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Solubilization of Sagopilone, a poorly water-soluble anticancer drug, using polymeric micelles for parenteral delivery

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ABSTRACT

Polymeric micelles were studied as a drug delivery system for Sagopilone, a poorly water-soluble anticancer drug, with respect to passive tumour targeting. Poly(ethylene glycol)-*b*-Poly(lactide) (PEG-*b*-PLA) and Poly(ethylene glycol)-*b*-Poly(ε-caprolactone) (PEG-*b*-PCL) were investigated to identify suitable copolymers and to assess the predictive value of solubility parameters. The impact of copolymer compositions (different hydrophobic/hydrophilic-ratios (w/w) from 0.3 to 1.3) and the preparation method (sonication; film formation) on the solubilization efficiency, size characteristics and micelle stability were studied. Thermal analysis was used to determine the apparent solid-state solubility. PEG_{2000}-*b*-PLA_{2200}, PEG_{2000}-*b*-PCL_{2600} and PEG_{5000}-*b*-PCL_{5000} were identified as the most suitable delivery systems for Sagopilone. They exhibited efficient solubilization (≥70%) yielding small (<100 nm), monodisperse, and spherical micelles. (80 ± 12), (93 ± 0.4) and (96 ± 6)% of the drug still remained solubilized after 24 h, respectively. Calculated solubility parameters were not predictive since they showed a reversed order of preference relative to experimental data. High solubilization after film hydration was accompanied with a 'supersaturation'. The reason for this well-known effect and the solubilization of Sagopilone within the block copolymer was elucidated by the evidence of glass solutions exceeding the solubilization capacity of the corresponding micelles. Overall, micellar drug delivery systems for Sagopilone were identified offering the potential for an improved cancer therapy.

1. Introduction

To date many potent drugs entering the developmental stage were selected from high throughput screening and passed through numerous pharmacodynamic evaluations *in vitro* as well as *in vivo*. These drug candidates frequently show poor or negligible water solubility. Sagopilone (Fig. 1) is a novel, potent derivative belonging to the group of epothilones, a new class of microtubule-stabilizing agents (Klar et al., 2006; Hoffmann et al., 2008). It is currently undergoing Phase II clinical trials for the treatment of various types of cancer (U.S. National Institutes of Health, 09). The parenteral administration of Sagopilone poses a challenge to formulation development due to its poor solubility in water (12 μg/mL). Furthermore, dosing of Sagopilone is limited due to the occurrence of peripheral neuropathy, a typical side effect of epothilones. Thus, the requirements of an ideal drug delivery system comprise (a) efficient and stable solubilization of the drug, (b) accumulation of the drug in tumour tissue, and (c) a reduction of drug related adverse effects at non-tumour sites.

Solubilizers currently used for parenteral administration like Cremophor® EL or polysorbate 80 have been implicated in clinically important adverse effects like hypersensitivity reactions and a highly increased systemic drug exposure along with a reduced cellular uptake (ten Tije et al., 2003). Among different approaches polymeric micelles were extensively studied and reviewed as drug delivery systems for the solubilization of hydrophobic drugs (Jones and Leroux, 1999; Aliabadi and Lavasanifar, 2006; Nishiyama and Kataoka, 2006; Torchilin, 2007; Bae and Kataoka, 2009) exhibiting no or marginal carrier-associated side effects after intravenous injection (Kim et al., 2004; Kawaguchi et al., 2009). Examples of anticancer drugs used for solubilization are Paclitaxel (Liggins and Burt, 2002), Doxorubicin (Kataoka et al., 2000) and Camptothecin (Opanasopit et al., 2004).

Furthermore, they offer the potential to alter the pharmacokinetic behaviour of anticancer drugs after parenteral administration achieving long circulation times and enhanced permeation and retention of micelles in solid tumours (EPR-effect) (Maeda et al., 2009). Thus, they may provide a promising approach for more efficient and patient-friendly cancer therapy (Bae and Kataoka, 2009). To date, approximately seven formulations based on this con-

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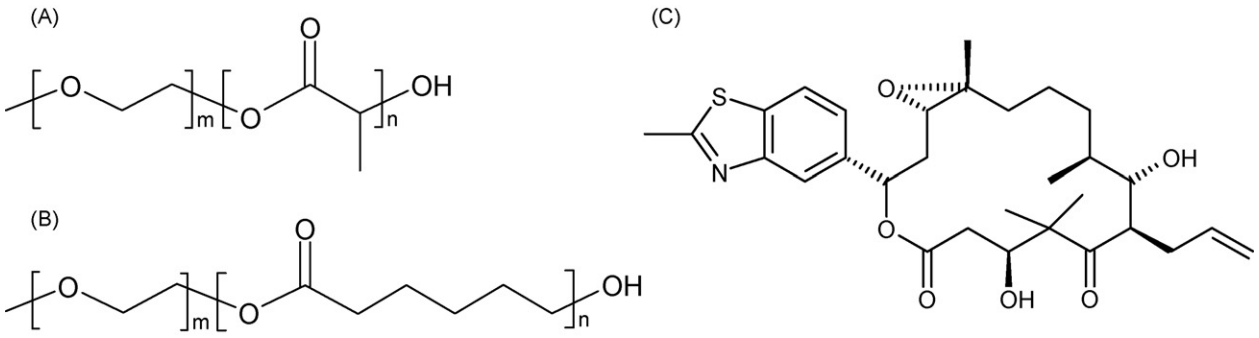

Fig. 1. Structural formulas of (A) PEG-*b*-PLA, (B) PEG-*b*-PCL and (C) Sagopilone.

cept have already entered clinical trials (Aliabadi et al., 2008). The amphiphilic copolymers used mostly contain poly(ethylene glycol) (PEG) as the hydrophilic block and can be classified into four categories according to the nature of the hydrophobic block, namely PEG-*b*-Poly(amino acids), PEG-*b*-Poly(ester), PEG-Phospholipids and Pluronics® (Kwon, 2003). The formulation of Paclitaxel in PEG-*b*-Poly(lactide) (PEG-*b*-PLA) micelles, e.g. in Genexol®-PM, is an impressive example for polymeric micelles as solubilization vehicles although clear evidence of an EPR-effect has not been provided to date. The key advantage of this formulation is a significantly increased maximum tolerated dose (MTD) in humans compared to Taxol® and the absence of carrier-related side effects (Kim et al., 2001; Kim et al., 2004; Kim et al., 2007; Lee et al., 2008). PEG-*b*-Poly(ε-caprolactone) polymers (PEG-*b*-PCL) offer very promising pharmacokinetics in terms of a drastically decreased clearance and consequential prolonged circulation times in preclinical studies (Shi et al., 2005; Liu et al., 2007) but have not been evaluated in clinical trials so far. Toxicity testing of PEG-*b*-PCL micelles revealed their biocompatibility in terms of low cytotoxicity and no acute toxicity after i.v. application *in vivo* (Shi et al., 2005; Wei et al., 2009). Apart from the polymer structure also other factors such as the micelle size and morphology were reported to affect circulation times and biodistribution after parenteral administration (Geng et al., 2007). Among the numerous literature examples, no reports appeared comparing PEG-*b*-PLA and PEG-*b*-PCL primarily with regard to their (a) solubilization capacity and (b) their effect on the pharmacokinetics of cytostatic drugs especially epothilones.

Thus, the aim of the present study was to investigate the solubilization of Sagopilone systematically using various PEG-*b*-PLA and PEG-*b*-PCL polymers (Fig. 1). Solubility parameters were calculated to assess their predictive value. The hydrophobic/hydrophilic-ratio (w/w) of the block copolymers was varied in a range from 0.3 to 1.3 to define the optimum polymer composition in terms of the formation of monodisperse, spherical micelles and efficient, stable drug loading. Furthermore, two different preparation methods were applied and compared to each other. Using the film method a solid film is formed after complete removal of the organic solvent from a single-phase system und subsequent drying. This film could be suitable for storage and the polymeric micelles are formed spontaneously upon film redispersion. However, 'supersaturation' with subsequent precipitation of the drug needs to be taken into account (Torchilin, 2007). For comparison, direct dissolution was employed using sonication.

Thermal analysis was performed to study the drug-polymer-compatibility and the apparent solid-state solubility of Sagopilone within the block copolymers.

Since the micellar morphology was reported to affect the biodistribution *in vivo* the selected candidates were investigated by transmission electron microscopy to identify micelle size and morphology.

2. Materials and methods

2.1. Materials

Sagopilone was obtained from Bayer Schering Pharma AG (Berlin, Germany). The block copolymers poly(ethylene glycol)-*b*-poly(ε-caprolactone) (PEG_{2000}-*b*-PCL_{500}, PEG_{2000}-*b*-PCL_{1400}, PEG_{2000}-*b*-PCL_{2600} and PEG_{5000}-*b*-PCL_{1600}, PEG_{5000}-*b*-PCL_{3600}, PEG_{5000}-*b*-PCL_{5000}), poly(ethylene glycol)-*b*-poly(D,L-lactide) (PEG_{2000}-*b*-PLA_{1200}, PEG_{2000}-*b*-PLA_{2200}) and poly(ethylene glycol)-*b*-poly(L-lactide) (PEG_{5000}-*b*-$PLLA_{2400}$, PEG_{5000}-b-$PLLA_{6000}$) were purchased from Polymer Source Inc. (Dorval, Canada). For the definition of the abbreviations used for these polymers see Table 1 as well as Section 3.2. All other ingredients were obtained in analytical quality.

2.2. Solubility parameter calculation

Solubility parameters of Sagopilone and different polymers were obtained using Hansen's approach (Barton, 1991). It assumes that the total solubility parameter (δ), almost equal to the Hildebrand parameter, arises from dispersive (δ_d), permanent dipole–dipole interactions (δ_p) and hydrogen bonding forces (δ_h) according to Eq. (1).

$$\delta^2 = \delta_d^2 + \delta_p^2 + \delta_h^2 \quad (1)$$

Calculation of the solubility parameters was done on the basis of the group contribution method by Hoy using the Solubility Parameter Software provided by Computer Chemistry Consultancy (Singen, Germany). Furthermore the difference of the three-dimensional solubility parameters between Sagopilone and various polymers ($\Delta\delta$) were calculated (see Eq. (2)).

$$\Delta\delta = \sqrt{(\delta_{d1} - \delta_{d2})^2 + (\delta_{p1} - \delta_{p2})^2 + (\delta_{h1} - \delta_{h2})^2} \quad (2)$$

2.3. Acid value determination

The Acid value is defined as the number that expresses, in milligrams the quantity of potassium hydroxide required to neutralize the free acids present in 1 g of the substance (Council of Europe, 2009). It was determined by anhydrous titration according to method A of the European standard EN ISO 2114 with minor changes regarding the solvent due to the polymer solubility. Briefly, the polymer was dissolved in acetonitrile, phenolphthalein solution was added and the solution was titrated with potassium hydroxide solution (0.01 M) using a GP-Titrino 736 (Metrohm AG, Switzerland) equipped with a Photometer 662 (Metrohm AG, Switzerland) measuring the transmission of light at 570 nm. Data were analysed with TiNet Software 2.4. All measurements were performed in triplicate.

Table 1
Characteristics of PEG-*b*-PCL and PEG-*b*-PLA block copolymers.

PEG-*b*-PCL[a]	M[b] (g/mol)	PDI[c]	AV[d] (mg/g)	PEG-*b*-PLA[a]	M[b] (g/mol)	PDI[c]	AV[d] (mg/g)
P2CL(0.3)	2000–500	1.08	7.4 ± 0.7				
P2CL(0.7)	2000–1400	1.20	4.9 ± 0.2	P2LA(0.6)	2000–1200	1.13	31.9 ± 0.8
P2CL(1.3)	2000–2600	1.15	9.1 ± 0.6	P2LA(1.1)	2000–2200	1.13	5.2 ± 0.4
P5CL(0.3)	5000–1600	1.07	8.4 ± 0.2				
P5CL(0.7)	5000–3600	1.10	5.6 ± 0.4	P5LLA(0.5)	5000–2400	1.04	7.1 ± 0.2
P5CL(1.0)	5000–5000	1.06	7.2 ± 0.5	P5LLA(1.2)	5000–6000	1.04	23.2 ± 1.0

[a] Polymer terminology, whereas the number in parentheses resembles the hydrophobic–hydrophilic-ratio (w/w).
[b] Values according to Polymer Data Sheets, determined by ^{1}H NMR.
[c] Values according to Polymer Data Sheets, determined by SEC.
[d] Acid values (n = 3).

2.4. Preparation of polymeric micelles

Two different preparation methods were employed to prepare loaded as well as unloaded polymeric micelles. (A) The sonication method consisted of the following steps, weighing of the polymer (30 mg) and Sagopilone (3.0 mg) into a screw-top glass vial, addition of 3.0 mL phosphate buffer (pH 7.4) and subsequent sonication using a Sonoplus HD2070 (Bandelin electronic, Berlin, Germany) at 100% power in an unpulsed mode for 10 minutes. (B) The film formation method was performed as follows. Block copolymer (30 mg) and Sagopilone (3.0 mg) were weighed into a round-bottomed flask and dissolved in 3 mL acetonitrile. The solvent was evaporated under reduced pressure at room temperature with subsequent drying at 0.1 mbar for 1 h. The resulting film was redispersed with 3.0 mL phosphate buffer (pH 7.4) under shaking without additional heating or sonication. Empty micelles were prepared according to the same protocol in the absence of Sagopilone. The initial drug–polymer-ratio was 1:10 for the drug loaded samples and the resulting polymer concentration was kept uniformly at 10 g/L for all samples allowing comparisons between unloaded and loaded samples. Blanks were prepared without the addition of the polymer. The samples were filtered using a syringe filter (Millex®-GV 0.22 μm, Millipore, USA) and the resulting micellar dispersions were used for further analysis.

2.5. Characterization of micelle size and size distribution

The micelle sizes and size distributions were determined by dynamic light scattering (DLS) using a Zetasizer Nano (Malvern Instruments Ltd., Worcestershire, UK). Briefly the principle is based on the measurement of the backscattered light fluctuations at an angle of 173° and the calculation of an autocorrelation function. The samples were measured undiluted at 25 °C adjusted to the temperature for 1 min prior to the measurement. The autocorrelation functions were analysed using the DTS v5.1 software provided by Malvern and the hydrodynamic diameter of the micelles (d_H) and their size distribution (PDI – polydispersity index) was calculated. Measurements were done in triplicate with 15 to 20 runs each and the calculated mean values were used.

2.6. Determination of the Sagopilone drug loading

The Sagopilone content of the micellar dispersions was determined by high performance liquid chromatography (HPLC) using an Agilent 1100 Series chromatography system (Agilent Technologies, Santa Clara, USA) consisting of a quaternary pump, an auto-injector, a column heater at 25 °C and a UV-detector. Two Chromolith® Performance RP-18e columns (100 × 4.6 mm, Merck, Germany) were used and a gradient was run from ACN/water (25/75 v/v) to ACN/water (45/55 v/v) in 10 min followed by isocratic elution for 15 min at a flow rate of 1 mL/min. Samples were diluted 5–10 times with ACN/water (50/50 v/v) prior to analysis. The injection volume of the samples was 10 μL and Sagopilone was detected at a wavelength of 220 nm. The data was analysed using Empower™ 2 software (Waters Corporation, Milford, USA) and the amount of Sagopilone was determined by an external standard calibration. The solubilization efficiency (SE) of Sagopilone was calculated according to Eq. (3).

$$SE(\%) = \frac{\text{mass of Sagopilone loaded in mg}}{\text{mass of Sagopilone fed in mg}} \times 100\% \quad (3)$$

2.7. Differential scanning calorimetry (DSC)

DSC measurements were carried out on a DSC822e (Mettler Toledo, Switzerland) at a heating rate of 20 K/min using dry nitrogen purge gas. The samples were first heated to 100 °C, subsequently cooled to −100 °C with liquid nitrogen and heated again to 180 °C. Polymeric films with varying Sagopilone weight fractions prepared by the film formation method were measured using aluminium sample pans. At least three individual samples were prepared with three individual measurements per sample and the data was analysed with STARe Software 9.10. The thermograms were normalized to the sample weight. The DIN midpoint of the slope change of the heat flow plot of the second heating scan was considered as the glass transition temperature (T_g). The melting (T_m) and crystallization (T_c) temperatures were taken as the maximum of the endothermic and the minimum of the exothermic peaks, respectively. Furthermore the heat capacity change (Δcp) at the T_g was determined for drug and polymers.

2.8. Cryogenic transmission electron microscopy (cryoTEM)

Samples were prepared for cryoTEM analysis by preserving in a thin layer of vitreous ice supported on C-Flat holey carbon films (Protochips, Inc.) on 400 mesh copper grids. Grids were cleaned in a Solarus plasma cleaner (10 s, 25% O_2, 75% Ar) immediately prior to vitrification using an FEI Vitrobot (4 °C, 95% RH). Vitrified grids were transferred into the electron microscope using a cryoholder (Gatan, Inc.) that maintains the temperature of the grid below −170 °C. Microscopy was performed using a Tecnai Spirit transmission electron microscope (FEI Co.) equipped with a 4k × 4k CCD camera. Images were acquired at nominal magnifications of 52,000× (0.21 nm/pixel) and 21,000× (0.50 nm/pixel) using the Leginon data acquisition software (Suloway et al., 2005) at a nominal underfocus of −6 μm (21,000×) and −3 μm (52,000×) with electron doses of 10–15 ($e^-/Å^2$). For shape investigations images were acquired at zero tilt (0°) as well as at a high tilt angle (55°). The alignment and classification process was done with the XMIPP processing package using the Kernel Probability Density Estimator Self-Organizing Map classification method as described in the literature (Pascual-Montano et al., 2001; Sorzano et al., 2004). Briefly, algorithms in this package align the selected particles and sort them into self-similar groups of classes. Afterwards the class average diameters were measured.

2.9. Statistics

Data were recorded as mean ± standard deviation. All experiments were done at least in triplicate as specified in Section 3. Means were analysed for statistical significance using unpaired student's *t*-test. Differences were considered significant at *p*-values <0.05. Linear regression analysis was processed using SigmaPlot 8.0 (Systat Software Inc., San Jose, CA).

3. Results

3.1. Solubility parameters

Following the theory that two solvents are miscible, when the difference in their solubility parameters is small enough, the theoretical drug–polymer-compatibility was estimated. The total solubility parameters (δ) of Sagopilone, poly(lactide) and poly(ε-caprolactone) were calculated to be 23.78, 21.78 and 20.64 $MPa^{1/2}$, respectively, resulting from dipole–dipole interactions, dispersive and hydrogen bonding forces.

Concerning the partial solubility parameters, PLA and PCL exhibit similar differences in their hydrogen bonding interactions to Sagopilone with $\Delta\delta_h$ of 0.71 and 1.00 $MPa^{1/2}$, respectively, but a distinct difference in the others. PCL reveals a high portion of dispersive/van der Waals interactions with Sagopilone compared to PLA with $\Delta\delta_d$ of 1.11 and 2.97 $MPa^{1/2}$, respectively. In contrast, permanent dipole–dipole interactions seem to be the major interaction forces between Sagopilone and PLA pursuant to $\Delta\delta_p$ of 0.25 $MPa^{1/2}$ compared to 3.72 $MPa^{1/2}$ for PCL.

According to the lower difference in the total solubility parameter to Sagopilone, poly(lactide) ($\Delta\delta$: 3.06 $MPa^{1/2}$) seems to be superior to poly(ε-caprolactone) ($\Delta\delta$: 4.01 $MPa^{1/2}$) suggesting a better compatibility. This gives rise to the expectation that PEG-*b*-PLA micelles exhibit higher solubilization of Sagopilone and stability compared to PEG-*b*-PCL micelles.

3.2. Characterization of block copolymers

A set of 10 commercially available block copolymers was used in this study. These can be divided in two groups, namely (1) PEG-*b*-PCL and (2) PEG-*b*-PLA, which can be further subdivided according to the molecular weight of PEG at (a) 2000 and (b) 5000 Da. They were abbreviated as P2CL, P5CL, P2LA and P5LLA, respectively, as shown in Table 1. Within the particular groups the molecular weight of the hydrophobic blocks was varied. The hydrophobic/hydrophilic-ratio, which is the quotient of the molecular weight of the hydrophobic and the hydrophilic block and stated in parentheses for the particular polymer, was varied in a range from 0.3 to 1.3 to ensure the formation of star-type micelles with a typical core-corona structure (Allen et al., 1999).

The block copolymers investigated were characterized by the supplier using size exclusion chromatography (SEC), ^{1}H nuclear magnetic resonance spectroscopy (^{1}H NMR) and differential scanning calorimetry (DSC). To establish an additional quality control for these excipients, not listed in the pharmacopoeia yet, acid values of the polymers were determined. An anhydrous titration according to the European standard (EN ISO 2114:2000) was used to determine the free carboxyl groups in the form of free acids or homopolymers and anhydrides present in the material. The PEG-*b*-PCL polymers exhibited low acid values (4.5–9.5 mg KOH per g polymer) displaying neither a dependency on the PEG-content, the PCL/PEG-ratio nor the polydispersity of the polymers (see Table 1). For comparison, the limit values of standard excipients like polysorbates, polyoxyethylene castor oil derivatives and sucrose ester are in a range of 2.0–6.0 mg KOH per gram raw material (Council of Europe, 2009). In contrast, the acid values of PEG-*b*-PLA were in a broader range (4–32 mg KOH per gram polymer) with remarkable high values of 32 and 23 mg KOH per gram polymer for P2LA(0.6) and the P5LLA(1.2), respectively.

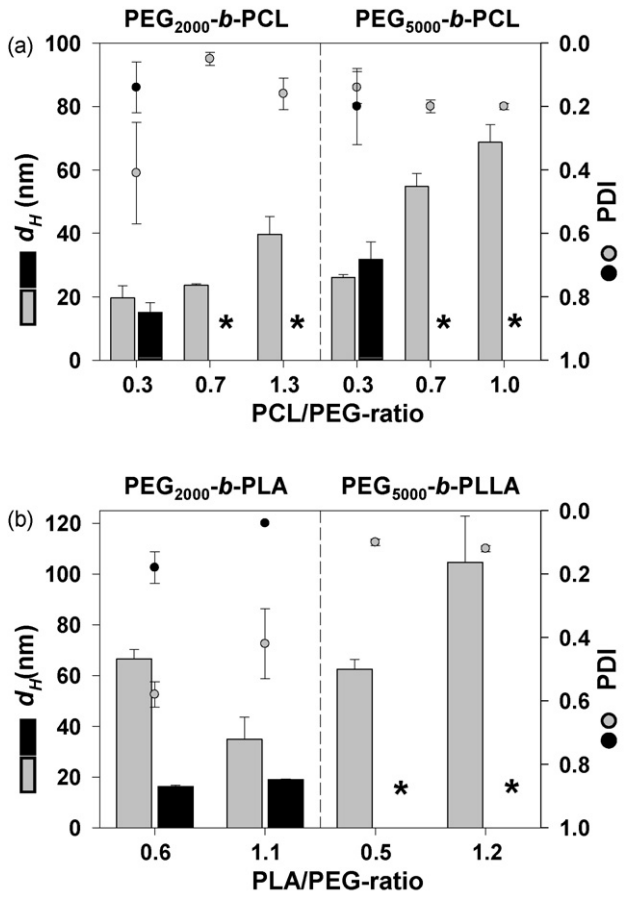

Fig. 2. Characteristics of polymeric micelles prepared by two different methods as a function of the hydrophobic/hydrophilic-ratio of the block copolymers (hydrodynamic diameter d_H (bars) and polydispersity index PDI (dots) of unloaded polymeric micelles prepared by either sonication (□ /○) or film formation (■ /●); * film redispersion not possible; n = 3–4).

3.3. Micelle preparation and characterization

The film formation and sonication method were investigated with regard to method applicability and solubilization efficiency. The results were compared to each other and correlated with the polymer properties.

The PCL/PEG-ratio had a stake in the applicability of the preparation method as well as the micelle sizes as shown in Fig. 2. At a constant molecular weight of PEG increasing PCL block lengths led to increasing micelle sizes in an almost linear manner (P2CL with R^2 = 0.94; P5CL with R^2 = 0.99) using the sonication method (Fig. 2). Independent of the molecular weight of PEG the PCL/PEG-ratio defined the redispersion behaviour of the polymeric films. A PCL/PEG-ratio of 0.3 allowed complete film redispersion (Fig. 2) and the corresponding polymeric films were clear with observable spherulites. In contrast, the films at higher PCL/PEG-ratios were turbid and not redispersible.

Comparing P2LA and P5LLA revealed a different behaviour (Fig. 2). P2LA polymers with a PLA/PEG-ratio of 0.6 and 1.1 resulted in small, monodisperse micelles after film redispersion, which are almost equal in size at 16 and 19 nm, respectively. In contrast, sonication was not feasible to form monodisperse micelles for these polymers, indicated by PDI values in the range of 0.4–0.6 (Fig. 2).

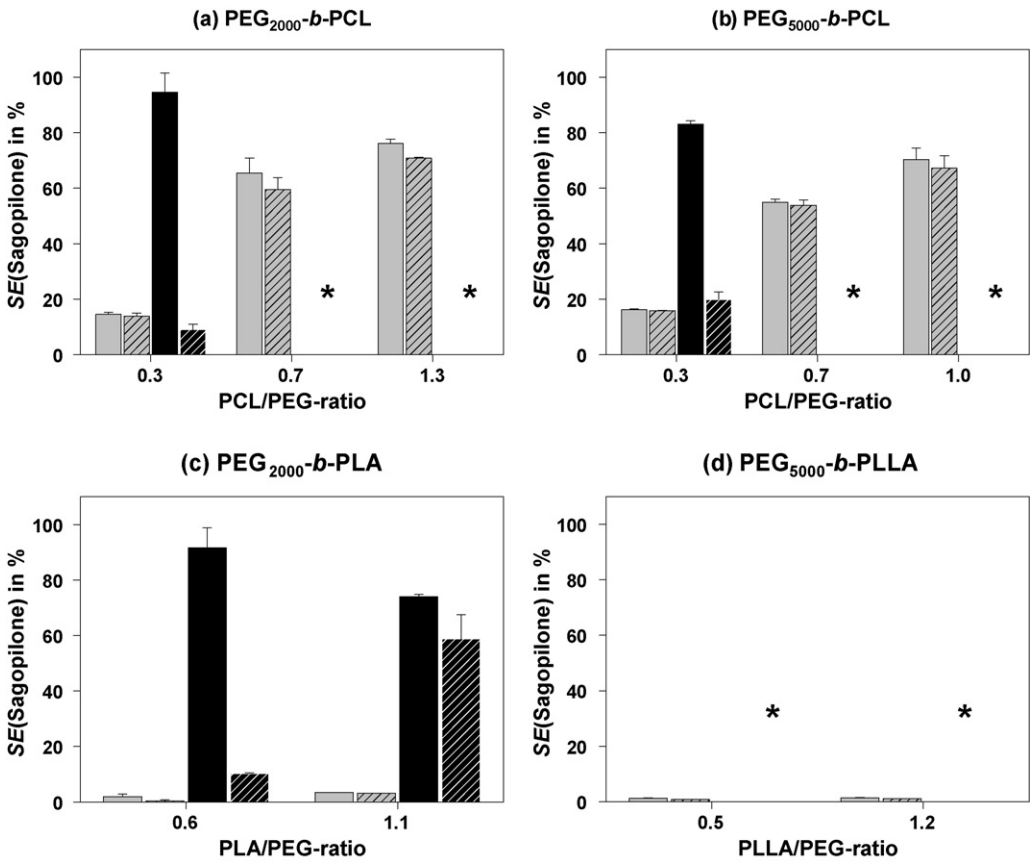

Fig. 3. Sagopilone solubilization and stability of polymeric micelles prepared by two different methods (solubilization efficiency (SE) after preparation either by sonication (□) or film formation (■) and after 24 h of storage at room temperature (▨/▩); *c*(polymer) of 10 g/L; * film redispersion not possible; $n = 3$).

Using the same methods, the behaviour of P5LLA polymers was vice versa. The polymeric films were turbid and not redispersible. On the other hand, sonication led to the formation of monodisperse (0.10 and 0.12), comparatively large micelles (63 and 105 nm) of P5LLA(0.5) and P5LLA(1.2) (Fig. 2).

3.4. Solubilization of Sagopilone

The impact of the mechanism of micelle preparation on the solubilization efficiency (SE) of Sagopilone was investigated (Fig. 3). Target concentration was constant at 1 g/L corresponding to a solubilization efficiency of 100%. The drug content as well as the physicochemical characteristics were determined after preparation and after storage at room temperature for 24 h.

P2CL and P5CL polymers showed similar results for the solubilization of Sagopilone (Fig. 3a and b). Addition of Sagopilone did not alter the redispersion behaviour of the polymeric films. Hence, no solubilization was observed for the polymers with a PCL/PEG-ratio of 0.7 and higher using the film method. Solubilization efficiencies as high as (95 ± 6.8) and (83 ± 1.2)% were achieved with P2CL(0.3) and P5CL(0.3), respectively, but a 'supersaturation' effect with subsequent precipitation of Sagopilone occurred (Fig. 3a and b). Using the sonication method the solubilization efficiency increased with the PCL/PEG-ratio in an almost linear manner within the group of P5CL ($R^2 = 0.98$) but without linear correlation for P2CL ($R^2 = 0.82$). The solubilization efficiency obtained with P2CL(0.3) and P5CL(0.3) of (15 ± 0.8) and (16 ± 0.2)%, respectively, was similar to the drug content remaining after precipitation of the 'supersaturated' dispersions. The sonicated dispersions were stable for at least 24 h in contrast to dispersions prepared by film formation. At the higher PCL/PEG-ratios of 0.7 and 1.0–1.3 solubilization efficiency of P2CL was higher than P5CL with (66 ± 5.4) and (76 ± 1.6)% compared to (55 ± 1.1) and (70 ± 4.2)%; respectively. Interestingly, particle sizes of P2CL were not affected by drug loading in contrast to an increase in size for the P5CL micelles without a change in the size distribution (data not shown). In addition, the sonicated P5CL micelles still contained more than 95% of initially loaded Sagopilone after 24 h compared to 91% and 93% within P2CL(0.7) and P2CL(1.3) micelles, respectively.

The P2LA polymers exhibited very low solubilization after sonication, namely (1.9 ± 0.9) and (3.5 ± 0)% for P2LA(0.6) and P2LA(1.1) (Fig. 3c). The application of the film formation method resulted in a high solubilization with (92 ± 7.1) and (74 ± 0.8)%, respectively. These micellar dispersions showed the typical 'supersaturation' effect and 90% and 20% of the initially loaded Sagopilone precipitated within 24 h at a PLA/PEG-ratio of 0.6 and 1.1, respectively (Fig. 3c). Micelles of P2LA(0.6) ($d_H = (16 \pm 1)$ nm) remarkably increased after Sagopilone loading ($d_H = (145 \pm 17)$ nm). The P5LLA polymers showed almost no solubilization of Sagopilone after sonication (Fig. 3d) with solubilization efficiencies as low as (1.2 ± 0.2) and (1.5 ± 0)% for P5LLA(0.5) and P5LLA(1.2), respectively, compared to (0.8 ± 0.02)% for the blank. Film formation was not applicable since drug loaded polymeric films were not redispersible at all.

As a result of this systematic screening, three polymers were selected as optimum materials for the solubilization of Sagopilone, namely P2LA(1.1), P2CL(1.3) and P5CL(1.0). As film formation was applied for P2LA(1.1), sonication was most suitable for P2CL(1.3) and P5CL(1.0). The well-known 'supersaturation' effect after film

redispersion has been observed as well independent of the polymer used.

3.5. Thermal analysis

Thermal analysis was used to study the 'supersaturation' effect accompanying the film formation method more in detail, to provide an evidence of the solubility of Sagopilone within the amphiphilic block copolymers and to determine the apparent solid-state saturation solubility. Therefore, blank as well as drug loaded polymeric films of P2CL and P2LA were analysed by DSC.

First, the thermodynamic transition points of the unloaded polymeric films, especially the glass transition temperatures of the hydrophobic blocks, were determined. As shown in Table 2 the polymeric films of P2CL(0.3) exhibited a glass transition as well as a melting. That was in good correlation with the observation of spherulites within the films, which are spherical semi-crystalline regions inside non-branched linear polymers by definition. With regard to the value of the glass transition temperature (T_g) at (-66.3 ± 0.5)°C, the amorphous phase was composed of PCL. An increase of the PCL/PEG-ratio to 0.7 and 1.3 led to an increase in the glass transition temperature and distinct shoulders of the PEG melting peak. Furthermore, the thermogram of P2CL(1.3) revealed two separate exothermic peaks during cooling indicating the formation of a third phase composed of crystalline PCL separately from PEG. This, again, correlated very well with the film turbidity of those polymers, in contrast to P2CL(0.3).

P2LA also exhibited a melting and a glass transition temperature as shown for P2LA(1.1) (Table 2). In contrast to P2CL, the measured T_g was an artefact due to the inhibition of the crystallization of the molten PEG phase during cooling at DSC measurement. The T_g of the PLA phase at approximately 38 °C could not be determined because of an overlap with the delayed crystallization and subsequent melting of PEG. Hence, the apparent solid-state solubility of Sagopilone was investigated for P2CL based on the alteration of the glass transition of PCL. The T_g of drug loaded polymeric films was determined and correlated to theoretical approaches. The films contained Sagopilone at a weight fraction equal to the solubilization experiments (Table 2).

$$\frac{1}{T_g} = \frac{w_1}{T_{g,1}} + \frac{w_2}{T_{g,2}} \quad (4)$$

$$T_g = \frac{w_1 T_{g,1} + K_1 w_2 T_{g,2}}{w_1 + K_1 w_2} \quad K_1 = \frac{\Delta cp_2}{\Delta cp_1} \quad (5)$$

If Sagopilone functions as a plasticizer T_g will be decreased as described by the Fox Approach (Eq. (4), Table 2). In contrast, the formation of a glass solution is indicated by an increasing value of T_g compared to the blank according to the Couchman–Karasz equation (Eq. (5), Table 2). The glass transition temperatures were significantly increased compared to the corresponding blanks, independent of the PCL/PEG-ratio (Table 2), and they correlated very well with the values calculated by the Couchman–Karasz equation. Hence, Sagopilone was solubilized within the glassy PCL region in terms of a glass solution. P2CL(0.3) showed the best correlation (deviation of 1.0%) in comparison to the higher PCL/PEG-ratios. No alteration was observed for the other thermodynamic transitions (data not shown) underlying the phase separation nature of these films.

P2CL(0.3) was investigated further in order to determine the saturation solubility of Sagopilone within this polymer (Fig. 4, left). The measured T_g correlated very well with the Couchman–Karasz equation at Sagopilone weight fractions of 0.09 and lower. At higher weight fractions the T_g was constant at approximately −60 °C, which was remarkably lower than the predicted values by Couchman–Karasz but still elevated in comparison to the blank film. Moreover, a Sagopilone melting peak appeared at weight fractions of 0.5 and higher. The T_g observed at the higher drug loading of approximately −60 °C was similar to the T_g of films comprising a Sagopilone weight fraction of 0.02 (Fig. 4, left). In addition, the micellar dispersions comprising a drug loading at this weight fraction (0.02) did not show the 'supersaturation' phenomenon as shown in the right graph of Fig. 4, indicating a saturated loading.

3.6. Characterization of micelle morphology

Morphology determination of the three selected micellar delivery systems was done by cryo transmission electron microscopy (cryoTEM) to preserve the three-dimensional structure of the micelles in their native hydrated state.

The images revealed that the micelles were spherical with a monodisperse distribution in the absence of larger aggregates independent of the polymer and preparation technique used (Fig. 5). The PCL-containing micelles exhibited a hexagonal arrangement with a high degree of order as seen for P5CL(1.0) in contrast to P2LA(1.1) micelles. The latter were randomly spaced apart. In addition, comparison of the values obtained by DLS and cryoTEM (Table 3) revealed a remarkable difference for the PCL-containing micelles although presumption for the size measurement by DLS, which is the existence of spherical particles, was fulfilled. This effect was pronounced most for P5CL(1.0). A cryoTEM tilt study of P5CL(1.0) micelles was performed to clarify the exact morphology since spheres may be simulated by single imaging of cylinders in a topview. The images were taken at a tilt angle of 0° and 55°. The micelles were proven to be spherical as shown in Fig. 6. Approximately 300 particles per image were selected and classified automatically resulting in 4 classes, which were represented below the full images. They were almost equal in size at the respective measurement angle as well as between the different tilts (25.8, 25.3, 25.4, 24.7 nm at 0° vs. 25.2, 26.0, 25.6, 24.6 nm at 55°). This

Table 2
Thermal properties of blank polymeric films of PEG_{2000}-*b*-PCL and PEG_{2000}-*b*-PLA compared to $mPEG_{2000}$ and drug loaded films of PEG_{2000}-*b*-PCL ($n = 3 \times 3$).

Films	Blank polymeric films					Sagopilone loaded films[a]			
	^1T_m (°C)	^2T_c (°C)	^3T_g (°C)	$^3\Delta cp$ (J g^{-1} K^{-1})	^3T_m (°C)	T_g (°C)	$^b T_g$ (°C)	$^c T_g$ (°C)	$T_g/^c T_g$
P2CL(0.3)	49.1 ± 1.2	5.4 ± 16	−66.3 ± 0.5	0.22 ± 0.03	47.6 ± 0.3	−50.6 ± 1.8*	−84.0	−51.1	0.99
P2CL(0.7)	50.5 ± 0.7	23.0 ± 2.1	−57.0 ± 0.6	0.29 ± 0.03	48.1 ± 0.2	−48.2 ± 3.3*	−70.7	−46.0	1.05
P2CL(1.3)	50.2 ± 0.3	18.7 ± 6.5; 11.4 ± 7.9	−52.3 ± 0.2	0.35 ± 0.04	43.8 ± 0.2; 45.4 ± 0.2; 49.7 ± 0.2	−46.0 ± 1.8*	−64.2	−43.5	1.06
P2LA(1.1)	40.7 ± 2.0	–	−37.1 ± 0.8	–	38.7 ± 0.3				
$mPEG_{2000}$	56.8 ± 0.3	27.4 ± 1.4	–	–	54.8 ± 0.3				
Sagopilone	–	–	50.2 ± 0.5	0.33 ± 0.04	–				

[1–3]Determined at first heating scan ([1]), at cooling cycle ([2]) and at second heating ([3]).
[a] Sagopilone weight fraction of 0.09.
[b] Theoretical T_g based on Fox Approach (see Eq. (4)).
[c] Theoretical T_g based on Couchman–Karasz equation (see Eq. (5)).
* Significant difference ($p < 0.05$) in T_g compared to blank films.

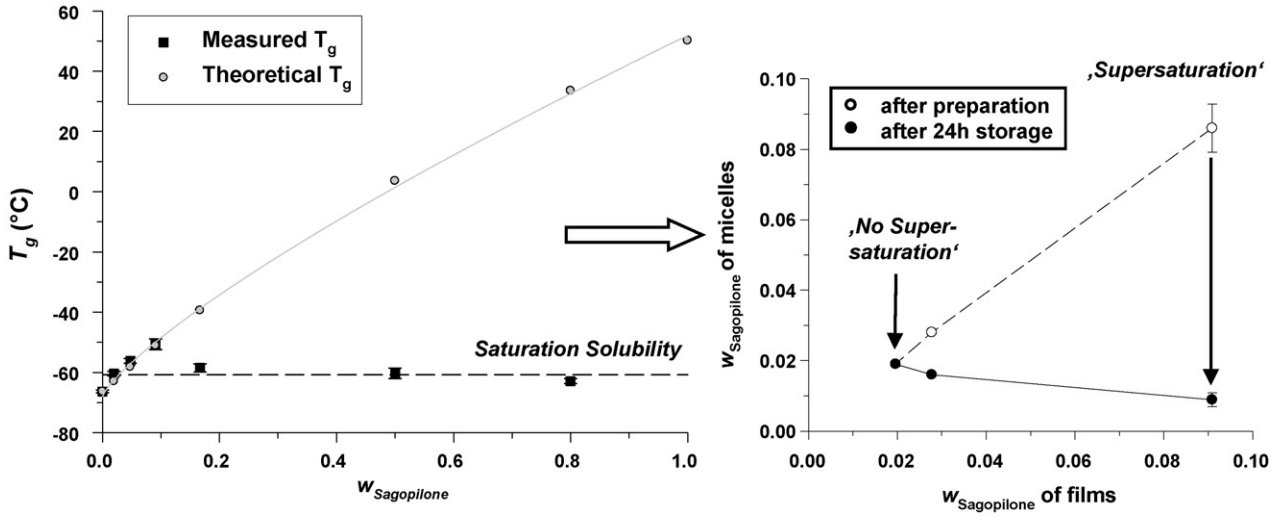

Fig. 4. Determination of the apparent solid-state solubility of Sagopilone (- - -) in films composed of P2CL(0.3) (left: Measured and theoretical T_g according to Couchman–Karasz as a function of drug weight fraction (w); right: Drug weight fraction (w) of micellar dispersions after film redispersion and after 24 h at c(polymer) of 50 g/L; n = 5).

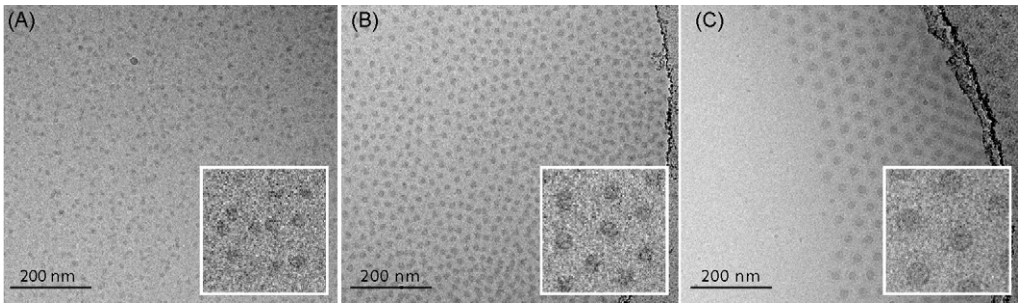

Fig. 5. CryoTEM images of polymeric micelles composed of (A) P2LA(1.1), (B) P2CL(1.3) and (C) P5CL(1.0) at a polymer concentration of 20 g/L. The inset shows a small region (100 nm^2) of the image at a larger scale.

Table 3
Comparison of particle characteristics obtained by DLS and cryoTEM of micellar dispersions.

Sample	Polymer	f (%)[a]	Preparation	c (g/L)	DLS		cryoTEM size[b] (nm)	ΔSize[c] (nm)
					d_H (nm)	PDI		
A	P2LA(1.1)	47.6	Film formation	20	20.0	0.016	12.9	7.1
B	P2CL(1.3)	43.5	Sonication	20	32.6	0.145	16.9	15.7
C	P5CL(1.0)	50.0	Sonication	20	71.9	0.215	22.3	49.6

[a] Hydrophilic fraction f of the block copolymer (w/w).
[b] Median of measurement of 200 micelles (4 different images with 50 micelles each).
[c] Difference between hydrodynamic diameter (d_H) and size at cryoTEM.

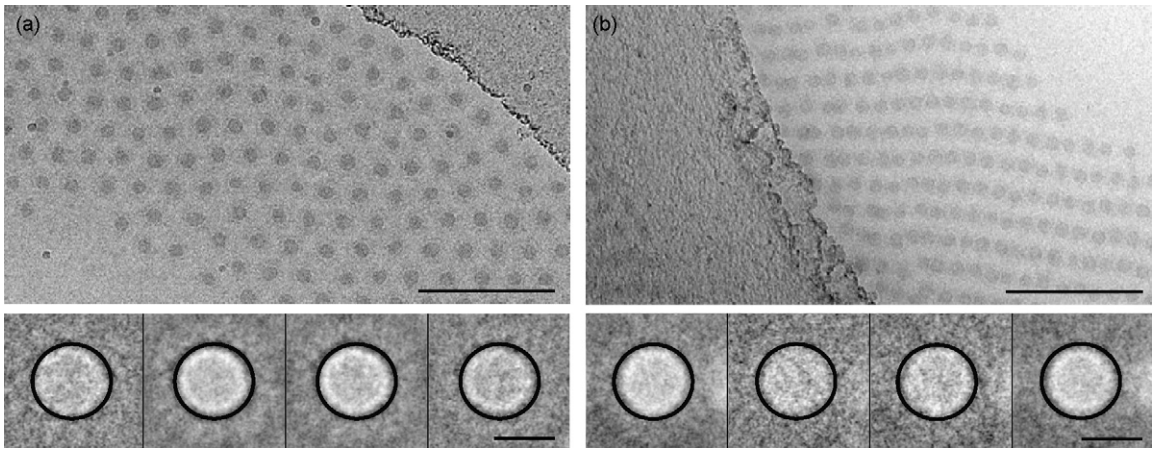

Fig. 6. Comparison of cryoTEM images of P5CL(1.0) micelles acquired at zero tilt (a) and at a high tilt angle of 55° (b) (bar = 200 nm). Underneath the respective class averages obtained by the described selection and classification process are represented (bar = 20 nm).

was additionally displayed by the overlay of a black circle of a fixed diameter of 26 nm.

4. Discussion

The method developed for acid value determination of amphiphilic block copolymers offers a fast and convenient test for purity assessment following standard tests of the European Pharmacopoeia. This is of high importance to their implementation as standardized excipients. Assuming the acid value is exclusively due to the cleavage of the polyester block the emerging acids constitute a maximum of 2% (w/w raw material) except for the outliers P2LA(0.6) and P5LLA(1.2). The latter may be due to the presence of possible by-products of the polymer synthesis such as poly(lactide) homopolymers and free lactic acid resulting from polymer degradation or cleavage of residual lactide present in the raw material. This fact has to be taken into account for data interpretation. Furthermore, future studies are needed to define threshold acid values for these novel excipients.

The nature of the hydrophobic block, the preparation technique as well as the composition of the block copolymers had a significant impact on the micelle formation and the solubilization of Sagopilone. Sonication was applicable to P2CL and P5CL exhibiting the same dependencies within the particular groups. In comparison with published data using a similar polymer, the micelles of P5CL(1.0) formed by sonication (69 nm, PDI 0.20) were smaller than micelles obtained by a co-solvent evaporation method (87.5 nm, PDI 0.198) (Aliabadi et al., 2005). But they were larger than those formed by solvent displacement with subsequent sonication (41.0 nm) (Shuai et al., 2004a). Likewise, the sonicated micelles of P5CL(0.7) exhibited remarkably smaller sizes (55 nm, PDI 0.20) compared to the use of a co-solvent evaporation method (71.8 nm) (Shuai et al., 2004b). The hydrophobic/hydrophilic-ratio was found be a crucial parameter for the applicability of the film formation for P2CL and P5CL. At values of 0.3 film redispersion was possible in contrast to the general statement that this preparation method is inappropriate for this kind of polymers (Aliabadi et al., 2005). These results support the published observations that the procedure of the micelle formation plays a significant role in determining the average diameter and size distribution aside from the block copolymer molecular weight (Vangeyte et al., 2004; Aliabadi et al., 2005). Larger micelle sizes reported for the co-solvent evaporation method may be due to the precipitation driven micelle formation in contrast to the self-assembly of amphiphilic block copolymers into micelles during direct dissolution using sonication. Comparison of the latter with micelles prepared by film formation, if applicable, revealed only small differences. This fact further corroborates the theory since film hydration is self-assembling driven as well.

The application of identical procedures for PEG-*b*-PLA polymers revealed a distinct difference between P2LA and P5LLA due to the different stereoisomers of the lactic acid monomers. Film redispersion was only feasible if the hydrophobic block was formed of poly(D,L-lactide). In contrast, only P5LLA polymers containing a poly(L-lactide) block produced monodisperse micelles when sonicated. It is known that steric factors play an important role in chain flexibility, chain packing and subsequent crystallization behaviour (Cowie and Arrighi, 2008). For this reason, P2LA micelles having an amorphous poly(D,L-lactide) core were almost similar in size (around 20 nm) independent from the MW of PLA. P5LLA micelles, comprising a semi-crystalline poly(L-lactide) core, were remarkably larger (63 and 105 nm) with a dependence on the PLA block length (Huh et al., 2003). These findings are in good agreement with literature values (Yasugi et al., 1999; Jongpaiboonkit et al., 2006; Zhang et al., 2007).

Solubilization efficiency of P2CL and P5CL micelles prepared by sonication increased with the particular PCL/PEG-ratio with comparable results between the two groups. This is in contrast to previous observations reporting an increase in the drug loading e.g. of Paclitaxel with the PCL block length independent of the molecular weight of PEG (Shuai et al., 2004b). However, the latter was observed for micelles prepared by co-solvent evaporation highlighting the impact of the preparation method. Using the co-solvent evaporation method clustering of Sagopilone with the hydrophobic blocks is promoted since the drug and the polymer are dissolved prior to the micelle formation and encapsulation event. On the contrary, drug and polymer dissolution, micelle formation and drug loading take place at the same time during the sonication method displaying an additional loading hindrance due to the shield of the hydrophobic core by a PEG corona. This is very likely to be the reason for the higher solubilization of the P2CL besides a partial degradation of the P5CL polymers. The latter can be excluded since it has not been observed in terms of higher acid values. Contrary to P2CL, the sizes of P5CL micelles increased after drug loading. This effect was not predictable since all possible alterations have been reported in the literature, namely an increase in size after solubilization of cyclosporine A for P5CL(1.0) (Aliabadi et al., 2005), no alteration in the size of Paclitaxel-loaded micelles of P5CL(0.8) (Shuai et al., 2004b) as well as a slight decrease in size of P5CL(1.0)-micelles together with an increase of P2CL(1.0)-micelles after doxorubicin loading (Shuai et al., 2004a). Possible explanations for this effect are the formation of a small amount of drug nanocrystals with diameters less than the pore size of the filter (0.22 μm) or measurement artefacts due to the presence of dust or an altered viscosity. However, the former have not been detected as a single size population at DLS and the corresponding blank samples exhibited negligible Sagopilone concentrations ($0.8 \pm 0.02\%$). Further studies are needed to elucidate the effect of the drug used, the polymer structure and concentration, and, in particular, the measurement settings.

Very high solubilization values after film hydration, if feasible, and the subsequent precipitation of Sagopilone coincide with the described 'supersaturation' effect of polymeric micelles especially obtained by a film formation method (Torchilin, 2007; Carstens et al., 2008). The same range of the effective solubilization after precipitation and after sonication without subsequent precipitation provides an indication of the loading capacity of the particular micelles.

A big discrepancy was observed in the solubilization capacity of micelles comprising a poly(D,L-lactide)- and poly(L-lactide)-core. This is not in accordance with the theory that the intermolecular forces between the hydrophobic drug and the core-forming block of the polymer are the major criterion for the solubilization capacity. The fact that Sagopilone was not solubilized by poly(L-lactide) was not predictable and may be due to a high degree of core crystallinity without molecular dispersion of Sagopilone. The high accommodation of Sagopilone into poly(D,L-lactide)-containing polymeric micelles indicates that the amorphous core structure is superior for drug solubilization. Using the film formation for P2LA a 'supersaturation' effect has been observed as well with a distinct lower amount of drug precipitation at the larger PLA/PEG-ratio. The instability of P2LA(0.6) may be additionally enlarged by hydrolytic degradation of the polymer as indicated by the high acid value.

Consequently, the hydrophobic/hydrophilic-ratio was best at approximately 1 regarding solubilization efficiency and stability. Thus, P2LA(1.1), P2CL(1.3) and P5CL(1.0) were selected as delivery vehicles for further studies along with the appropriate method of preparation.

Correlation of the effects described with the calculated solubility parameters revealed that the latter were not predictive. According to the theoretical estimation PLA exhibits a better compatibility

compared to PCL, whereas direct comparison of P2LA(1.1)- and P2CL(1.3)- micelles revealed similar to higher solubilization and, most notably, more stable drug loading of P2CL(1.3). This is in contrast to findings of Jubo et al. (2004) demonstrating a good correlation between solubility parameters and drug formulation characteristics like drug loading for PEG-*b*-PLA and PEG-*b*-PCL micelles.

As shown by the partial solubility parameters the portions of the different interaction forces between Sagopilone and PCL differ from those of PLA. Structurally, both polymers consist of a polyester backbone with possible hydrogen bonding between their carbonyl functions and free hydroxyl groups of Sagopilone. This is congruent with the similar difference in the particular hydrogen bonding parameter. Hence, the better compatibility of PEG-*b*-PCL is very likely to be due to the higher portion of dispersive/van der Waals forces between Sagopilone and PCL as well as between PCL chains itself resulting in higher micelle stability.

Further evidence of the non-correlation was shown by the inability of P5LLA to solubilize Sagopilone compared to sufficient solubilization of P5CL micelles. The distinct solubilization capacities of poly(L-lactide) and poly(D,L-lactide) as core-forming blocks were not covered by this method since the solubility parameter does not distinguish between different stereoisomers. However, the difference in the stereochemistry of the lactic acid monomer results in different aggregation behaviour/crystallinity of the resulting polymer, and this in turn highly affects the solubilization. Thus, the theoretical methods need to be adapted to accommodate this type of difference.

Thermal analysis revealed a highly phase-separated composition of the polymeric films. As shown for P2CL(0.3–1.3) the films consisted of crystalline PEG, amorphous PCL and crystalline PCL. The formation of the latter was essentially influenced by the PCL/PEG-ratio instead of the mere PCL molecular weight and determined the film redispersion behaviour. This is in good correlation with findings that nanoscale confinement of normally semi-crystalline PCL within blends with 100 nm dispersed phases impedes the crystallization of PCL, yielding liquid-state PCL domains at room temperature (Kim et al., 2008). Thermo-analytical investigations of drug loaded films indicated the presence of glass solutions comprising Sagopilone and PCL besides crystalline regions of the film. The good correlation with the theoretical approach describing glass solutions (Couchman–Karasz Approach) provided clear evidence that Sagopilone was molecularly dispersed in the amorphous PCL phase. Complete molecular dispersion of the drug was obtained with P2CL(0.3) indicated by a deviation of only 1.0% from the theoretical value. This finding is in contrast to a described plasticizer effect of drugs like propranolol after loading into PCL nanoparticles indicated by a decrease of T_g (Ubrich et al., 2004). The findings of this study provide an explanation of the repeatedly described 'supersaturation' effect of polymeric micellar dispersions after film hydration. Sagopilone was shown to be completely dispersed in the liquid-like PCL phase of P2CL(0.3)-films at a maximum drug weight fraction of 0.09. This value was not consistent with the solubilization capacity of the micelles as observed by the 'supersaturation' effect. Further thermal analysis revealed a saturation solubility of the films at a weight fraction of 0.02. This value correlated well with the solubilization capacity of the corresponding micelles indicated by the absence of any "supersaturation" (Fig. 4). This is a very promising approach for the determination of the solubilization capacity and has to be proven for further drugs. To expand this approach to PEG-*b*-PLA the measurement parameters have to be optimized with respect to a reliable determination of the T_g of PLA.

Depending on the hydrophilic fraction *f* self-assembly of amphiphilic block copolymers leads to the formation of micelles with spherical ($f > 50\%$) or worm-like ($f = 40$–50%) morphologies or vesicular structures called polymersomes ($f = 25$–40%) (Discher and Ahmed, 2006). Additionally, the preparation method has an impact on the formation of a specific morphology (Cai et al., 2007). Thus, worm-like as well as spherical micelles may be expected for the three micellar dispersions selected comprising block copolymers with *f*-values $\leq 50\%$ using the particular preparation methods (Table 3).

Particle Analysis by cryoTEM provided clear evidence of spherical micelles independent of the type of the block copolymer. This was additionally confirmed in a cryoTEM tilt study to exclude misinterpretation of images of worm-like structures in a top view pretending spheres as well. Interestingly, an additional bright shell surrounding the particles was visualized after the addition of several pictures during the retrospective classification procedure. This shell was not detected at pictures of single measurements because they were taken with short times of electron beam to avoid liquidation of the vitrified sample. This finding provides a good explanation for the observed difference in the particle sizes obtained by DLS and cryoTEM (Table 3) since the latter describes the size of the core of the polymeric micelles. The size difference of approximately 50 nm as seen for P5CL(1.0) correlates very well with the theoretical thickness of the PEG-shell of 25 nm. The latter could be estimated by the constant particle interspaces of 50 nm observed within the hexagonal arrangement, the densest packing of spheres. The small difference in the core size between P5CL(1.0) and P2CL(1.3) (22 and 17 nm, respectively) is in good correlation with their almost similar solubilization capacity of Sagopilone. The remarkably larger hydrodynamic diameter of P5CL(1.0) ($d_H = 71.9$ nm) compared to P2CL(1.3) ($d_H = 32.6$ nm) was due to the higher molecular weight of PEG. Furthermore, the distinct higher PDI values of P2CL(1.3)- and P5CL(1.0)-micelles at DLS in spite of the spherical, monodisperse morphology imaged with cryoTEM may be due to the dense sphere packing and subsequent hindrance of the free Brownian-motion of the micelles. This theory is encouraged by the absence of such a long range order for P2LA(1.0)-micelles along with a remarkably lower polydispersity at DLS. At least, the formation of larger aggregates increasing the PDI value at DLS has not been observed in any of the images taken.

5. Conclusion

The poorly water-soluble anticancer drug Sagopilone was sufficiently solubilized by PEG-*b*-PCL and PEG-*b*-Poly(D,L-lactide) micelles with an optimum hydrophobic/hydrophilic-ratio of approximately 1 regarding the solubilization efficiency and stability. No solubilization was observed for PEG-*b*-Poly(L-lactide). Sonication was most suitable for polymers with a high PCL/PEG-ratio (≥ 0.7). Film formation was superior for poly(D,L-lactide)-containing polymers and those comprising a PCL/PEG-ratio of 0.3 at the most. Drug loading into PEG-*b*-PCL micelles was superior to PEG-*b*-PLA due to the absence of a 'supersaturation' effect after sonication. Thermal analysis revealed the molecular dispersion of Sagopilone in the liquid-like PCL phase of the polymeric films in form of a glass solution. Contrary to previous publications, calculated solubility parameters were not suitable as predictive parameters. Against theoretical prediction PCL was superior to PLA and the serious difference between the two stereoisomers of PLA in their ability to solubilize Sagopilone was disregarded by this approach. The three selected drug delivery systems composed of P2LA(1.1), P2CL(1.3) and P5CL(1.0) consist of small (<100 nm), monodisperse and spherical micelles with slightly different core sizes and distinct differences in their hydrodynamic shell. The impact of the core material as well as the PEG-shell at a constant Sagopilone loading and micelle morphology on the *in vitro* as well as *in vivo* behaviour is under further study with the aim to enhance the therapy of Sagopilone.

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