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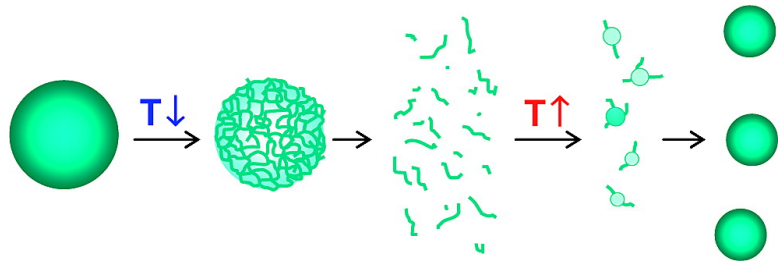

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Preparation of Monodisperse Block Copolymer Vesicles via a Thermotropic Cylinder−Vesicle Transition

Anja Rank,[†] Stephan Hauschild,[‡] Stephan Förster,[‡] and Rolf Schubert*,[†]

Department of Pharmaceutical Technology and Biopharmacy, Albert-Ludwigs-University Freiburg, D-79104 Freiburg, Germany, and Institute of Physical Chemistry, University of Hamburg, D-20146 Hamburg, Germany

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In aqueous solution, poly(2-vinylpyridine-*b*-ethylene oxide) spontaneously forms bilayer vesicles, the size of which can be tailored by extrusion through polycarbonate membranes. However, their size can be even more precisely influenced by subjecting them to a specific cooling/warming process proceeding through a cylinder−vesicle shape transition. The thermotropic alterations of the polymer aggregates and the topological pathways of the cylinder−vesicle transition were followed by dynamic light scattering (DLS) and cryo-electron microscopy (cryo-TEM). Upon cooling the vesicles to 4 °C, there is a transition of the vesicles to basketlike aggregates and their further disintegration to wormlike micelles. Rewarming of the dispersion results in the reformation of vesicles via intermediate discoid and octopus-like structures. The variation of incubation times at 4 and 25 °C, heating rate, polymer concentration, and ionic strength allows tailored preparation of unilamellar and almost monodisperse vesicles with diameters between 60 and 500 nm. Furthermore, fluorescently labeled dextrans, which were used as model drugs of differing molar mass, could be easily and stably encapsulated during the thermotropic formation of vesicles from wormlike micelles.

1. Introduction

Vesicles are spherical lamellar structures consisting of amphiphilic compounds, such as lipids,[1] surfactants,[2] or block copolymers,[3] that are potential candidates for use as drug delivery vehicles or as cell membrane models. Vesicles formed from amphiphilic block copolymers are known as "polymersomes".[4,5] Their entirely synthetic nature allows the control of their physicochemical properties and targeted drug release in the organism.[6−12] Various morphologies of dispersed polymer aggregates can be obtained by changing the composition of copolymer, the nature of the solvent, the water content, the amount of ions, or the temperature.[13−20] The use of polymer nanocontainers as drug delivery systems or cell membrane models requires an easy way to control the size and shape of the aggregates at the nanometer scale. Many methods have been used for preparing unilamellar narrow-sized vesicles from phospholipids including sonication,[21] extrusion,[22] and high pressure homogenization[23] and via the use of organic solvents[24] or detergents.[25] Many of these methods are suitable for polymersomes as well.[24,26]

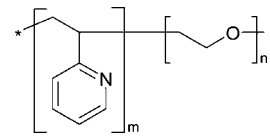

Figure 1. Poly(2-vinylpyridine-*b*-ethylene oxide) with block lengths of $m = 66$ and $n = 46$; $* =$ initiator.

The present study focuses on the preparation of monodisperse and tailored-sized vesicles of P2VP-PEO[27] diblock copolymer (Figure 1). Interestingly, it is found that the specific cooling/warming processes involve a cylinder−vesicle shape transition. Details of temperature-dependent alterations in the aggregation behavior of dilute aqueous polymer dispersions and the topological pathways of the cylinder−vesicle transition were investigated by using dynamic light scattering (DLS) and cryo-transmission electron microscopy (cryo-TEM).

* To whom correspondence should be addressed. Address: Institute of Pharmaceutical Sciences, Dept. of Pharmaceutical Technology and Biopharmacy, Hermann-Herder-Str. 9, D-79104 Freiburg, Germany. Telephone: +49 761 203 6336. Fax +49 761 203 6366. E-mail: rolf.schubert@pharmazie.uni-freiburg.de.

† Albert-Ludwigs-University Freiburg.

‡ University of Hamburg.

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2. Experimental Section

2.1. Materials. Poly-2-vinylpyridine$_{66}$-*b*-poly(ethylene oxide)$_{46}$ block copolymer (P2VP$_{66}$-PEO$_{46}$, mean $M_w = 9277$) was synthesized by sequential living anionic polymerization and had a narrow polydispersity in molecular weight of $M_w/M_n = 1.02$, where M_w and M_n are the number- and weight-averaged molecular masses. The synthesis of P2VP-PEO and polymer characterization by MALDI-TOF-MS, GPC, and ^{1}H NMR are described in detail elsewhere.[27,28] The dry polymer melt was stored in the freezer at −27 °C before use. Fluorescein-isothiocyanate labeled dextrans (FITC-dextrans) with molar masses of 4, 148, or 40 kDa were obtained from Fluka (Buchs, Switzerland) or Sigma (Steinheim, Germany), respectively. Sepharose CL-4B for gel chromatography was from Pharmacia Biotech (Uppsala, Sweden).

2.2. Vesicle Preparation. The polymer was dissolved in chloroform in a round-bottom flask, and the solvent was removed by a stream of nitrogen gas. The resulting polymer film was then dried under high vacuum for at least 1 h. After adding aqueous HBS (HEPES buffered saline: 10 mM HEPES, 150 mM NaCl, pH 7.4) and vigorously stirring, the polymer film lifted off the flask wall and formed a mixture of particles and a milky suspension. After approximately 24 h at room temperature, no larger polymer particles were visible anymore.

Extrusion of small sample volumes was performed using a hand-held device (LiposoFast, Avestin, Ottawa, Canada). A 1 mL sample of the hydrated polymer vesicle dispersion was pressed 21 times each through polycarbonate membranes with defined pore sizes of 600 and 400 nm (Whatman Nuclepore, Pfullingen, Germany). Repeated extrusion was found to be essential for downsizing and achieving a low polydispersity of the vesicles. By using this particular extrusion device, an odd number of extrusion steps ensured that no unextruded vesicles remained in the final preparation.

For the down-sizing of polymer vesicles by controlled shifts in temperature, glass tubes containing the dispersions were shaken in temperature-controlled water baths. The standard procedure was to first cool the dispersion to 4 °C and then incubate it for 24 h, after which it was warmed up to 25 °C.

For the encapsulation of hydrophilic model drugs, FITC-labeled dextrans with different molar masses (4, 40, or 148 kDa) were added to the vesicle dispersions before subjecting them to controlled temperature changes. Encapsulation efficiency, that is, the portion of vesicle-associated fluorescence compared with the total fluorescence of the dextrans in the dispersions, was determined fluorimetrically after separation of the polymer vesicles from nonencapsulated dextrans by gel chromatography using Sepharose CL-4B.

2.3. Dynamic Light Scattering. Vesicle size (*z*-average) and size distribution (PDI: polydispersity index) were measured by DLS at 25 °C (Zetamaster S, Malvern Instruments Ltd., Malvern, U.K.) at $\lambda = 633$ nm with a scattering angle of 90°. For monitoring the hydrodynamic diameter during temperature changes from 4 to 25 °C, a dynamic/static light scattering system (DLS/SLS-5022 ALV, Langen, Germany) was used to measure at different scattering angles from 120 to 150°, where the hydrodynamic radius was angular independent. At lower scattering angles, in many cases, a strong increase of the hydrodynamic radius is observed due to polydispersity and coexistence of different micellar and vesicular structures.

In order to yield an appropriate count rate, the samples were diluted with particle-free filtered buffer (0.22 μm Minisart, Sartorius AG, Göttingen, Germany).

2.4. Cryo-Transmission Electron Microscopy. Cryo-TEM investigations were performed with a LEO 912 OMEGA electron microscope (Zeiss, Oberkochen, Germany) operating at 120 kV and "zero-loss" conditions. After placing a droplet (approximately 5 μL) of the sample on a copper grid (Quantifoil S7/2 100 × 400 mesh, Quantifoil Micro Tools GmbH, Jena, Germany), most of the liquid was absorbed by a filter paper, so that only a thin (100−500 nm) liquid film remained. The sample was then immediately shock-frozen by plunging it into liquid ethane.[29] The vitrified sample was stored at 90 K in liquid nitrogen until it was loaded into a cryogenic sample holder (D626, Gatan Inc., Pleasanton, CA). The specimens were examined at −174 °C. Digital images were recorded under low dose conditions (MDF) with a slow-scan CCD camera system (Oxford, 1024 × 1024 pixel) and at minimal underfocus of the microscope objective lens to provide sufficient phase contrast.[30] For the determination of size distribution, dimensions of at least 350 particles were measured by using the iTEM software (Olympus, Muenster, Germany).

3. Results and Discussion

If polymersomes are used as membrane models or even as drug delivery systems, the possibility of being able to tailor their size and homogeneity will of course be of crucial interest. P2VP$_{66}$-PEO$_{46}$ (Figure 1) initially forms a turbid dispersion consisting exclusively of vesicles when it is hydrated in an excess of buffer. We first decreased their size by repeated extrusion through porous polycarbonate membranes. Interestingly enough, we found that, upon storage of the crude dispersion or the extruded vesicles in the refrigerator, the dispersions became clear. Moreover, they turned into very homogeneous dispersions of small vesicles when warmed up again to room temperature. We have studied this thermotropic behavior in more detail, because it offers the possibility of producing homogeneous vesicles of a suitable size and gives information about their optimal storage condition. In addition, it was found that the thermotropic behavior results from a temperature-induced cylinder−vesicle transition whose topological pathway could be studied in detail. The same behavior was also found for other vesicle-forming P2VP-PEO block copolymers.

3.1. Hydration of Dry Polymer at Room Temperature. A thin and dry polymer film was formed in a round-bottom flask, and isotonic HBS (10 mM HEPES, 150 mM NaCl, pH 7.4) was then added for hydration to yield a polymer dispersion of 10 mg/mL. Additional stirring supported the shearing of the swollen polymer aggregates. This hydration step of amphiphilic block copolymers requires about 1 day and takes much longer[31] than the hydration process of amphiphilic phospholipids, which is normally finished after some minutes. Cryo-TEM pictures show that, by simple hydration, vesicles that are almost exclusively unilamellar and have a broad size range are formed, with the large vesicles showing folded membranes (Figure 2a).

Additional dynamic light scattering measurements at an angle of 90° showed an average vesicle size of approximately 400 nm, with pronounced polydispersity (PDI > 0.6).

3.2. Sizing by Extrusion. The downsizing of these initial polymersomes was performed by repeated hand extrusion through 600 and 400 nm pores, whereas extrusion through 200 nm pores, in contrast to phospholipid vesicles, failed when applying reasonable pressures. The reason for this is probably due to the high rigidity of the polymer bilayers, which prevents the formation of highly curved bilayer parts which are able to enter the pores of the extrusion membranes. Nevertheless, the final pore size of 400 nm yielded vesicles with a mean diameter of approximately 200 nm as measured by DLS (90°) (PDI = 0.16). This was confirmed by cryo-TEM (see Figure 2b), where a size distribution of 190 ± 100 nm was determined.

3.3. Changes in Size of Block Polymer Aggregates upon Cooling and Warming. When the extruded sample was cooled down to 4 °C, the opalescent dispersion became almost clear

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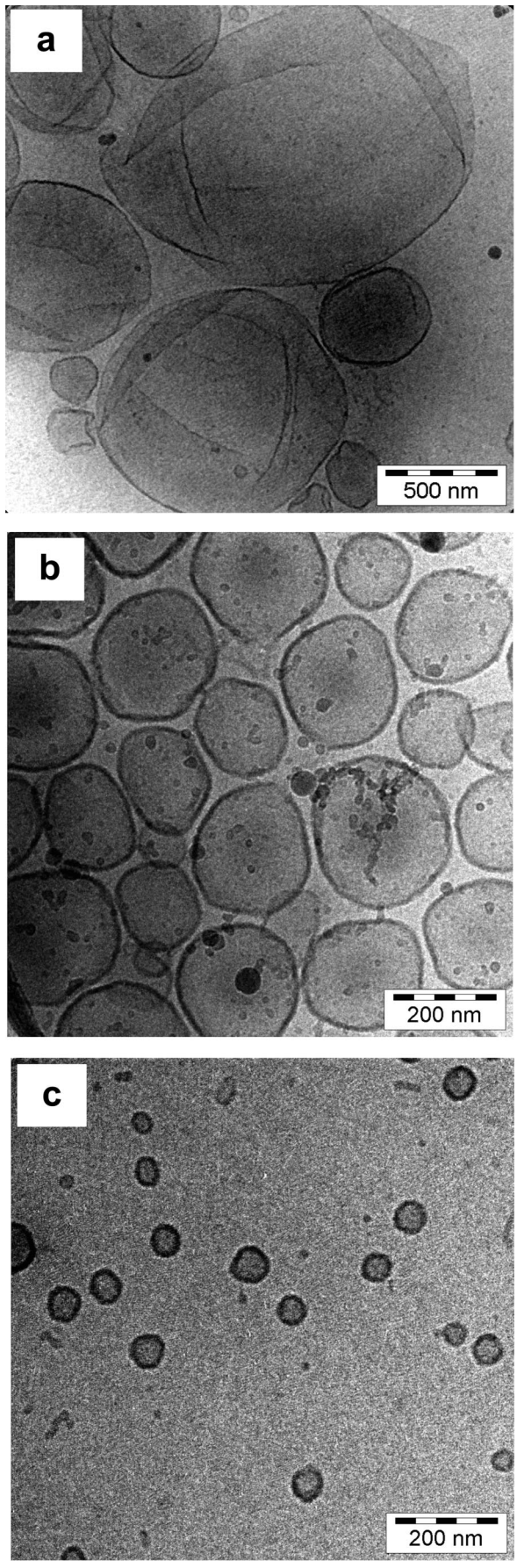

Figure 2. P2VP$_{66}$-PEO$_{46}$ polymersomes prepared by different procedures; (a) dried polymer hydrated for 24 h, (b) hydrated polymers after successive extrusion through 800 and 400 nm pores, and (c) polymer dispersion after cooling to 4 °C and warming up to 25 °C.

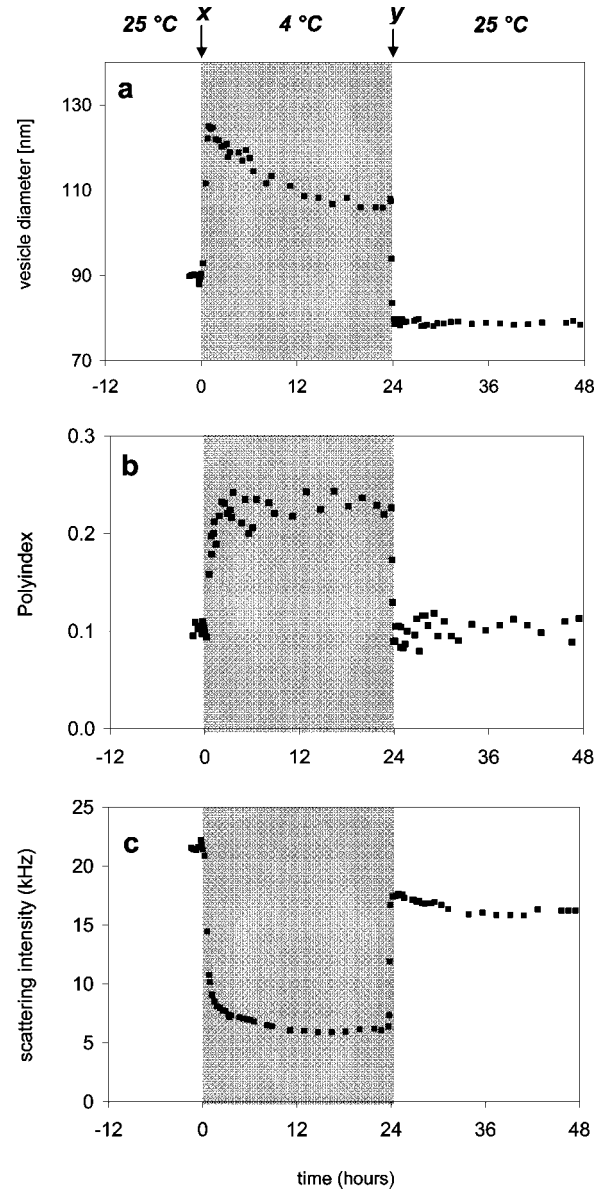

Figure 3. DLS data (scattering angle 120−150°) of P2VP$_{66}$-PEO$_{46}$ dispersion after temperature jumps from 25 to 4 °C (*x*) and after 24 h (gray areas) from 4 to 25 °C (*y*): (a) aggregate size starting with vesicles of 90 nm, (b) polydispersity index, and (c) scattering intensity.

within 30 min. After incubating for 24 h at this low temperature, the dispersion was rapidly warmed up to 25 °C and incubated for an additional 48 h to form 90 nm vesicles. Figure 3 shows DLS measurements (mean of 120−150°) when these vesicles were cooled again to 4 °C (time point *x* at 0 h). This temperature jump initially led to a fast increase in size (a) and polydispersity index (PDI, b) of the forming new aggregates within minutes, while the count rate (scattering intensity) strongly decreased rapidly (c). Further incubation at 4 °C slightly reduced the particle size, whereas the PDI and the count rate remained constant. The data at 4 °C were strongly dependent on the scattering angle, which is indicative of the coexistence of large and small objects or elongated objects[32,33] such as wormlike micelles, which were rapidly formed after the temperature drop to 4 °C. A subsequent temperature shift after 24 h back to 25 °C (time point *y*) resulted

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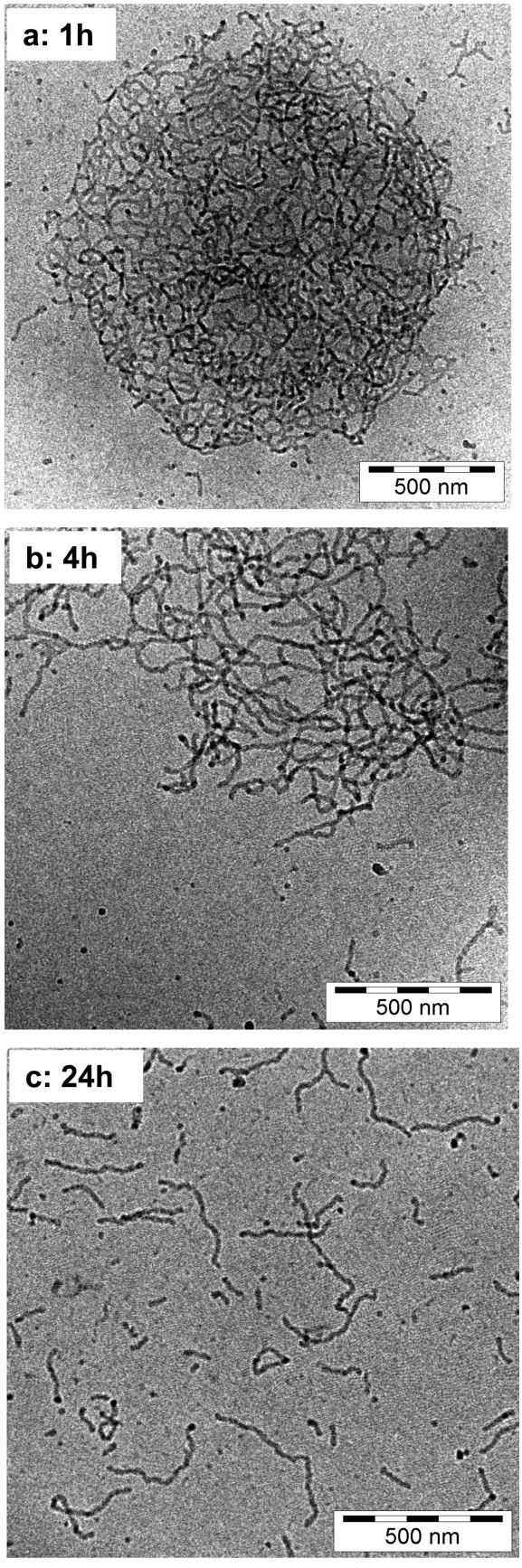

Figure 4. Cryo-TEM pictures of $P2VP_{66}$-PEO_{46} in HBS at different incubation times after a temperature jump from 25 to 4 °C: (a) basketlike network of wormlike micelles formed from an initial unilamellar large vesicle after 1 h at 4 °C, (b) coexisting wormlike, branched and small spherical micelles after 4 h at 4 °C, and (c) coexisting wormlike and small spherical polymer micelles.

in a parameter change in the direction of the initial values. The mean particle size as determined by DLS was then 82 nm (PDI < 0.1)

An intermediate incubation at 16 °C for 24 h before further warming to 25 °C resulted in slightly larger particles with a *z*-average of 112 nm (PDI = 0.13)

3.4. Structural Changes upon Cooling. The thermotropic transition processes were studied in detail by cryo-TEM. First, the kinetics of structural alterations during incubation of the vesicle dispersion at 4 °C was examined. Starting from an extruded dispersion containing exclusively unilamellar vesicles (Figure 2a), the bilayer membranes were found after 1 h to have transformed into a spherical basketlike network of entangled linear and branched wormlike micelles (Figure 4a) coexisting with some isolated wormlike and very small spherical micelles. Some gray areas between the micelles indicate residual flat bilayer sheets (disklike micelles), which have been described in the literature[34,35] as an intermediate state in vesicle−worm transition. The basketlike network continues to decompose during further cooling at 4 °C (Figure 4b) for 4 h, and partially branched or circular micelles can still be found. After 24 h, the sample predominantly contains separated wormlike micelles coexisting with a few very small spherical micelles (Figure 4c). As determined by cryo-TEM, the undulated wormlike micelles have a mean length of 250 nm with a size range between 50 nm and 1.5 μm. The diameter of the worms is not uniform (16 ± 2 nm) and varies within a single worm as pictured in Figure 5a (left). The endcaps of the worms and the spheroid nubs have larger diameters of about 21 ± 2.5 nm and appear darker than the inner parts of the worms. It is noticeable that some worms contain other darker punctated regions, which may be the breaking points at which the worms were shortened or may be short tips at cross-links that extend vertically leading to a darker appearance in the projection.

3.5. Structural Changes upon Warming Up. When the polymer dispersions had been incubated for at least 24 h at 4 °C and then rapidly warmed up directly to 25 °C, after 15 min, no structures other than vesicles and a few wormlike micelles could be found (not shown). After 1 h, the dispersion was composed of predominantly closed unilamellar vesicles with a narrow size distribution and a mean diameter of around 62 ± 15 nm, as measured by cryo-TEM. Sporadically, spherical micelles of a smaller size or wormlike micelles were also detected.

To discover intermediate structures during the worm−vesicle transition, an additional incubation step at 16 °C was included. For this purpose, the sample, preincubated for at least 24 h at 4 °C, was incubated at 16 °C and the time course of structural changes of the intermediate aggregates were studied by cryo-TEM.

Figure 6a shows the sample after 1 h at 16 °C which reveals mostly wormlike structures, although some disklike micelles already occur (gray areas). After 2.5 h (Figure 6b), intermediate structures together with wormlike micelles and a few smaller vesicles were found. In cryo-TEM, flat micellar areas can be distinguished from closed or still opened vesicles due to the absence of projections of darker vesicle edges.[36] After 25 h (Figure 6c), some closed vesicles are found in equilibrium with octopus-like open vesicles showing attached wormlike micelles as tentacles, similar to structures which have been found with other block copolymers.[33,37] To complete the worm−vesicle transition, the dispersion had to be slightly warmed up from 16 to 25 °C,

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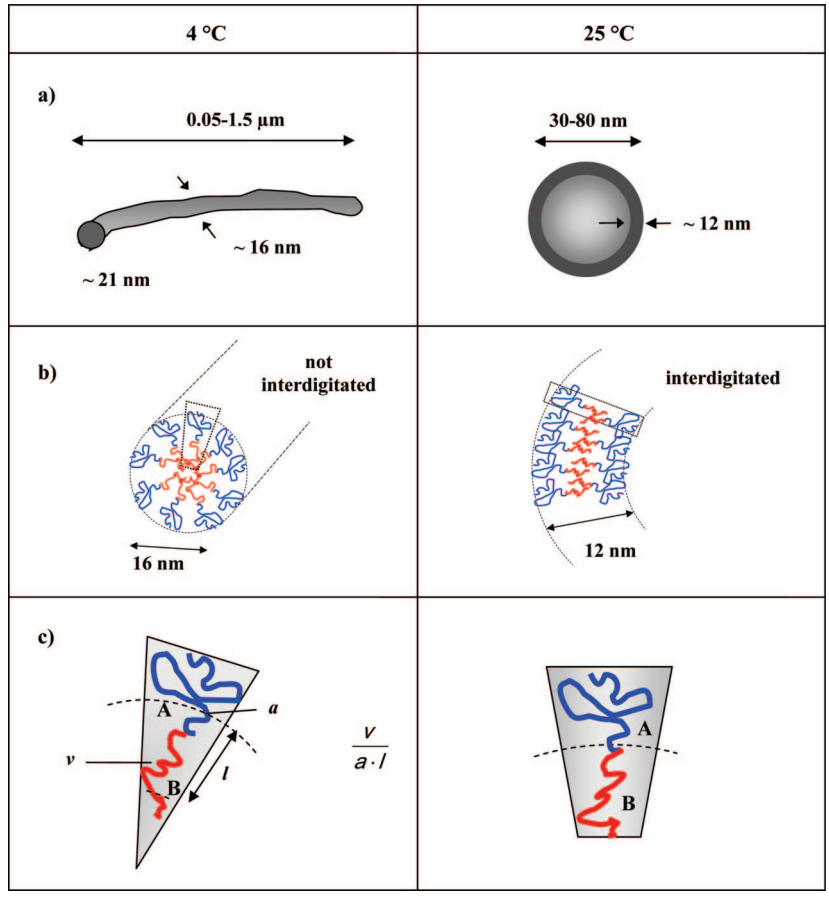

Figure 5. Schematic drawing of the dimensions of P2VP$_{66}$-PEO$_{46}$ in wormlike micelles at 4 °C (left) and bilayers at 25 °C (right): (a) dimensions of a wormlike micelle (4 °C) compared to a vesicle (25 °C), (b) self-assembly of the polymer amphiphiles in micelle or bilayer membrane in detail, and (c) shape of single polymer molecules in worm micelles and bilayers, and estimation of the surfactant packing parameters: $v/(al)$, with v being the volume, l being the length of the hydrophobic part, a being the interfacial area, A being the hydrophilic block, and B being the hydrophobic block.

resulting almost exclusively in closed vesicles after even only 1 h (Figure 6d).

The structures found in the cryo-TEM study are schematically pictured in Figure 7. During heating from 4 to 25 °C, a part of the wormlike micelle (Figure 7a) changes into a flat bilayer sheet whose area grows (Figure 7b). If a critical disk radius is reached, for example, if R_{disk} is more than twice the final vesicle radius $R_{vesicle}$, as seen in the cryo-TEM picture in Figure 6b, the rim energy for the flat lamellae is higher than their curvature energy,[35] favoring their bending to an open vesicle (Figure 7c). The residual wormlike arms are still attached at the curved bilayer edges, and upon their disappearance they contribute to an increase in surface area and radius $R_{vesicle}$ of the finally closed vesicle (Figure 7d).

If it is supposed that in sufficiently dilute solution a single cylinder transforms into a single vesicle, an estimate of the relation between cylinder length and vesicle radius can be given. The volume taken up by the vesicle bilayer, V_b, is given by $V_b = (4\pi/3)(R^3 - (R - d_b{}^3)$, where R is the outer radius of the vesicle and d_b the thickness of the vesicle wall. We assume that R is n-times the bilayer thickness d_b, that is, $R = nd_b$, where n for small vesicles as in our study is typically in the range of 2−5. The volume of the cylinder, V_c, is given by $V_c = (\pi d_c{}^2 L)/4$, where L is the cylinder length and d_c the cylinder diameter. From simple geometrical considerations of the packing parameter (see below), it is expected that $d_c = 2d_b$. This trend is observed experimentally (see Figure 5b and c, left).[38] Assuming that bilayer and cylinder volume are the same, we obtain a simple relation between cylinder length L, vesicle radius R, and the factor n, that is, $L/R = (4/3n)(n^3 - (n - 1)^3) \approx 4n$. With a mean length of the cylinders of $L = 500$ nm (Figure 6b) and an average radius of the vesicles of $R = 50$ nm (Figure 6d), we calculate a factor of $n \approx 2.5$ corresponding to a bilayer thickness of $d_b \approx 20$ nm which is in a reasonable range compared to the experimental value of $d_b = 12$ nm (Figure 5).

Notably, we observe that the curvature of the vesicle wall is not as perfectly smooth as that, for example, at lipid membranes. This buckling of the polymer bilayer is typical and results from the high glass transition temperature of the hydrophobic block ($T_g \sim 100$ °C). By cryo-TEM, the estimated membrane thickness has an average of about 12 nm and is similar to data of earlier small-angle neurtron scattering studies (13.5 nm).[28]

The reason for the thermotropic behavior of P2VP-PEO block copolymers is the temperature-dependent solubility of the PEO block. With increasing temperature, the solubility of PEO in water decreases. With decreasing solvent quality, the volume of the water-swollen PEO layer decreases, thereby reducing the interfacial area at the hydrophilic/hydrophobic interface. Within the packing parameter concept introduced by Israelachvili for surfactant self-assembly (Figure 5c),[32,34,38−41] this corresponds to a reduction of the interfacial area per chain (a_0) which increases

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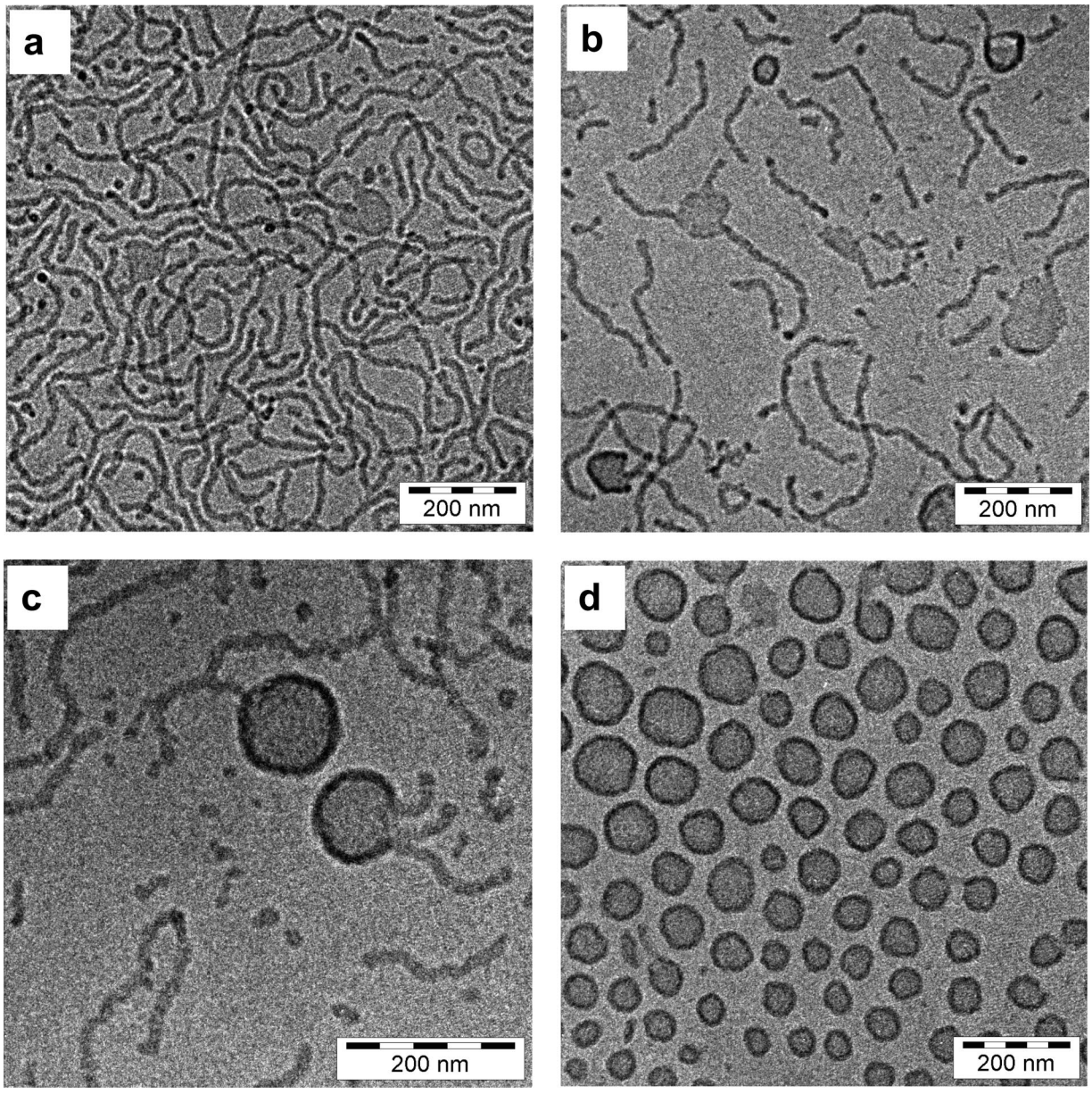

Figure 6. Cyro-TEM of intermediate structures during warming up of $P2VP_{66}$-PEO_{46} micelles (a) 1 h, (b) 2.5 h, or (c) 24 h after a temperature jump from 4 to 16 °C and (d) 1 h after a temperature jump from 16 to 25 °C.

the packing parameter P defined as $P = V/a_0l$. V is the hydrophobic chain volume, and l is the hydrophobic chain length. Cylinders have a packing parameter of $P = 1/2$, bilayers have a value of $P = 1$, and vesicles have a slightly smaller value depending on their radius. Accordingly, an increase of the packing parameter, for example, by increasing temperature leads to the observed shape transition from cylinders to vesicles.

We have also found thermotropic cylinder−vesicle transitions for other vesicle-forming P2VP-PEO block copolymers. Employing the same cooling/warming cycles as described above, the hydrodynamic radius could, for example, be decreased from 176 to 56 nm for $P2VP_{29}$-PEO_{15}, from 199 to 61 nm for $P2VP_{38}$-PEO_{24}, and from 207 to 95 nm for $P2VP_{47}$-PEO_{29}.

3.6. Encapsulation of Hydrophilic Model Drugs. Entrapment of hydrophilic molecules during thermotropic micelle−vesicle transition of $P2VP_{66}$-PEO_{46} was estimated using FITC-labeled dextrans of 4, 40, or 148 kDa. These mimic pharmacologically highly active substances such as peptides, proteins, or nucleic acids. The size of the vesicles was not influenced by the dextrans to be encapsulated. After separating encapsulated from nonentrapped dextrans by gel chromatography, the measured encapsulation efficiency was compared with the portion of the aqueous vesicular interior. This can be calculated from the mean vesicle diameter, the molar concentration of the bilayer-forming polymer molecules, the thickness of the bilayer, and the mean lateral area in the membrane of a single polymer molecule.[42] The latter was estimated to be approximately 1.5 nm^2 as determined for other diblock copolymers.[10,31,43] The measured encapsulation efficiency of 40 and 148 kDa dextrans corresponded with the calculated value and remained unchanged over several days of storage at 25 or 37 °C, indicating the stable encapsulation of these hydrophilic model drugs. The association of the 4 kDa dextran with the liposomal fraction was about 10-fold compared with the calculated encapsulated portion. By incubating preformed empty polymer vesicles with the 4 kDa dextran, and separating free dextran from vesicle-associated dextran, this phenomenon could be revealed as being due to adsorption to the outer vesicle surface. After 8 h, half of the adsorbed dextran was found to

(42) Schubert, R.; Wolburg, H.; Schmidt, K. H.; Roth, H. J. *Chem. Phys. Lipids* **1991**, *58*, 121.

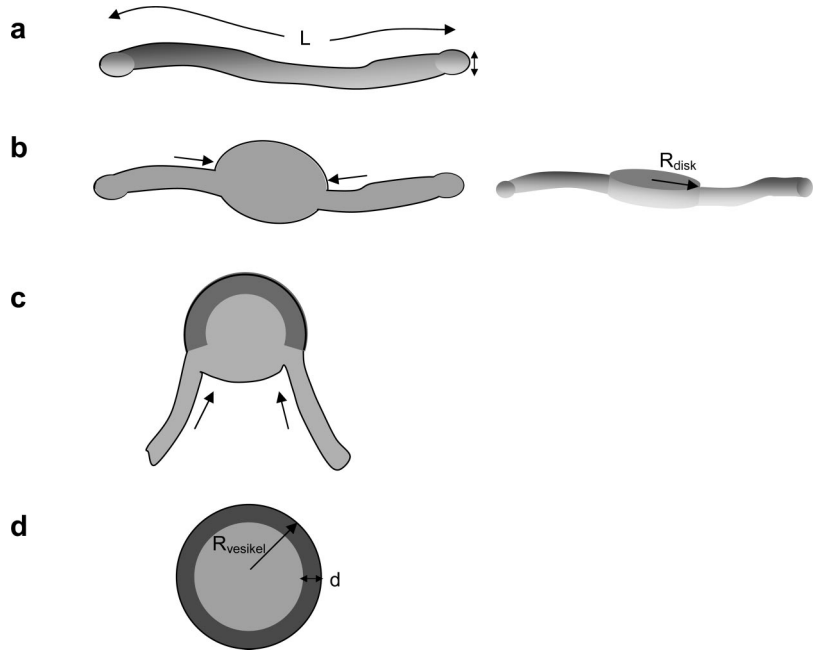

Figure 7. Schematic drawing of the different states of micelle−vesicle transition. The wormlike micelle at 4 °C (a) transforms locally into a disklike bilayer (b), which at a critical disk radius forms a curved bilayer and an open vesicle (c). Residual wormlike micellar parts are adsorbed, and the vesicle is closed (d).

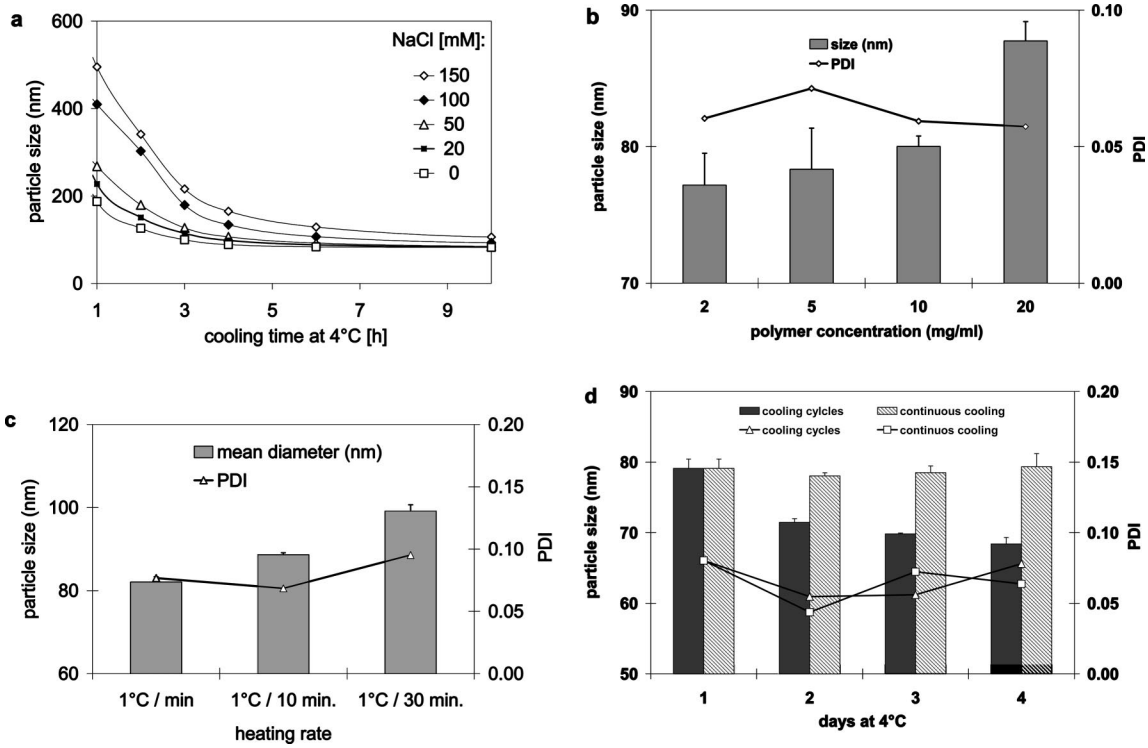

Figure 8. Hydrodynamic diameter and polydispersity index (PDI) of P2VP$_{66}$-PEO$_{46}$ at 25 °C and various conditions, measured by DLS (scattering angle 90°). (a) Kinetics of the decrease in particle size after a temperature jump from 25 to 4 °C of a polymer dispersion (10 mg/mL) in 10 mM HEPES-buffer pH 7.4 with different amounts of sodium chloride; (b) vesicle size in HBS containing different polymer concentrations 24 h after a temperature jump from 25 to 4 °C ($n = 3$); (c) vesicle size of a polymer dispersion (10 mg/mL in HBS) at 25 °C after warming from 4 °C with different heating rates ($n = 3$); and (d) vesicle size of a polymer dispersion (10 mg/mL in HBS) at 25 °C after continuous cooling at 4 °C compared to cooling/warming cycles: 24 h, 4 °C/24 h, 25 °C; $n = 3$

be detached from the vesicles again. Obviously, a part of the small dextran molecules interacts with the hydrophilic PEO chains of the block copolymer.

3.7. Parameters Influencing Vesicle Size during Thermotropic Formation. Several parameters which could potentially affect the resulting vesicle size after thermotropic sizing were investigated. First, cooling time and the influence of ion strength were assessed. The polymer (10 mg/mL) was hydrated at room

(43) Napoli, A.; Totelli, N.; Wehrli, E.; Hubbell, J. A. *Langmuir* **2002**, *18*, 8324.

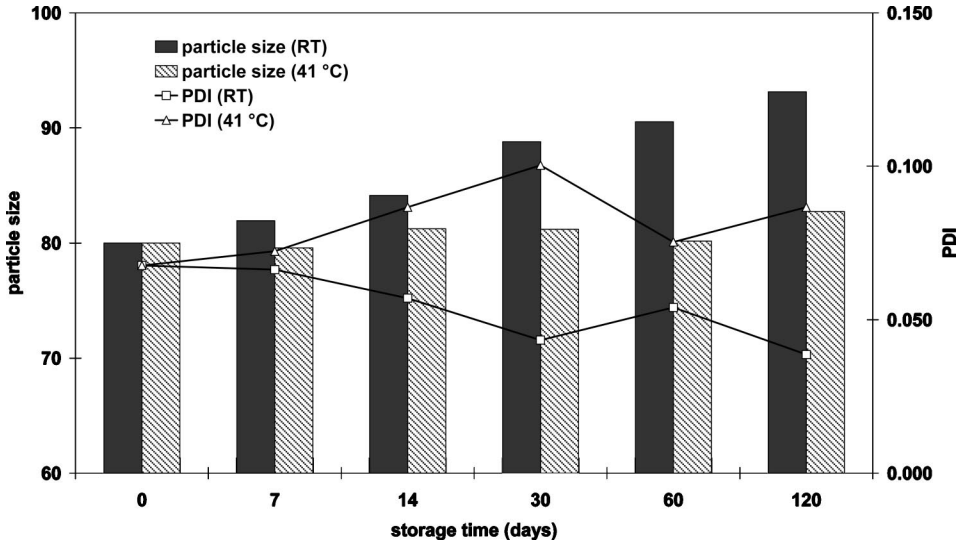

Figure 9. Size stability of $P2VP_{66}$-PEO_{46} vesicles (10 mg/mL in HBS) at different temperatures over a storage period of 4 months ($n = 3$).

temperature with Hepes buffer (10 mM, pH 7.4) containing various amounts of sodium chloride. The dispersions were then rapidly cooled down to 4 °C and incubated at this temperature for different lengths of time before they were rapidly warmed up again to 25 °C to form almost exclusively unilamellar vesicles. As shown in Figure 8a, the vesicle size decreased with increasing cooling time up to 10 h. After incubating longer than 24 h at 4 °C, the vesicles adapted the same sizes as those after the warming step (data not shown). Obviously, the close vicinity of the wormlike micelles formed from the vesicles after 1 h at 4 °C (see Figure 4a) favors the growth in area of the intermediate disks upon rewarming. This then results in larger vesicles compared to that is seen with longer incubation at 4 °C, when the worms are diffused apart.

In general, for shorter exposure time at 4 °C, decreasing ion strength correlated with decreasing vesicle size after warming up. Therefore, controlling the choice of specific ion strength combined with the time of incubation at 4 °C offers the possibility of tailoring the polymersomes over a wide range of sizes.

The second parameter to be studied was polymer concentration (Figure 8b). Using the standard procedure (hydration in HBS, pH 7.4 at 25 °C and cooling for 24 h before rewarming), higher polymer concentration yielded an increase in the size of the vesicles with an almost constant and low PDI. Obviously, a higher amount of wormlike micelles at 4 °C is then inserted into the intermediate bilayer fragments before vesicle formation upon warming up.

The third variable examined was heating rate, which showed that after standard pretreatment that vesicle size increased upon slower heating (Figure 8c). This again indicates that the intermediate insertion of wormlike micelles in the growing disklike bilayers and forming vesicles (see Figure 7) is the rate-limiting step of micelle−vesicle transformation.

Finally, the reversibility of the cooling and warming steps was studied. When samples were warmed up rapidly to 25 °C on days 1 to 4 from a polymer batch that had been continuously cooled at 4 °C, the resulting vesicle size was essentially the same as expected. However, when the sample was subjected to cooling and warming cycles of 24 h each, the resulting vesicle size decreased significantly (Figure 8d). This phenomenon tends to suggest that at 25 °C the vesicles are still not in a stable equilibrium. An advantage of using cooling/warming cycles is that polymer vesicles with a size below 70 nm can also be made.

Vesicle instability at 25 °C is also shown in Figure 9, showing vesicle size after thermotropic standard preparation and upon storage for up to 120 days at room temperature or at 41 °C. The slight increase in vesicle size at room temperature, combined with a slight decrease in polydispersity, suggests that vesicles are still in equilibrium with a small fraction of cylinders. In contrast, vesicle size remains constant upon storage at 41 °C, indicating the complete absence of cylinders in accordance with the temperature-increasing packing parameter as discussed above.

4. Conclusion

The copolymers of the $P2VP_m$-PEO_n type show pronounced thermotropic behavior. As an example, the aggregate $P2VP_{66}$-PEO_{46} structures formed upon cooling and rewarming were studied in detail in the present study. Other polymers related to $P2VP_m$-PEO_n with shorter block lengths *m*/*n* of 47/29, 38/24, or 29/15 showed thermotropic behavior similar to $P2VP_{66}$-PEO_{46} with a disappearance of turbidity at 4 °C in addition to revesiculation at 25 °C. Interestingly enough, for copolymers with block lengths of 55/38 and 54/34, precipitation was found at 4 °C, which rearranged to vesicles at 25 °C. The structural alterations of these polymers were not studied in detail.

As shown in detail for $P2VP_{66}$-PEO_{46}, a controlled temperature program ranging from 25 to 16 °C can be used for preparing vesicles of a tailored size. At around body temperature, the vesicles are stable. This is a prerequisite for targeted drug delivery of nanoparticulate systems after intravasal application to tissues of interest, such as tumors. Furthermore, the temperature controlled process offers the possibility of efficient encapsulation of active compounds and may even facilitate the reconstitution of membrane proteins without the use of additional excipients. As has been shown recently,[28] a series of $P2VP_m$-PEO_n vesicles offers the additional possibility of releasing the compounds in an acidic environment, which is important for the intracellular release from vesicles in the lysosomal organelles. $P2VP_m$-PEO_n may therefore be a good candidate for the development of formulations of highly specific and controlled drug delivery.

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