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Research Article

Stability of polymersomes with focus on their use as nanoreactors

The increased membrane stability of polymersomes compared to their liposomal counterparts is one of their most important advantages. Due to this benefit, polymer vesicles are intended to be used not only as carrier systems for drug delivery purposes but also as nanoreactors for biotechnological applications. Within this work, the stability of polymersomes made of the triblock copolymer poly(2-methyloxazoline)₁₅-poly(dimethylsiloxane)₆₈-poly(2-methyloxazoline)₁₅ (PMOXA₁₅-PDMS₆₈-PMOXA₁₅) toward mechanical stress, typically prevailing in stirred-tank reactors being the most often used reactor type in the biotechnological industry, was characterized. Dynamic light scattering and turbidity measurements showed that stirrer rotation causing a maximum local energy dissipation of up to 1.23 W/kg⁻¹ did not result in any loss of vesicle quality or quantity. Nevertheless, most probably due to local membrane defects, 6.6% release of the previously encapsulated model dye calcein was recognized at 25°C within 48 h. Moreover, increased temperature, leading to decreased membrane viscosity and increased membrane fluidity, respectively, led to a higher molecule leakage. Besides, the stability of polymersomes in two-phase systems was investigated. Although alkanes and ionic liquids were shown not to lead to complete vesicle damage, no efficient calcein retention was achieved in either case.

Keywords: Compartmentalization / Nanobiotechnology / Polymersomes / Stability / Two-phase system

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1 Introduction

The possibility of encapsulating guest molecules in the interior of hollow spheres has led to growing interest in vesicular structures in the recent years. Especially nanoscale membrane vesicles can be used in a wide field of applications. In medicine, these compartments are intended to be used as drug delivery systems [1–3] or for imaging of tissues *in vivo* [4]. By loading them with enzymes, they can also be turned into biocatalytically active nanoreactors, which are of great interest in the field of biotechnology [2, 5–9]. Membrane vesicles are made of amphiphilic self-assembling molecules. The most famous examples are lipid vesicles, so-called liposomes. However, a serious disadvantage when using liposomes for biotechnological applications is based on the intrinsic softness of the phospholipid membrane, leading to comparatively low stability and hardly

controllable retention of the encapsulated molecules. A promising alternative are polymer vesicles (polymersomes [10]) made of amphiphilic block copolymers. Polymersomal membranes are typically thicker (3–40 nm [2]) than phospholipid membranes of liposomes (3–5 nm [11]) and therefore show enhanced stability.

Recently, a very efficient polymersome production process in stirred-tank reactors, which is the most frequently used reactor type in biotechnology, was developed by our group [12, 13]. It was shown that uniform polymer vesicles made of the triblock copolymer poly(2-methyloxazoline)₁₅-poly(dimethylsiloxane)₆₈-poly(2-methyloxazoline)₁₅ (PMOXA₁₅-PDMS₆₈-PMOXA₁₅) can be produced in miniaturized stirred-tank reactors [12] and in standardized reactors at the liter scale [13]. Polymersomes with narrow particle size distribution, shown by a low polydispersity index (PDI) of less than 0.2 [14], and a z-average (harmonic intensity based particle diameter [15, 16]) of about 180 nm were produced within 1 h and in only one production step [12, 13]. PMOXA-PDMS-PMOXA was used as an exemplary amphiphile since this polymer does not evoke inflammatory response by biological systems and shows good biocompatibility and low cytotoxicity [17–19]. Besides, this polymer is very suitable for polymersomes intended to serve as nanoscale reactors for enzymatic reactions since the membrane functionalization, for example in form of integration of several

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Abbreviations: BMIM, 1-butyl-3-methyl-imidazolium; HMPL, 1-hexyl-1-methyl-pyrrolidinium; HPYR, *N*-hexylpyridinium; IL, ionic liquid; NTF2, bis(trifluoromethylsulfonyl)imide; PDI, polydispersity index; PDMS, poly(dimethylsiloxane); PMOXA, poly(2-methyloxazoline)

transmembrane proteins [6, 20–26] or the permeabilization of the membrane with a photoreactive permeabilization agent [27], and the simple surface functionalization with immobilized proteins [28] is feasible. During the development of the process, the formation of a small amount of undesired tiny polymer flocs at process times longer than 4 h was recognized [12]. It was proposed that the continuous impact of shear forces induced by the stirrer destroyed the membrane vesicles and it seemed likely that the membrane segments of destroyed vesicles were not able to reassemble to new polymersomes but formed aggregating morphologies. Since the particle size distribution predominantly was narrowed down from the greater particle sizes toward the smaller ones over the process time, it was further assumed that larger vesicles are more fragile than small ones and were therefore destroyed faster/earlier [12].

These observations gave reason for further and deeper investigations on the stability of polymersomes with focus on their use as nanoreactors for biotechnological applications. As mentioned above, the stirred-tank reactor is by far the most used reactor type in the biotechnological industry to enable sufficient mixing for example during biotransformations [29, 30]. However, shear forces induced by stirrer rotation cause mechanical stress on the stirred particles. Thus, the resistance of the polymersomal membranes toward mechanical stress typically prevailing in stirred-tank reactors has to be characterized.

For some biocatalytic applications, the instability of involved substrates and products in aqueous media and their poor water-solubility represent a great challenge. To overcome these drawbacks, different in situ substrate feeding and product removal strategies can be applied, for example in the form of liquid–liquid biphasic reaction systems [31]. In this case, the aqueous phase containing the biocatalyst and the second water-immiscible phase in which both the substrates and the products are highly soluble are brought into contact. The possibility of using polymer vesicles as nanoreactors in two-phase systems requires the investigation of the stability of polymersomal membranes toward the second, water-immiscible phase.

For these reasons, the aim of this work was the characterization of the stability of polymersomes in stirred-tank reactors. Besides, the stability of polymersomal membranes toward several water-immiscible solvents or ionic liquids ([ILs] as alternative to commonly used solvents [31, 32]) was also studied. Investigations were done with the focus on changes of the vesicle quality (*z*-average, PDI) and quantity (relative turbidity, see Section 2.1.3) of unloaded polymersomes. Moreover, the retention of the model dye calcein (chosen due to its very low detection limit) in polymersomes under process conditions typically applied for biotechnological applications was also investigated.

2 Materials and methods

2.1 Polymersomes

2.1.1 Triblock copolymer

Polymersomes were produced by using the ABA triblock copolymer PMOXA₁₅-PDMS₆₈-PMOXA₁₅, purchased from Polymer-source (Dorval QC, Canada). The polymer with a molecular mass of 7600 g/mol shows a narrow mass distribution given by

$M_w/M_n = 1.23$ (M_w : mass average molecular mass; M_n : number average molecular mass).

2.1.2 Polymersome production

A recently developed process for polymersome production in miniaturized stirred-tank reactors is described elsewhere [12]. In brief, polymersomes were produced by the ethanol method. A 20% w/v ethanolic polymer solution was injected into the aqueous phase (double distilled water for unloaded polymersomes or calcein solution for calcein-loaded polymersomes, see Section 2.1.5) to get a total volume of 12 mL of a 1% w/v vesicle dispersion. The dispersion was stirred by using the so-called S-stirrer [33, 34] at 4000 rpm in unbaffled reactors. Sampling (0.1 mL) was carried out at the top of the reactor.

2.1.3 Qualitative and quantitative polymersome evaluation

The quality of the polymersome dispersion was measured via dynamic light scattering, described in detail elsewhere [12, 13]. Samples were 10-fold diluted with double distilled and filtered water. No further sample preparation (extrusion, centrifugation) was carried out. The polymersome size (*z*-average) and the size distribution (PDI) were determined by using a Zetasizer NS (Malvern, Worcestershire, UK). Quantification of polymersomes was carried out by turbidity measurement at 280 nm in a microplate reader (infinite M200, Tecan, Männedorf, Switzerland). A volume of 50 μ L was measured in 384-well flat bottom UV-assay plates (Brand, Wertheim, Germany). The relative quantity of intact vesicles was evaluated by the ratio of the turbidity of the dispersion after a certain period of mechanical stress or contact with the second phase T_t and of the initial polymersome dispersion T_0 . A value of 1 represents a polymersome preservation of 100%.

$$rel. T = \frac{T_t}{T_0} \quad (1)$$

2.1.4 Viscosity measurements

The viscosity of the polymersome dispersion (1% w/v in double distilled water) was measured in a cylindrical double slit viscometer (RheolabQC, Anton Paar, Ostfildern, Germany). The viscosity of 13 mL of the vesicle dispersion was measured at 8°C, 25°C, and 40°C in the temperature controlled measuring cell. The rotational rheometer was controlled using the relating software Rheopuls and linear increasing shear rates (10–4000/s) were employed.

2.1.5 Polymersome loading and purification

To perform leakage experiments polymersomes were loaded with calcein (0.6 kDa) at a self-quenching concentration (15 mM in 0.1 M Tris-HCl, pH 8.14). To encapsulate the fluorescent dye, polymersomes were formed in the calcein solution according to the method described above (Section 2.1.2). Afterwards, the calcein-loaded polymersomes were separated from the free molecules by dialysis, using a dialysis tube (cut off of 12–14 kDa).

The vesicle dispersion was dialyzed at 4°C under gentle stirring against 100-fold excess of buffer (0.1 M Tris-HCl, pH 8.14). Dialysis was performed several times until no calcein was traceable (fluorescence measurement, Section 2.4) in the dialysis fluid.

2.2 Investigations on the mechanical stability of polymersomes

The mechanical stability of the polymersomes was investigated by agitating the dispersion in unbaffled miniaturized stirred-tank reactors by using the S-stirrer [33, 34]. Stirrer speeds from 100 to 4000 rpm were realized by a magnetic inductive drive. Temperature control (8°C–60°C) was carried out by a heat exchanger integrated in the reactor block, whereas a reflux cooler in the headspace (4°C) reduced volume loss by evaporation [35, 36]. Changes of polymersome quality and quantity (Section 2.1.3) were followed up to 48 h.

2.3 Polymersome stability in two-phase systems

2.3.1 Organic solvents and ionic liquids

The polymersome dispersions were brought into contact with 23 water-immiscible organic solvents (analytical grade, various suppliers). The impact of different solvent classes (alcohol, ether, ester, ketone, aromatic/aliphatic/halogenated hydrocarbons) was investigated. To create a two-phase system with the aqueous polymersome dispersion, the ILs *N*-hexylpyridinium bis(trifluoromethylsulfonyl)imide [HPYR][NTF2], 1-butyl-3-methyl-imidazolium [BMIM][NTF2], and 1-hexyl-1-methylpyrrolidinium [HMPL][NTF2] (Merck, Darmstadt, Germany) were used as well. The stability of unloaded polymersomes in two-phase systems was investigated at the microliter scale, at the milliliter scale (Sections 2.3.2 and 2.3.3) and in miniaturized stirred-tank reactors (Section 2.3.4). The integrity of calcein-loaded polymer vesicles was investigated in miniaturized stirred-tank reactors solely.

2.3.2 Microliter scale

At the microliter scale, the polymersome stability was investigated in 200 μ L reaction vessels at a total volume of 100 μ L. The aqueous polymersome dispersion and the second phase (10% v/v) were brought into contact by gentle shaking (3 s⁻¹, 3 min, 25°C) in a vibration mill (MixerMill MM200, Retsch, Haan, Germany). Afterwards, the two phases were separated by centrifuging the reaction vessels (16 200 g, 6 min). Finally, a sample volume of 50 μ L was taken from the aqueous phase for the subsequent quality and quantity determination of the polymersomes (Section 2.1.3).

2.3.3 Milliliter scale

At the milliliter scale, the aqueous polymersome dispersion was brought into contact with the water-immiscible phase (10% v/v or 20% v/v of the organic solvent or the IL) in cylindrical glass reaction vessels (5 mL nominal volume) at a total volume of 1 mL. The two-phase system was gently agitated (150 rpm, 25°C) up to

24 h by using a magnetic two cross-arm stirrer. Afterwards, the two-phase system was transferred to a 1.5 mL reaction vessel and the phase separation was carried out by centrifugation (16 200 g, 6 min). After phase separation a sample volume of 100 μ L was taken from the aqueous phase for the subsequent quality and quantity determination (Section 2.1.3).

2.3.4 Miniaturized stirred-tank reactors

In the miniaturized stirred-tank reactors, the aqueous polymersome dispersion and the water immiscible phase (20% v/v) were stirred at 500 rpm by using the S-stirrer. During the experimental time several samples (100 μ L) were taken from the stirred two-phase system, transferred to 1.5 mL reaction vessels and centrifuged (16 200 g, 6 min) for phase separation. The aqueous phase served for quality/quantity determination of unloaded vesicles (Section 2.1.3) and for calcein release determination of loaded polymersomes (Section 2.4).

2.4 Determination of calcein leakage

The calcein leakage was determined by measuring the sample fluorescence (485 nm/515 nm) in 96-well, chimney, black polystyrene assay plates (Greiner bio-one, Kremsmünster, Austria). The fluorescence of 75 μ L sample was measured in an Infinite M200 microplate reader (Tecan). Measurements were performed for the initial polymersome dispersion F_0 and the dispersion after a certain period of mechanical stress or contact with the second phase F_t . Besides, the fluorescence of disintegrated vesicles was measured as well ($F_{\infty,0}$, $F_{\infty,t}$). Vesicles were disintegrated by the addition of 1% v/v of the lysis detergent *n*-octyl-poly-oxyethylene (Bachem, Bubendorf BL, Switzerland). Finally the calcein release was determined by equation (2):

$$\text{Release, \%} = \left(\frac{F_t}{F_{\infty,t}} - \frac{F_0}{F_{\infty,0}} \right) 100\%. \quad (2)$$

3 Results

The mechanical stability of polymersomes in stirred-tank reactors and their stability in diverse two-phase systems were investigated. All results and relating SDs derive from threefold determinations, unless stated otherwise.

3.1 Mechanical stability of polymersomes in stirred-tank reactors

3.1.1 Influence of the agitation speed

During the development of a polymersome production process in miniaturized stirred-tank reactors, the formation of a small amount of undesired tiny polymer flocs at process times longer than 4 h was observed under otherwise optimum process conditions (S-stirrer, 4000 rpm) [12]. In Fig. 1A the homogeneous polymersome dispersion with its typical turbidity after 1 h process time is compared to the heterogeneous appearance of the respective dispersion after 24 h. The latter shows a considerable

Figure 1. (A) Visual appearance of the polymersome dispersion after 1 and 24 h process time. (B) Relative turbidity of the polymer-some dispersion over process time at different agitation speeds at 25°C.

amount of sedimented polymeric aggregates and a lower turbidity due to the decrease of intact vesicles. Consequently, the relative turbidity, Eq. (1), is an appropriate measure to quantify the amount of intact vesicles. Thus, a value of 1 shows that no vesicle loss occurred.

To investigate the impact of different agitation speeds on the stability of unloaded polymersomes, a 1% w/v dispersion with an initially narrow size distribution ($PDI = 0.18$) and an initial z -average of 177 nm was mechanically stressed by driving the S-stirrer at 1000–4000 rpm at 25°C in an unbaffled reactor. The resulting relative turbidity over process time is shown in Fig. 1B. No significant change was recognized at 1000 rpm. In contrast, at agitation speeds from 2000 to 4000 rpm, the relative turbidity decreased more rapidly the faster the stirrer was driven. A relative turbidity of 0.12 ± 0.04 indicates that the majority of the polymersomes was destroyed within 48 h at 4000 rpm. At this agitation speed both the z -average and the PDI decreased continuously within the first 30 h until minimum values of 156 ± 3 nm and 0.15 ± 0.01 , respectively, were reached (data not shown). The decrease of the particle diameter confirms the hypothesis stated during the development of the polymersome production process in miniaturized stirred-tank reactors [12] that larger vesicles are more fragile than smaller ones and therefore are destroyed faster. In this way, the particle size distribution is narrowed

from the right side, leading to lower mean vesicle size and lower polydispersity. However, after 48 h the polydispersity and the particle diameter increased again (z -average = 266 ± 89 nm, $PDI = 0.27 \pm 0.08$, data not shown). This leads to the conclusion that the polymersomes were completely destroyed under such a heavy mechanical stress for such a long time, leading to the occurrence of undesired, rather large polymer aggregates. On the contrary, at 1000 rpm neither the vesicle quality (z -average = 173 ± 2 nm, $PDI = 0.18 \pm 0.02$, data not shown) nor the relative turbidity (1.03 ± 0.08) changed significantly within 48 h. This suggests that the unloaded polymersomes were not destroyed at the lowest investigated agitation speed. Consequently, the retention of encapsulated calcein was investigated at agitation speeds of maximum 1000 rpm (Section 3.1.3).

3.1.2 Influence of the temperature

It is well-known that the fluidity of natural (lipid) membranes increases with increasing temperature, while the membrane viscosity decreases at the same time. Since any change in membrane properties may result in changed mechanical membrane behavior, the stability of empty polymersomes was investigated at different temperatures. To estimate the impact of the temperature on the membrane viscosity of $PMOXA_{15}$ - $PDMS_{68}$ - $PMOXA_{15}$ polymersomes, the dynamic viscosity of a polymer vesicle dispersion (1% w/v in double distilled water) was measured. Figure 2A shows the difference between the dynamic viscosity of the vesicle dispersion and pure double distilled water at 8°C, 25°C, and 40°C. The difference of the dynamic viscosity decreased from 0.26 mPa s at 8°C to 0.14 mPa s at 40°C. Consequently, it was demonstrated that the dynamic viscosity of polymersomal $PMOXA_{15}$ - $PDMS_{68}$ - $PMOXA_{15}$ membranes decreases with increasing temperature, such as it is known for lipid membranes.

Investigations on the impact of the agitation speed on the polymersome stability led to the assumption that shear forces induced by the S-stirrer at 1000 rpm (Section 3.1.1) do not lead to the disintegration of the vesicles. Therefore, the impact of different temperatures on the mechanical stability of polymersomal membranes was investigated at this agitation speed. As shown in Fig. 2B, the relative turbidity did not change significantly at any temperature within 48 h and was approximately 1, showing that no loss of vesicle quantity occurred. Moreover, the z -average and the PDI remained constant over time (z -average about 175 nm, PDI about 0.17, data not shown), showing that no loss of quality was recorded as well. Thus, increased temperature, going along with decreased membrane viscosity and increased membrane fluidity, did not lead to higher sensitivity toward mechanical stress.

3.1.3 Retention efficiency of loaded polymersomes

To use polymersomes for biotechnological applications, preserving their macroscopic integrity alone is insufficient. The efficient retention of encapsulated molecules is required as well. The model dye calcein was encapsulated to investigate the stability of loaded polymersomes (z -average about 165 nm, $PDI < 0.2$), and the resulting release at different mechanical and thermal stresses was investigated afterwards. Calcein release due to passive diffusion was determined to be less than 0.22% after 48 h at 25°C and

Figure 2. (A) Difference of the dynamic viscosity between a PMOXA₁₅-PDMS₆₈-PMOXA₁₅ polymersome dispersion (1% w/v in double-distilled water) and pure double-distilled water at different temperatures. (B) Relative turbidity of the polymersome dispersion over process time at different temperatures.

was therefore neglected. Based on the results of the investigations on empty polymersomes, calcein-loaded polymer vesicles were stressed by agitation speeds from 100 to 1000 rpm (at 25 °C) and by temperatures from 8 °C to 40 °C (at 200 rpm). In any case, the vesicle quality (z-average, PDI) stayed approximately constant (mean deviation < 5%, data not shown) within 48 h. Nevertheless, no complete calcein retention was achieved. Figure 3A shows that calcein was released continuously at any investigated

agitation speed. After 48 h the release ranged from $3.2 \pm 0.1\%$ at 100 rpm to $6.6 \pm 0.2\%$ at 1000 rpm. The shear stress caused by the stirrer is not distributed evenly. To characterize the stress on particles in stirred-tank reactors, the maximum local energy dissipation ε_{max} is the appropriate measure [37, 38]. When using the S-stirrer in an unbaffled reactor at moderate agitation speeds n up to 2100 rpm, the empirically determined equation $\varepsilon_{max} [W/kg] = \exp\left(\frac{n[rpm] - 884.3rpm}{550.3rpm}\right)$ [39] can be used to calculate the prevalent maximum local energy dissipation. Hence, ε_{max} in the miniaturized stirred-tank reactors ranged from 0.24 W/kg at 100 rpm to 1.23 W/kg at 1000 rpm. In Fig. 3B, the calcein release is shown at constant process times, depending on the maximum local energy dissipation. Within relatively short process times up to 8 h, the molecule loss seemed to be independent of ε_{max} . At longer process times, the calcein release increased with increasing maximum local energy dissipation. For instance, at 48 h process time the release doubled from $3.2 \pm 0.1\%$ to $6.1 \pm 0.1\%$ when doubling the maximum local energy dissipation (0.24 W/kg at 100 rpm to 0.50 W/kg at 500 rpm). A further increase of ε_{max} to 1.23 W/kg (1000 rpm) resulted in only a slight increase of the release to $6.6 \pm 0.2\%$. Thus, although it was shown that under the investigated process conditions neither vesicle rupture nor calcein release due to passive diffusion occurred, continuous calcein leakage was observed even at low mechanical stress. Most probably, occurring local membrane defects that are caused by the mechanical stress are leading to the continuous calcein release without destroying the vesicles. The higher release at higher agitation speed can be explained by a more frequent or more pronounced concurrence of the membrane defects at higher mechanical stress.

To use polymersomes as universal reaction compartment the efficient retention of the guest molecules has to be feasible over a wide temperature range. Thus, the mechanical stability of calcein-loaded polymersomes was investigated at different temperatures from 8 °C to 40 °C (at 200 rpm). Figure 3C, displaying the temperature dependent calcein release over the process time, shows the high impact of the temperature on the retention efficiency of PMOXA₁₅-PDMS₆₈-PMOXA₁₅ polymersomes. At 8 °C only $2.8 \pm 0.0\%$ molecules were released within 48 h, whereas $5.0 \pm 0.0\%$ were lost at 25 °C. At 40 °C the calcein release was almost twice as much ($9.6 \pm 0.2\%$). A direct correlation of the discussed temperature-dependent membrane viscosity (fluidity) (Section 3.1.2) and the retention behavior of the polymersomes is apparent. It seems likely that an increased polymer chain flexibility at increased temperatures, which goes along with decreased membrane viscosity, resulted in higher calcein leakage. Figure 3D shows the calcein release at constant process times depending on the measured membrane viscosity (difference between the dynamic viscosity of the polymersome dispersion and pure water, Section 3.1.2). Up to 8 h process time, no clear dependence of the release and the membrane viscosity was recognized. In contrast, at longer process times (24, 48 h) a linear decrease of the calcein release with increasing membrane viscosity was observed.

Summing up, since calcein release was observed even at process conditions not leading to complete vesicle disintegration, not only the integrity of the vesicle itself, but also the membrane properties have to be considered to enable efficient retention of the guest molecules.

Figure 3. (A) Calcein release over process time at different agitation speeds at 25 °C and (C) at different temperatures at 200 rpm. (B) Calcein release at constant process times depending on the maximum local energy dissipation and depending on (D) the dynamic membrane viscosity.

3.2 Stability of polymersomes in two-phase systems

Commonly, organic solvents are used in biphasic biotransformations to build a two-phase system with the aqueous phase. Since ILs were shown to have a lower negative impact on the membrane stability of natural cells, they are promising alternatives compared to common solvents [29–31,40]. For these reasons, the stability of unloaded and calcein-loaded polymersomes was investigated in diverse two-phase systems with organic solvents and with ILs.

3.2.1 Organic solvents

The influence of 23 solvents, belonging to different solvent classes (alcohol, ether, ester, ketone, aromatic/aliphatic/halogenated hydrocarbons) on empty polymersomes (initial *z*-average: 160 nm, initial PDI: 0.15) was investigated at the shaken microliter scale. A solvent phase fraction of 10% v/v was applied. In Fig. 4A, showing the resulting *z*-average of the polymersome dispersion after contact with the respective organic solvents, the impact of the *n*-octanol/water partition coefficient (*log P*) becomes obvious. With decreasing *log P*, being synonymous with increasing hydrophilicity, a decrease of the *z*-average was observed. For instance, the contact of the polymersome dispersion with 2-butanol (*log P* = 0.78) led to a *z*-average of about 27 nm, showing

that the vesicles were transformed to undesired micelles [12,13]. Moreover, the contact to solvents with *log P* < 3 mainly resulted in a broadening of the particle size distribution, shown by an increase of the PDI (Fig. 4B). Thus, independent of the solvent class, solvents with higher hydrophilicity were shown to lead to stronger polymersome damage. For instance, using the alcohols 2-butanol and 1-butanol led to an increase of the PDI from 0.15 to 0.26 ± 0.06 and to 0.31 ± 0.04 , respectively. In contrast, using hydrophobic alkanes with *log P* > 3, did not lead to great changes neither of the vesicle diameter nor of the polydispersity of the particle size distribution. Both, the *z*-average (Fig. 4A) and the PDI (Fig. 4B) stayed approximately constant when using this solvent class. Moreover, the relative turbidity of the polymersome dispersion did not change either (data not shown), showing that no vesicle loss occurred. Consequently, due to the low-damaging behavior alkanes were chosen for further investigations.

After successfully verifying the transferability of the results from the microliter to the milliliter scale for five solvents with *log P* < 3 (2-butanol, ethyl acetate, cyclohexanone, methyl *tert*-butyl ether) and for three solvents with *log P* > 3 (cyclohexane, *n*-hexane, *n*-dodecane) (changes in *z*-average and PDI before and after contact with the solvents were comparable at both scales, data not shown), further investigations were carried out at the stirred milliliter scale system, being more relevant for biotechnological applications than the shaken microliter scale.

Figure 4. (A) z-average after contact with water-immiscible solvents of different solvent classes depending on their *n*-octanol/water partition coefficient $\log P$. (B) PDI after contact with the water-immiscible solvents, presented with increasing $\log P$. The dotted lines represent the initial z-average and the initial PDI, respectively, before contact with the solvent at the microliter scale.

The use of *n*-hexane and *n*-dodecane being two representatives for the low-damaging alkanes was deeply investigated at low stirrer speed (150 rpm, magnetic two cross-arm stirrer) to reduce the influence of the mechanical stress. Both alkanes were brought into contact with unloaded polymersomes (1% w/v in double distilled water) for 1 h. No significant difference of the resulting vesicle quality was observed when using 10% v/v and 20% v/v solvent phase fraction (data not shown), showing that a higher contact area between the polymer vesicles and the solvent phase (a higher solvent phase fraction leads to higher specific solvent surface when stirring is applied) did not affect the vesicle stability. Since the latter is often used for two-phase systems for biocatalytic applications [40], 20% v/v *n*-hexane and *n*-dodecane were used to investigate the influence of the contact duration up to 24 h, shown in Fig. 5. It can be seen that the z-average (Fig. 5A) did not change significantly up to a contact duration of 4 h when using *n*-hexane and *n*-dodecane, whereas a slight increase of the PDI (Fig. 5B) from 0.12 to 0.16 ± 0.02 was recognized when using *n*-dodecane for the same period of time. However, rather high deviations (especially with regard to the PDI when using *n*-dodecane) rule out a clear statement about the usability of the two solvents at longer process times. Consequently, the use of calcein-loaded polymersomes in a two-phase system with alkanes was taken into consideration for short process times of up to 4 h only. Unfortunately, the contact of calcein-loaded polymersomes with 20% v/v *n*-dodecane in miniaturized stirred-tank reactors for less than 1 h only resulted in undesired interactions of the polymer vesicles and the solvent. This led to an unclear phase boundary, ruling out the required proper phase separation. Moreover, a large amount of calcein-loaded polymersomes was lost, which was recognized by the typical color (calcein orange) within the initially transparent upper solvent phase, shown in Fig. 5 C. Thus, it can be finally stated that PMOXA₁₅-PDMS₆₈-PMOXA₁₅ polymersomes are not stable enough to be used as nanoreactors in a two-phase system with organic solvents.

3.2.2 Ionic liquids

The applicability of ILs as alternative to common solvents in biphasic reaction systems was investigated. The use of the three ILs [HPYR][NTF2], [BMIM][NTF2], and [HMPL][NTF2] was exemplarily studied, since they were shown to be useful in biphasic whole-cell biocatalysis [30, 41]. To compare the suitability of the named ILs, unloaded polymersomes (1% w/v in double distilled water) were brought into contact with 10% v/v of the water-immiscible phase at the milliliter scale for 1 h. The z-average and the PDI before and after the contact with the respective IL are shown in Fig. 6A and B, respectively. Particularly noticeable was the change of the particle diameter (z-average). Independent of the used IL, a decrease of the z-average from 185 nm to about 160 nm was recognized. In contrast, no notable change of the initial polydispersity ($PDI = 0.16$) was observed, as shown in Fig. 6B. Thus, no major advantage of one of the ILs was found. Nevertheless, due to the comparably poor reproducibility when using [HPYR][NTF2] and due to the lowest changes in vesicle size and polydispersity when using [HMPL][NTF2], the latter one was chosen for further investigations. No deterioration of the resulting polymersome quality (z-average, PDI) was observed at the milliliter scale when using 20% v/v [HMPL][NTF2]

Figure 5. (A) z-average and (B) PDI after contact with 20% v/v *n*-hexane (black) and *n*-dodecane (gray) in dependence of the contact duration. The dotted lines represent the initial z-average and the initial PDI, before contact with the solvent at the milliliter scale. (C) Unclear phase boundaries in-between the upper solvent phase and the aqueous polymersome dispersion. The orange color in the initially transparent alkane layer shows the undesired interaction of the calcein-loaded polymersomes and the solvent phase.

compared to 10% v/v (data not shown). Therefore, the investigations on the impact of the contact duration between polymersomes and IL were carried out at 20% v/v, which is a commonly used phase fraction of ILs for biocatalytic applications [40]. Figure 6C and D show the resulting z-average and the resulting PDI, depending on the contact time. A threefold determination at the milliliter scale as well as one exemplary experiment in the miniaturized stirred-tank reactor were carried out. The significant decrease of the z-average becomes obvious once more at both scales. Within 1 h only, the z-average decreased from 165 nm to 133 ± 1 nm (mL-scale) and from 160 to 140 nm (miniaturized stirred-tank reactor) but did not change notably afterwards, as shown in Fig. 6C. Besides, whereas a constant increase of the PDI was observed within 24 h at the milliliter scale (from $PDI = 0.12$ to $PDI = 0.15 \pm 0.01$), no trend of the polydispersity was seen in the miniaturized stirred-tank reactor (PDI about 0.13).

Summing up, the rapid decrease of the z-average at the first contact of the aqueous polymersome dispersion and the IL seemed to be phenomenological. A change of the thermodynamic equilibrium due to the presence of the second phase might be a reason for this behavior [42–50], leading to a smaller stable vesicle diameter. Despite the significant decrease of the z-average, the use of loaded polymer vesicles in the presence of ILs was taken into consideration since the polymersomes seemed to be macroscopically intact. Therefore, the influence of 20% v/v [HMPL][NTF2] on calcein-loaded polymersomes was investigated in the miniaturized stirred-tank reactor. Figure 7A shows that a clear phase boundary was obtained and no undesired interactions of the polymersomes in the upper aqueous phase with the lower IL phase were visually apparent. Due to this promising result, the calcein release was measured over process time. In Fig. 7B, the release caused by contact with the IL is shown. Calcein release caused by the present mechanical and thermal conditions (S-stirrer, 500 rpm, 25°C, compare Fig. 3A) have been subtracted. It becomes obvious that a very rapid release of the calcein occurred. Within 1 h only, $11.6 \pm 0.1\%$ of the previously encapsulated molecules have leaked. It seems likely that a direct correlation of the phenomenological decrease of the z-average and the pulse-like calcein release at the first IL contact exists. Most possibly, the occurrence of membrane defects, caused by the rearrangement of the polymer chains when decreasing the vesicle diameter led to the high molecule loss within the first hour. Within 48 h, the molecule loss increased to $36.1 \pm 0.1\%$. Thus, the very poor molecule retention rules out the reasonable use of PMOXA_{15} - PDMS_{68} - PMOXA_{15} polymersomes in the presence of [HMPL][NTF2].

4 Discussion

One of the most important advantages of polymersomes, compared to lipid vesicles, is the enhanced membrane stability [10, 51–54]. To investigate the mechanical stability of membrane vesicles, the method of micropipette aspiration is often used [55, 56]. Here, a single vesicle is sucked into the channel of a micropipette. The length of the membrane area sucked-in can be measured in dependence on the suction pressure. Finally, by calculating the membrane area sucked-in shortly before vesicle rupture occurs, conclusions on the membrane stability can be

Figure 6. (A) z-average and (B) PDI after 1 h contact with 10% v/v IL at the milliliter scale. (C) z-average and (D) PDI after contact with 20% v/v [HMPL][NTF2] in dependence of the contact duration at the milliliter scale (black, $N = 3$) and in the miniaturized stirred-tank reactor (gray, $N = 1$). The dotted lines represent the initial z-average and the initial PDI, before contact with the IL.

drawn. In this manner, it was able to show that membrane stability of polymersomes is proportional to the molecular mass of the membrane forming polymer chains [52, 53]. This method seems to be very useful to select suitable polymers for polymersome production, since the stability of polymer vesicles made of different polymers can be compared easily. Nevertheless, the investigation on the stability of polymersomes, made of one certain polymer type, in dependence on defined mechanical stress is not possible. Moreover, to apply polymer vesicles as nanoreactors for biotechnological applications, the precise knowledge about their stability in stirred-tank reactors, representing the most often used reactor type in biotechnology, is required. Thus, characterizing the vesicle stability in dependence on the maximum local energy dissipation, being a descriptive measure to evaluate the stress on particles in stirred-tank reactors, is a reasonable approach as it was shown in this study.

Regarding the use of polymersomes for biocatalytic applications, the poor water solubility of some substrates and products is often challenging as well as their low stability in aqueous media. A common method to overcome these drawbacks is the application of a suitable in situ substrate feeding and product removal strategy, for example, the use of liquid–liquid biphasic reaction systems with organic solvents. Thus, the knowledge about the stability of polymersomes in the presence of different solvents is needed. For lipid vesicles, the increasing destructive

effect with decreasing *n*-octanol/water partition coefficient $\log P$ of the solvent is already known [57]. Within this work the same effect could be confirmed for polymer vesicles for the first time. The use of alkanes with high $\log P$ was shown to have only low impact on the quality and quantity of unloaded polymer vesicles but led to a high release of previously encapsulated calcein. It was found that longer polymer chains form polymersomes of higher membrane stability with higher resistance toward detergents [58] and possibly higher resistance toward solvents. Nevertheless, longer PMOXA-PDMS-PMOXA chains are not expected to allow the efficient retention of guest molecules in the presence of common organic solvents. Compared to organic solvents, water-immiscible ILs are known to cause lower negative impact on the membrane stability of natural cells [29, 30, 40]. For this reason and since polymersomes made of poly(butadiene-*b*-ethylene oxide) and polymer vesicles formed from zwitterionic block copolymers were successfully produced in ILs [59–61], they seemed to be a good alternative. Unfortunately, in this work more than 10% of the encapsulated calcein were lost at the first contact with the IL [HMPL][NTF2]. It was assumed that the pulse-like release correlated with the accompanying phenomenological decrease of the vesicle diameter of about 20 nm. Using a cross-linkable PMOXA-PDMS-PMOXA polymer with acrylate end groups could prevent polymersomes from shrinking, since the shape of the vesicle is frozen by postpreparative radical

Figure 7. (A) Unclear phase boundaries in-between the upper solvent phase and the aqueous polymersome dispersion. The orange color in the initially transparent alkane layer shows the undesired interaction of the calcein-loaded polymersomes and the solvent phase. (B) Calcein release over process time.

polymerization. Moreover, cross-linked PMOXA-PDMS-PMOXA polymersomes show increased stability in general [22, 62, 63] and could therefore possibly enable the use of polymersomes in a two-phase system with IL or even organic solvent. As an alternative, the stability of polymersomes in solid–liquid reaction systems with adsorbent resins as second phase could be studied because this SFPR strategy has also been applied successfully in diverse biocatalytic reactions [41, 64, 66].

5 Concluding remarks

The main focus of this work was on the feasibility of using polymer vesicles as nanoreactors for biotechnological applications. The stability of PMOXA₁₅-PDMS₆₈-PMOXA₁₅ polymersomes toward mechanical stress was studied in stirred-tank reactors since this is the most often used reactor type in the biotechnological industry. The particle stress induced by stirrer rotation

was evaluated by the maximum local energy dissipation. Thus, it was shown that agitation speeds causing a maximum local energy dissipation of up to 1.23 W/kg did not lead to loss of vesicle quality or quantity. However, most possibly due to occurring membrane defects not resulting in complete vesicle disintegration, a continuous release, of the previously encapsulated model dye calcein was observed, which was increasing with increasing maximum local energy dissipation. Nevertheless, only 6.6% calcein leaked at the highest investigated maximum local energy dissipation (1.23 W/kg at 25°C) within 48 h. Calcein release was not only triggered by increasing mechanical stress, but also by increasing temperature, leading to lower membrane viscosity and higher membrane fluidity, respectively. Besides, the usability of polymersomes in two-phase systems would be a great benefit. However, although it was shown that alkanes with rather high *n*-octanol/water partition coefficient lead to only low vesicle damage, both organic solvents and ILs led to high losses of the encapsulated molecules. Nevertheless, it should be mentioned that calcein chosen as model dye is a rather small molecule (0.6 kDa) and is much smaller than any enzyme which could be encapsulated to turn the polymersomes into biocatalytically active nanoreactors. Thus, calcein can be expected to cross the membrane barrier more easily than much larger enzymes of several tens of kDa, certainly having more difficulties to pass the local membrane defects. Concluding, due to its low detection limit and due to its small size, calcein is the perfect candidate to precisely investigate the membrane stability. Moreover, calcein is perfectly suited for worst-case studies, since efficient enzyme retention can be attempted if no or only low calcein release is detected at the same process conditions. First experiments on the release behavior of the model enzyme *N*-acetylneuraminidase (4 × 34.8 kDa) encouraged this assumption. Further investigations on the release behavior of enzymes as well as the use of cross-linkable polymers for the production of more stable polymersomes would be of great interest.

Practical application

Due to the considerably higher membrane stability of polymersomes compared to their liposomal counterparts, a huge diversity of polymersome applications was developed. Polymer vesicles are not only intended to be used as carrier system in the field of medicine, but shall also be used as nanoreactors for biotechnological applications. To bring this vision closer to reality, the precise knowledge about polymersome stability at relevant process conditions is required. Within this work, the stability of polymersomes toward mechanical stress and their stability in two-phase systems is investigated in the stirred-tank reactor, being the most important reactor type in the biotechnological industry. The mechanical stability was valued by the maximum local energy dissipation, being the appropriate measure to evaluate particle stress in stirred-tank reactors. It is shown that preserving the macroscopic integrity of the vesicles alone is insufficient. Parameters influencing membrane properties (e.g., temperature), have to be considered as well.

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Nomenclature

F_t	[-]	Fluorescence of unlysed polymersomes after a certain time t
F_0	[-]	Fluorescence of the initial unlysed polymersomes at $t = 0$
$F_{\infty,t}$	[-]	Fluorescence of lysed polymersomes after a certain time t
$F_{\infty,0}$	[-]	Fluorescence of the initial lysed polymersomes at $t = 0$
n	[rpm]	Agitation speed
$rel.T$	[-]	Relative Turbidity
T_t	[-]	Turbidity of the polymersome dispersion after a certain time t
T_0	[-]	Turbidity of the initial polymersome dispersion at $t = 0$
ε_{max}	[W/kg]	Maximum local energy dissipation

6 References

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