.

Selection of Appropriate Solvent for Cosolvent Evaporation. The following water miscible organic solvents were included in a solvent screen for DXM: THF, ACN, acetone, DMSO, DMF, methanol, and ethanol. The screen was carried out by using the following procedure at 25°C: 1) Dissolving 1 mg of drug in 100 μL organic solvent, 2) stepwise addition of 10 μL water to this solution, 3) determination of drug precipitation by optical visibility of particles. Precipitation was investigated against a black background with sample illumination by cold light. The solvent in which the drugs exhibited the highest optical solubility was selected for preparation in the solvent evaporation step.

Cosolvent Evaporation. Drug-loaded micelles were prepared by the combination of solvent evaporation followed by dialysis. 10 mg block copolymer and 2 mg drug were dissolved in 6 mL THF or 8 mL acetone; this solution was mixed with 2 mL water. The mixture was transferred into a round bottom flask and evaporated by applying vacuum of 30 mbar at 25°C. To remove the maximum amount of solvent, this vacuum was kept constant for 10 min. The remaining aqueous formulation was then transferred into a Float-A-Lyzer G2 tube for dialysis to remove residual solvents. The formulation was dialyzed against 5 L DXM saturated water for 24 h. Finally the formulation was filtered through a 0.2 μm membrane and mass adjusted to 2 g.

Drug Load Determination

100 μL of the final micellar formulation was dissolved in 900 μL acetonitrile. This solution was injected in a HPLC system (Merck Hitachi La Chrom Elite) equipped with a UV detector (detection wavelength: 282 nm) and an Agilent Eclipse Plus C18 column (3.5 μm coarse, 5 cm length) at 35°C. A gradient method was used for separation where the mobile phase A consisted of 90% (v/v) ACN/ 10% (v/v) ammonium acetate buffer (pH 4.5, 10 mM); mobile phase B had the opposite composition. DXM concentration was determined by drug substance calibration curve. The polymer concentration was calculated from the initially used polymer feed prior to the preparation of micelles.

Drug load was calculated according to Eq. 1:

$$\text{Drug load}[\%] = \frac{\text{Drug concentration}\left[\frac{\text{mg}}{\text{mL}}\right]}{\text{Polymer concentration}\left[\frac{\text{mg}}{\text{mL}}\right]} \cdot 100\% \qquad (1)$$

Particle Size Measurement

Dynamic light scattering (DLS) was used to determine the hydrodynamic diameter of the nanoparticles. Therefore, a Zetasizer-ZS from Malvern Instruments, Worcestershire, UK, was selected and used in the backscattering mode. Measurements were performed in triplicate.

Storage Stability of Drug-Loaded Micelles

DXM-loaded PEG-PVPy micelles with 0.5% polymer concentration prepared by cosolvent evaporation from THF were stored at 4°C, 25°C and 40°C over 4 weeks. Particle sizes and drug loads were monitored once per week of storage. All experiments were done in triplicate.

Lyoprotector Screening via Differential Scanning Calorimetry (DSC)

10%w/v aqueous solutions of the selected lyoprotectors served as reference and were filled into 100 μL aluminum pans. Micellar formulations of DXM-loaded PEG-PVPy (0.5%w/v) were mixed with the lyoprotector solutions (10% w/v) 1:1 to reach a final lyoprotector concentration of 5%w/v. Each aluminum pan was filled with 80 μL and sealed. Thermoanalysis was performed on a Mettler Toledo DSC 821e (Mettler Toledo GmbH, Giessen, Germany). The samples were frozen from 25°C to −50°C with a cooling rate of 10 K/min followed by an isothermal step of 5 min. The samples were heated to 25°C with a heating rate of 10 K/min. After an isothermal step at 25°C of 5 min the whole procedure was repeated. Onset of glass transitions were evaluated in the thermograms of the heating step. For stabilizer screening each experiment was carried out in triplicate.

Lyoprotector Screening via Lyophilization of Micelles

Polymeric micelle formulations (0.5%w/v polymer) were prepared employing the cosolvent evaporation method from THF, including either the DXM-loaded PEG-PVPy micelles or drug-free PEG-PDLLA, PEG-PLGA and PEG-PCL micelles. The dispersions were premixed with adequate amounts of β-cyclodextrin derivative solutions to reach final lyoprotector concentrations of 5%w/v. The prepared solutions were transferred into 2R-glass vials; the

filling height was adjusted to 1.0 mL for all samples. Lyophilization was carried out on a Christ Epsilon 2-6 D freeze-dryer (Martin Christ GmbH, Osterode am Harz, Germany). The samples were frozen over 2 h to −40°C, primary drying was achieved at −38°C (according to previous T_g' determination) and 0.05 mbar over 48 h and followed by a secondary drying step at 30°C and 0.005 mbar for 24 h. After drying was finished, all sample vials were sealed under nitrogen atmosphere at 500 mbar. The lyophilized samples were reconstituted with 500 µL water and analyzed by DLS measurements. Data evaluation was carried out by the calculation of factors of particle size increase as proposed by Abdelwahed *et al.* (20). Following their proposal the size increase factors were calculated as the ratio between initial particle sizes prior to lyophilization and particle sizes upon reconstitution of the samples.

Influence of Lyoprotector Concentration on Micelle Reconstitution

Investigation of the influence of lyoprotector concentrations on the drug-loaded micelles was carried out by preparation of lyophilizates with varying β-cyclodextrin concentrations. For this purpose the dexamethasone-loaded PEG-PVPy micelles were freeze-dried with 0.5%; 1%, 2.5% and 5% of HPßCD and SBEßCD. Reconstitution properties were investigated by DLS.

Cryo-Transmission Electron Microscopy (Cryo-EM)

Drug-free and dexamethasone-loaded PEG-PVPy nanoparticles as well as nanoparticles upon reconstitution of the lyophilizates with SBEßCD as cryoprotector were subjected to cryo-EM analysis. To this end, 4 µl of sample were pipetted onto a glow-discharged holey-carbon copper grid, blotted with filter paper and plunge-frozen in liquid ethane. EM grids were stored in liquid nitrogen until imaging. Images were taken under cryo conditions in a Tecnai T12 (FEI, Eindhoven, The Netherlands) electron cryomicroscope equipped with a MultiScan 794 CCD camera (Gatan, Pleasanton, CA, USA) using a side-entry cryoholder (Gatan). Individual nanoparticles were selected from the raw images, and the dimensions of the nanoparticles were determined by comparing with reference binary discs of different diameters using a multi-reference alignment (21). The determined particle sizes were used to calculate cumulant frequency curves for each sample. From these cumulant curves, the d_{10}, d_{50}, d_{90} values were calculated as the size distribution parameters of the samples. The preparation and visualization of micelles resulting from the cosolvent evaporation is described in the Supplementary Material. For this purpose, dechloro-4-iodo-fenofibrate synthesized according to Breyer *et al.* (22) was used as a model drug.

RESULTS

Solvents for Cosolvent Evaporation

Based on previous reports that the preparation of drug-loaded micelles (14) requires a high solubilization capacity of the selected organic solvents, the drug solubility was screened in common fully water-miscible organic solvents. Figure 1 shows the maximum volume of water which could be added to 7 solvents without drug precipitation. From this screening it was obvious that THF had the best solubilization properties in connection with water. Consequently we selected this solvent as the most appropriate one for the cosolvent evaporation. However, for comparison purposes the preparation was additionally carried out using acetone which had a still reasonable solubilization capacity and low toxicity (23).

Preparation of Drug-Loaded Micelles

The micelles were prepared and drug loaded according to the three selected methods. Table I reports the respective drug loading and size distributions.

Storage Stability of Drug-Loaded Micelles

The liquid micellar formulation loaded with dexamethasone was stored under different conditions to investigate stability of micelles and drug encapsulation. For this purpose every week drug loads, particle sizes and particle size distributions were analyzed. As portrayed in Fig. 2a the drug loads were decreasing over storage time independently of the storage conditions. The amplitude of decrease followed the temperature of storage inversely (from high decrease to low): 40°C > 25°C > 4°C.

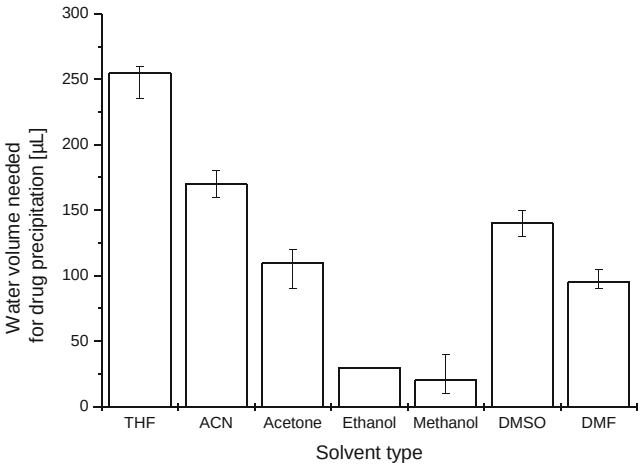

Fig. 1 Solubility screening of 1 mg DXM in 100 µL of varying organic solvents upon addition of water. Drug precipitation was detected visually against a black background and illumination of the sample with cold light. The experiments were carried out in triplicate (mean ± range).

Table I DXM Incorporated in PEG-PVPy [5-b-20] Micelles in Dependence of the Preparation Techniques. All Experiments were Done in Triplicate (mean ± SD)

Polymer conc. w/v [%]	Drug/pol ratio feed	Preparation technique	Solvent and conditions	Drug load w/w [%]	d_h [nm]	PdI
0.5	1:10	O/W emulsion	DCM, preformed micelles from acetone	8.74 ± 0.03[a]	52 ± 2	0.15 ± 0.01
1.0	1:10	O/W emulsion	DCM, preformed micelles from acetone	7.81 ± 0.18[a]	69 ± 4	0.19 ± 0.03
0.5	4:10	O/W emulsion	DCM, preformed micelles from acetone	13.50 ± 5.05[a]	52 ± 1	0.19 ± 0.02
0.5	1:10	Direct dialysis	Acetone	1.71 ± 0.15[a]	56 ± 7	0.18 ± 0.06
1.0	1:5	Direct dialysis	Acetone	0.62 ± 0.60[a]	67 ± 2	0.16 ± 0.01
0.5	1:5	Cosolvent evaporation	Acetone	12.07 ± 1.21[a]	41 ± 3	0.14 ± 0.01
1.0	1:5	Cosolvent evaporation	Acetone	10.84 ± 2.64[a]	45 ± 5	0.12 ± 0.01
0.5	1:5	Cosolvent evaporation	THF	18.67 ± 0.21	37 ± 1	0.21 ± 0.01
1.0	1:5	Cosolvent evaporation	THF	19.25 ± 0.54	52 ± 1	0.26 ± 0.01

[a] Drug precipitation occurred during preparation. Precipitates were removed by filtration through a 0.2 μm filter membrane prior to analysis of drug loads and particle sizes

Upon storage at 4°C the drug load of the system was reduced after 4 weeks by ~2% whereas for 25°C and 40°C it decreased by far more (>>10%). It was remarkable that the particle sizes and size distributions (Fig. 2b) did not follow this trend. Unexpectedly, sizes and PdI remained nearly unchanged over the storage time in liquid formulation.

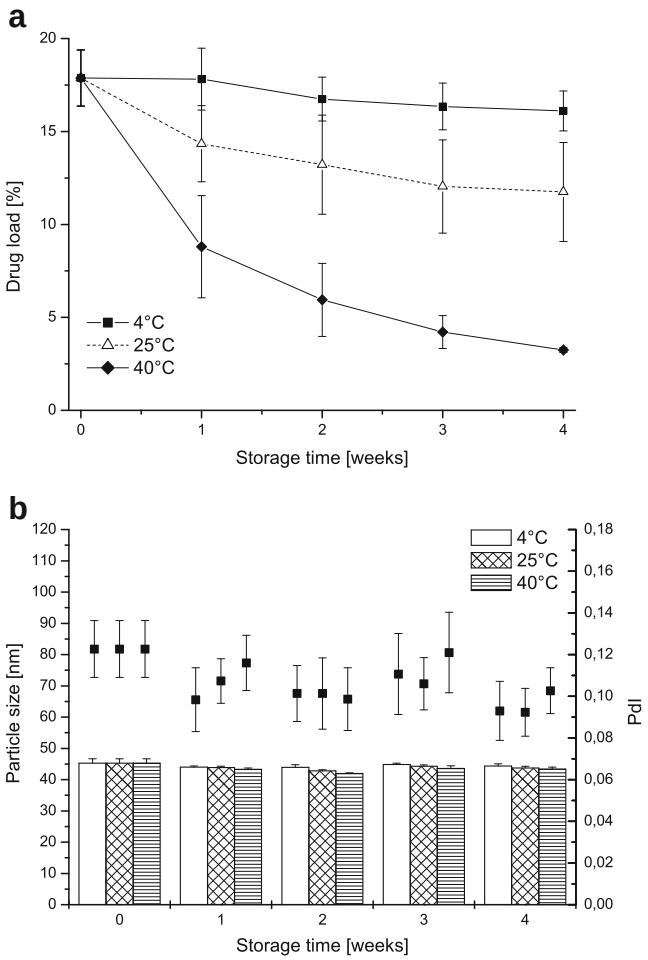

Fig. 2 DXM-loaded PEG-PVPy [5-b-20] micelles under different storage conditions. (**a**). Drug load of micelles determined by HPLC-UV upon storage under different conditions: 4°C (*squares*), 25°C (*triangles*) and 40°C (*diamonds*). (**b**). Particle sizes (*bars*) and polydispersity indices (PDI, *squares*) obtained from DLS upon storage.

Lyoprotector Screening via DSC and Lyophilization of Placebo Micelles

DXM-loaded PEG-PVPy micelles prepared by cosolvent evaporation technique from THF were selected for freeze-drying due to their high drug content compared to micelles made by other methods. Prior to lyophilization the lyoprotector solutions were prepared, mixed with micelles and analyzed by DSC measurements for their thermal properties. The thermograms revealed the glass transition temperatures of the maximally freeze concentrated solutions T_g'(24) (Fig. 3). The T_g' values of the pure excipient solutions and of the excipient micelle mixtures are reported. Sucrose had the lowest T_g' with ~−33°C; the highest was reached with HPßCD at ~−9°C. When the influence of nanoparticle presence was investigated by mixing DXM-loaded PEG-PVPy micelles with lyoprotector solutions, the maximum deviation in T_g' comparing with and without micelles was found for SBEßCD, where the micelles led to 2 K lower glass transition temperatures. Based on these results the primary drying temperature for a generic lyophilization process was set to −38°C (−33°C for lowest T_g' and 5 K safety margin). This maintained the "glassy" state of the freeze-dried solution and prevented influence of physical changes in the lyoprotector structure which could lead to micellar rupture and non-redispersibility.

The DXM-loaded PEG-PVPy micelles were dried in a lyophilization process and screened with the selected lyoprotectors for reconstitution properties. The screening results are shown in Fig. 4a. As a quality parameter the particle size increase factor was calculated to compare the

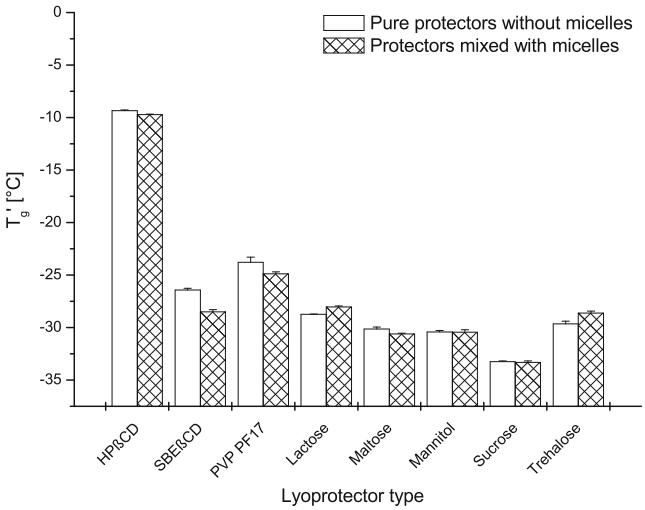

Fig. 3 T_g' determination for the lyoprotectors included in the study (results: mean ± SD, $n=3$) determined by DSC.

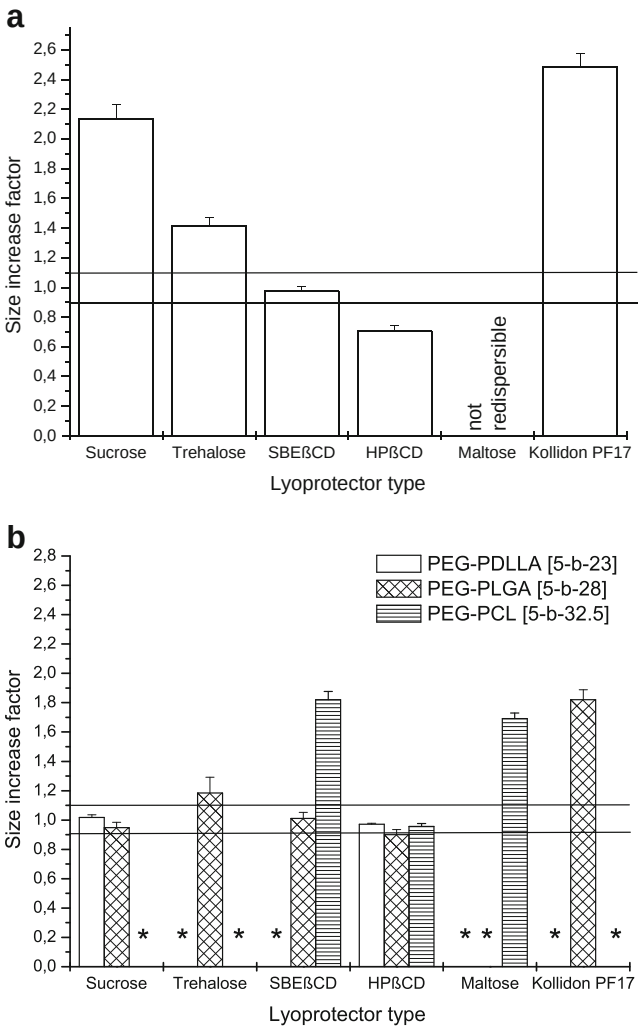

Fig. 4 (**a**) Size increase factors upon reconstitution of the lyophilizates of DXM-loaded PEG-PVPy [5-b-20] micelles prepared with varying lyoprotectors (results: mean ± SD, $n=3$). (**b**) Size increase factors upon reconstitution of the lyophilizates of drug-free PEG-PDLLA [5-b-23], PEG-PLGA [5-b-28] and PEG-PCL [5-b-32.5] micelles prepared using different lyoprotectors (results: mean ± SD, $n=3$). Asterisk indicates non-resdispersible formulations.

stabilizing properties of each lyoprotector with the investigated micelles, a method that was proposed by Abdelwahed *et al.* (20). As evaluation criteria a change of hydrodynamic diameter of 10% compared to the micellar size before lyophilization was considered to be acceptable.

The PEG-PVPy micelles increased in size by more than a factor of two when sucrose or Kollidon 17PF was used for stabilization. Maltose led to non-redispersable lyophilizates whereas trehalose did not stabilize the micelles sufficiently which can be seen by an increase in the size factor to 1.4. The investigation revealed that HPßCD and SBEßCD stabilized micelles quite well, apart from HPßCD that decreased particle size by ~30% after reconstitution. SBEßCD was able to stabilize the micelles excellently. Our observations raised the question whether or not the results of lyoprotector screening were only valid for PEG-PVPy micelles and each polymeric micellar species requires a new screening. To answer this question three different micelle forming block copolymers were selected and screened accordingly on their reconstitution properties (Fig. 4b). The results showed that HPßCD stabilized PEG-PDLLA, PEG-PLGA and PEG-PCL micelles excellently, whereas SBEßCD only stabilized PEG-PLGA micelles sufficiently. Moreover, sucrose led to full reconstitution of freeze-dried PEG-PDLLA and PEG-PLGA, but could not stabilize PEG-PCL micelles. Trehalose, maltose and the Kollidon derivative did not stabilize any of the investigated micelles. Based on these results we performed further mechanistic investigations of DXM-loaded PEG-PVPy micelles with both β-cyclodextrin derivatives HPßCD and SBEßCD in varying concentrations.

Optimum Concentration of Lyoprotector

The lyoprotector concentration played an important role for particle reconstitution. Consequently varying concentrations of HPßCD and SBEßCD were screened to determine the optimal ratio. As a result drug-loaded PEG-PVPy micelles were reconstitutable in the selected concentration range between 0.5% and 5.0%w/v of lyoprotector concentrations (Fig. 5). The hydrodynamic diameter of the reconstituted micelles decreased for both stabilizers from 0.5% to 5.0%. It was remarkable that the micelles stabilized with 0.5% HPßCD exhibited strongly scattering particle sizes (reported as size increase factors) upon reconstitution whereas the micelles with 5.0% HPßCD were found to have strongly scattering particle size distributions. Lyoprotector concentrations between 1.0% and 2.5% were ideal to achieve reproducible hydrodynamic diameter and particle size distribution values.

Furthermore, we analyzed which influence the presence of β-cyclodextrins had on the recovery of dexamethasone

before and after lyophilization. The results (data not shown) revealed that the HPLC method yielded a ~102% recovery rate of dexamethasone in presence of β-cyclodextrins in liquid formulation. Moreover, upon reconstitution of lyophilizates the recovery decreased to ~93%–95% from the initial liquid formulation.

Morphological and Size Analysis by Cryo-EM

Previous studies indicated that the PEG-PVPy material was able to form various particle morphologies (25,26). For elucidation of the particle morphologies in our experiments we employed cryo-EM. As shown in Fig. 6a–c (left and middle), cryo-EM revealed spherical particles in the nanometer range for all analyzed samples. Drug-free and DXM-loaded particles revealed a similar morphology, and the shape of the micelles was independent of sample processing, i.e. drug-loading *versus* lyophilization with lyoprotectors. Upon measuring the diameter of the particles, we plotted the distribution of sizes. In line with the visual impression, this analysis confirmed that drug-free, drug-loaded and reconstituted PEG-PVPy micelles exhibited comparable sizes (Fig. 6a–c, right). In particular, particles obtained by lyophilization and reconstitution of particles revealed similar particle sizes and size distributions. Size distributions obtained from micrograph analysis are summarized in Table II.

Moreover, we analyzed micelles loaded with dechloro-4-iodo-fenofibrate, an iodine containing model drug, in order to study the distribution of electron density within the micelles. Micelles loaded with this model drug exhibited an irregular border that was considerably less electron dense compared to the inner core (see cryo-EM micrographs in the Supplementary Material). These borders were less pronounced and only rarely seen in the other, non-iodine containing samples.

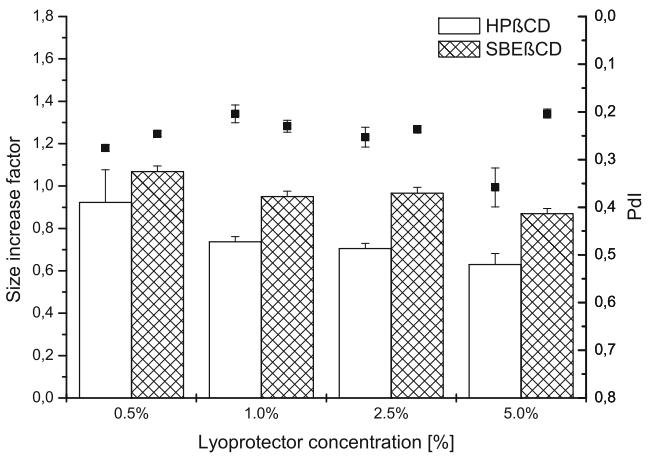

Fig. 5 Size increase factors of dexamethasone-loaded PEG-PVPy micelles upon reconstitution of lyophilizates with varying HPßCD and SBEßCD concentrations (results: mean ± SD, $n=3$).

DISCUSSION

The purpose of this study was to gain mechanistic insights into the drug-loading procedure and the stabilizing mechanisms of β-cyclodextrins during lyophilization of polymeric micelles.

Three different preparation techniques for the DXM-loading of PEG-PVPy micelles were studied. All these methods had in common that organic solvents were used to load the micelles whereas the loading principles were completely different.

The type of organic solvent was known to have an influence on particle sizes of resulting micelles: Vangeyte *et al.* (27) investigated PEG-poly-(ε-caprolactone) and PEG-poly-(γ-methyl-ε-caprolactone) in terms of particle sizes as a function of organic solvent type and mixture ratios. In their preparation THF, DMF, dimethylacetate (DMAc) and dimethylsulfoxide (DMSO) was included. The nanoparticles were prepared by 3 different methods: 1.) direct dialysis, 2.) addition of water to organic solution and 3.) addition of organic solution to water. The group showed, that direct dialysis did not work reproducibly resulting in large particles in submicron range to micron range, whereas the solvent evaporation techniques worked well (sizes <100 nm). The group of Aliabadi *et al.* picked up these thoughts about solvent influences and investigated the impact of the solvent type on drug loading into polymeric micelles (14). Cyclosporine A which was encapsulated into PEG-poly-(ε-caprolactone) served as a model payload. Selected cosolvents were acetone, THF and acetonitrile. For micellar preparation, either the organic/water mixture was injected into water or it was prepared *vice versa*. In line with the studies of Vangeyte *et al.* (27), Aliabadi *et al.* (14) found solvent dependent particle size of drug-free micelles. The authors selected acetone as the solvent of choice due to the nearly unimodal size distribution of the formed micelles from this solvent. The authors argued for a positive effect of gradually increasing the water content in a micellar system during preparation that prevented the hydrophobic drug from precipitation and consequently enabled encapsulation. Jette *et al.* (28) employed this cosolvent evaporation technique from acetonitrile/water to load fenofibrate into PEG-PCL micelles and drew similar conclusions on this method. Summarizing these studies, it was obvious that the organic solvent selected for the preparation of a micelle had a strong influence on drug load as well as particle sizes. Furthermore, the preparation technique was decisive for the successful encapsulation even if the solvent was selected appropriately. In this context the cosolvent evaporation technique employed e.g. by Aliabadi *et al.* (14) seemed to be superior over the other techniques due to the wide spectrum of organic solvents that can be used and its increased controllability by measurable physical factors during preparation.

However, there are a number of aspects these studies did not cover. This inspired us to further look into the drug

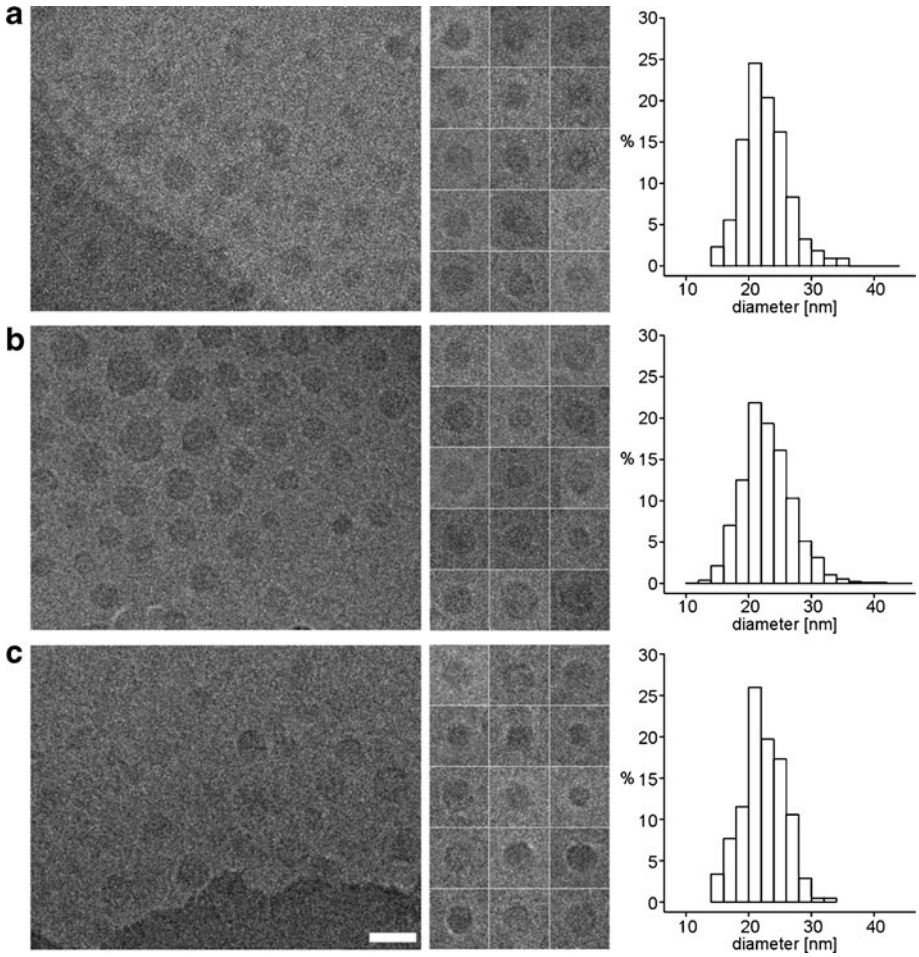

Fig. 6 (**a**) Cryo-EM analysis of PEG-PVPy micelles. On the left side, a representative cryo-EM micrograph is shown, the central panel shows a gallery of selected particles, and on the right side, the distribution of diameters is plotted in a histogram. The scale bar of the overview images (left) corresponds to 40 nm, and the box length of the single-particles (middle) corresponds to 53 nm. (**a**) Drug-free PEG-PVPy micelles. (**b**) DXM-loaded PEG-PVPy micelles. (**c**) PEG-PVPy micelles loaded with DXM upon reconstitution of lyophilizates stabilized with SBEßCD.

loading techniques for polymeric micelles. Most important is the influence of organic solvents and preparation methods on the drug load of micelles. We wished to clarify which preparation technique, i.e. the organic solvent within the preparation technique or the drug-polymer compatibility, facilitates drug loading of micelles best. To address this point we selected three different preparation methods for this purpose: 1) O/W emulsion technique, 2) direct dialysis and 3) cosolvent evaporation technique and assayed their potential for loading polymeric micelles with drug.

The O/W emulsion technique is based on the use of water non-miscible solvents. By applying this method the organic phase (Fig. 7a) containing the dissolved drug was injected into an aqueous phase under constant agitation (e.g. stirring, ultrasound). Due to mechanical agitation the organic phase is distributed as small emulsion droplets in the aqueous phase. Consequently this effect increased the surface area of the organic phase. At these interfaces between the organic and aqueous phase the distribution of the drug from organic emulsion droplets toward the micellar cores was possible. Furthermore, the slow evaporation of the organic solvent increased the time for this distribution process compared to simple mixing of both phases with small interface areas. This method resulted in quite high drug loads for DXM in PEG-PVPy micelles (up to ~13% w/w). Variation of the initial drug-polymer ratio from 1:10 to 4:10 increased the drug load from ~10% w/w to ~13% w/w. This was concomitantly found to be the upper limit for this method due to the occurring drug precipitation upon solvent evaporation. A completely different loading mechanism exhibited the direct dialysis

Table II Particle Size Distribution Parameters Obtained from cryo-EM Micrograph Analysis

Sample	d_{10} [nm]	d_{50} [nm]	d_{90} [nm]	Number of particles evaluated from micrograph analysis
Drug-free PEG-PVPy	18	22	27	216
DXM-loaded PEG-PVPy	18	22	28	1881
DXM-loaded PEG-PVPy SBEβCD lyo	18	22	27	208

method (Fig. 7b): Once the organic drug/polymer solution was filled into the dialysis bag, the overall system immediately came into contact with an aqueous phase during dialysis. The exchange of organic solvent at the interface of the dialysis tube could be very fast and less controllable. Finally the solution conditions of the drug decreased strongly which led to immediate drug precipitation and resulted in a low drug load (~2% w/w) independent of the drug-polymer ratio. Once precipitated, the drug was not available for encapsulation anymore. For the cosolvent evaporation mechanism there was a variety of water-miscible organic solvents available in which the drug solubility was screened prior to micelle preparation (Fig. 1). Compared to the direct dialysis, premature drug precipitation was avoided during the cosolvent evaporation by the selection of a suitable solvent for the drug DXM (Fig.7c). The cosolvent evaporation was carried out from an initial solvent/water ratio which was able to still solubilize both, drug and polymer. During evaporation the micelles were preformed but simultaneously the drug was still dissolved at this point. This procedure enabled the distribution from the outer aqueous/organic phase into the micellar core. By continuing the evaporation the drug solubility decreased in the organic/water phase which finally shifted the distribution equilibrium further toward the micellar core. At the end of this step the formulations contained only low amounts of residual solvents which were further cleared by dialysis. The dialysis step was implemented to overcome the disadvantage of cosolvent formation: azeotropic mixtures (28). These mixtures made it impossible to evaporate the solvents completely. Overall the superior properties of cosolvent evaporation are reflected by our results: the drug load of the system increased up to 19% w/w DXM for 1% PEG-PVPy w/v.

As a next step, stabilization of this high drug load inside the micelles was a focus of this study. Freeze-drying was selected as the method of choice to gain a storage stable product. Maintaining full reconstitution properties of micelles with high drug load is generally very challenging: during freezing of micellar dispersions varying stress factors could appear which are of physical or chemical nature and which are well investigated for therapeutic proteins (24). For nanoparticles, these stress factors include: 1) particle aggregation due to the loss of repulsive properties of steric stabilizers (29), 2) nanoparticle reassembly and remodeling after reconstitution (30), 3) loss of encapsulated drug due to changes in the physical status of the nanoparticle (31) as well as 4) chemical degradation of the carrier polymer and the encapsulated drug (20). Prevention of these stresses and full reconstitution properties can be achieved by the addition of cryo- and lyoprotectors to the formulation (24). There were two well established hypotheses for the explanation of the efficacy of protectors as discussed by Abdelwahed *et al.* (20): the water substitution model explains protection during the drying step whereas the vitrification model emphasizes on the stability during freezing (20). The water substitution model suggests that water molecules are substituted by OH-groups from molecules which are similarly hydrophilic e.g. polyols like sugars which are widely used as lyoprotectors. This is known to assure chemical and physical integrity of proteins (24) and consequently should be one approach to stabilize nanoparticles. The vitrification model describes nanoparticle embedding into an amorphous stabilizer matrix which finally prevents aggregation (20,32). Moreover, Allison et. al (33) brought the "particle isolation hypothesis" (33) into play which argued for the isolation of particles in the non-frozen fraction by polyols. It is worth mentioning that the above cited studies investigated nanoparticles such as liposomes or nanocapsules and did not highlight on the mechanisms of freeze-drying of polymeric micelles. Since this is an important issue it will be discussed below in more detail.

According to Abdelwahed *et al.* (20) we selected various sugars (maltose, sucrose, trehalose) as cryo- (prevents from freezing stress) and lyoprotectants (prevents from drying stress), a stabilizer and "collapse temperature modifier" (20) vinylpyrrolodin macromolecule (PVP PF 17) and cyclic oligosaccharides (HPßCD, SBEßCD) (also "collapse temperature modifier" (20)) for a lyoprotector screening. As a first step all considered lyoprotectors were screened via DSC concerning their optimal freezing-properties. T_g' was selected as a reporting parameter for the optimization of the lyophilization process. Whereas HPßCD revealed a T_g' of ~−10°C which could assure a short, effective and less costly lyophilization process, all the T_g' values for the other investigated lyoprotectors scattered between ~−22°C and ~−33°C. Consequently to run a generic lyophilization process for all stabilizers the primary drying temperature was selected at −38°C. Interestingly the presence of micelles did not strongly change the freezing properties of lyoprotectors.

Obviously β-cyclodextrin derivates seemed to have excellent stabilization properties for various micellar species (Fig. 4b) and seemed to be versatile lyoprotectors. Support of this hypothesis came from the investigations of the other PEGylated block copolymers PEG-PDLLA, PEG-PLGA and PEG-PCL (Fig. 4b). HPßCD stabilized all investigated micelles very well and led to completely redispersable formulations, while SBEßCD excellently stabilized PEG-PLGA and PEG-PVPy micelles. Especially the results acquired by using HPßCD led to the assumption that for β-cyclodextrin derivatives there might be an additional mode of action concerning stabilization besides the water molecule substitution and vitrification model. All investigated polymeric micelles present PEG chains on their surfaces. It is known from the literature that PEG and cyclodextrins are able to form inclusion complexes called pseudopolyrotaxanes (34,35). Less was known if this phenomenon appeared on micellar surfaces as well. Joseph *et al.* described inclusion complexes of a cyclodextrin derivative with Pluronic® block copolymer molecules which they proved by small angle

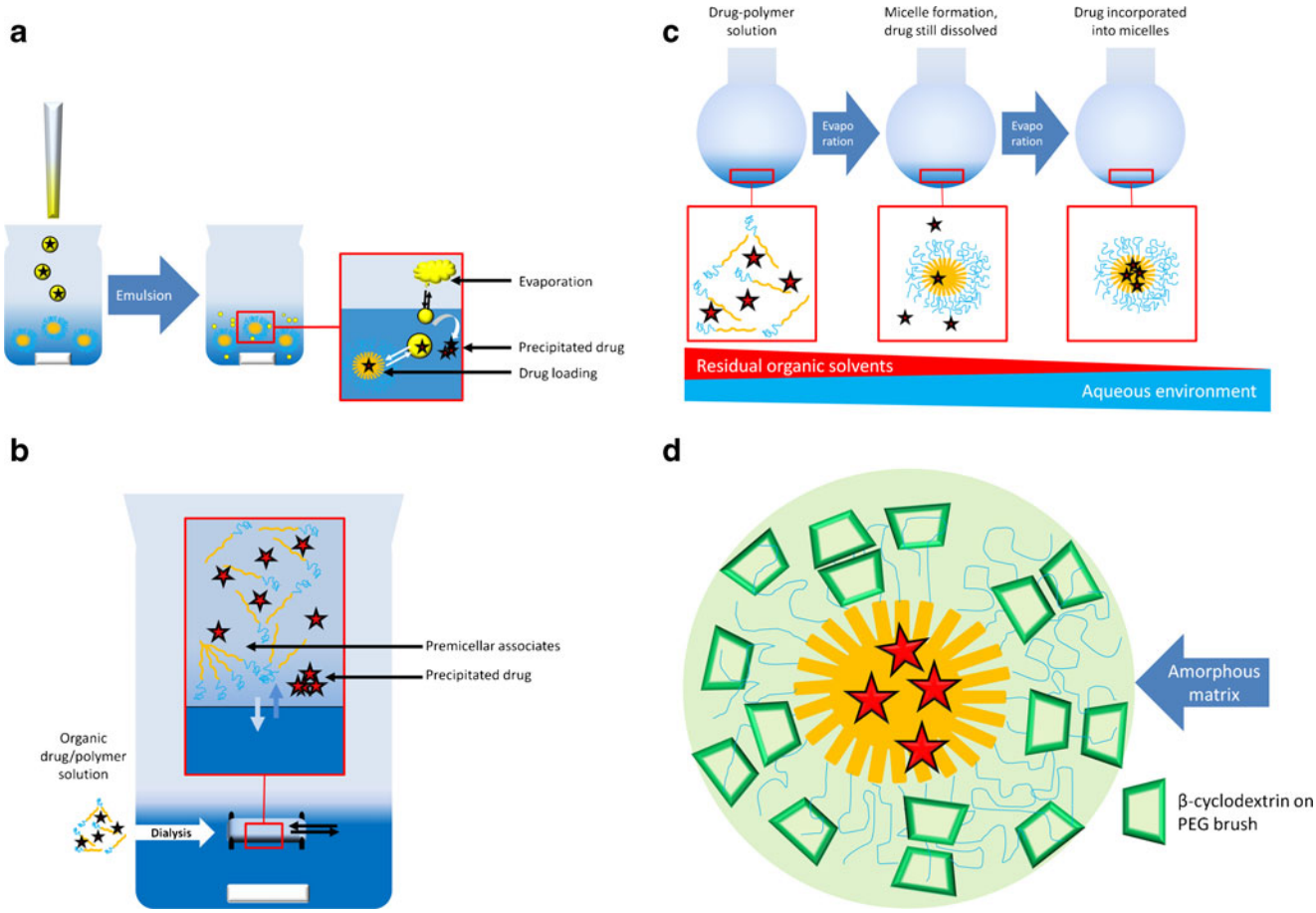

Fig. 7 Schematic drawings of: (**a**) O/W emulsion process, (**b**) Direct dialysis process and (**c**) Cosolvent evaporation process. (**d**) β-cyclodextrin inclusion complex which was able to stabilize micelles during lyophilization.

neutron scattering (SANS) experiments (36). The authors hypothesized that the β-cyclodextrin derivative preferentially covered the more hydrophobic PPO block of the polymer. This observation was found for single polymer chains and for micellar aggregates, but increasing β-cyclodextrin concentrations led to increased complexation and finally to dissociation of the micelles. These phenomena were highly temperature dependent; below room temperature pseudopolyrotaxane formation appeared whereas above 50°C precipitation/gelation occured. Qin *et al.* investigated the interaction of β-cyclodextrin with varying Pluronic® derivatives as well and confirmed the pseudopolyrotaxane formation by two-dimensional NMR spectroscopy (37). A strong dependency of cyclodextrin concentration and temperature on the aggregate size was found. Moreover, such an inclusion phenomenon was found to be very fast (<3 min), preferentially below 25°C and could be disturbed by changing the water cluster structure by chaotropic solutes (e.g. urea) (38). The presence of pseudopolyrotaxanes on PEG brushes was at first described by He *et al.* in 2005 (39).

The general question remains if pseudopolyrotaxane formation is beneficial for the lyophilization of polymeric micelles and what makes them superior compared to the other polyols. In this connection the influence of solutes on the water cluster structure comes into play. Generally solutes can have "structure-making (kosmotropic)" (40) and "structure-breaking (chaotropic)" (40) properties on the water cluster structure. Sugars lead to kosmotropic structures as has been shown for trehalose, maltose and sucrose (41). Trehalose is known to be a very effective cryoprotectant and superior to the other two sugars. Investigation of the reasons for this observation was performed by Lerbret *et al.* (41). These three sugars were investigated and compared to each other on their changes in water cluster structure by means of molecular dynamic simulations. As a result the group found trehalose to form larger water cluster sizes compared to the other sugars. Consequently more water atoms were associated by hydrogen bonding to trehalose molecules and the amount of bulk water which induces nucleation during freezing was reduced. The group concluded on less drying stress and less risky ice formation to explain the superior cryoprotective properties of trehalose. Coming back to the polypseudorotaxane formation of PEGylated block copolymer micelles with β-cyclodextrins, the facts appear in a

different light: Generally cyclodextrin derivatives are known to be strong kosmotropic solutes (42). In contrast, Sano *et al.* (42) revealed by Raman spectroscopy that the formation of polypseudorotaxane recovered the original cluster structure of pure water and denied a kosmotropic influence of cyclodextrin derivatives. Consequently the water cluster model cannot be used for polypseudorotaxanes to explain the results obtained from lyophilization. An alternate explanation is provided by Liu *et al.* (43) who investigated the change of material properties of such inclusion complexes. The group prepared guest-host complexes from an ABA triblock copolymer (poly[(*R*,*S*)-3-hydroxybutyrate]-poly(ethylene glycol)-poly[(*R*,*S*)-3-hydroxybutyrate] [PHB-PEG-PHB]) containing PEG as block B. They confirmed the selective formation of the inclusion complexes with block B (central block) with an α-cyclodextrin derivative by differential scanning calorimetry (DSC), ^{1}H-NMR and X-ray diffraction (XRD) studies. As a result the partial degree of crystallinity of PEG was strongly reduced and could not be detected after formation of polypseudorotaxanes. Consequently the material properties were altered and the PEG part was embedded into an amorphous matrix. We, therefore, postulate that the PEG shield which covers the hydrophobic cores in our systems is undergoing polypseudorotaxane formation. Consequently freezing stress and crystallization of water that challenges the core stability could be prevented by creating this kind of amorphous shield. Furthermore, desiccation stress from the particles can be reduced by the amorphous matrix as well.

Based on our results and previous studies from literature we feel that there is mounting evidence for a direct interaction between β-cyclodextrins and PEGylated block copolymers (Fig. 7d) which could explain the excellent results from lyophilization. The formation of PEG-β-cyclodextrin inclusion complexes had a beneficial effect on the reconstitution properties of PEGylated block copolymer micelles. Consequently we propose these molecules as versatile lyoprotectors for such micellar systems.

CONCLUSION

In this study we compared three different drug loading mechanisms for hydrophobic drugs into polymer micelles. Among those the cosolvent evaporation technique was superior compared to O/W emulsion technique and direct dialysis technique. The reasons for these observations were the possibility of appropriate solvent selection as well as a better control of the evaporation step during preparation. This combination kept the drug as long as necessary in solution during micelle formation and prevented premature precipitation. This high drug load can be stabilized by final micelle freeze-drying. We found that β-cyclodextrins are versatile lyoprotectors for PEGylated polymeric micelles due to a possible direct interaction between PEG corona chains and the hydrophobic β-cyclodextrin cavity. The special stabilization properties of β-cyclodextrins was seen to be based on 1) embedding of the PEG parts into an amorphous matrix, 2) prevention from formation of crystalline PEG during freezing and 3) stabilization of the hydrophobic core during freezing by prevention of water crystallization in the hydrophilic shell.

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