Sarah Theresa Poschenrieder
Sina Katharina Schiebel
Kathrin Castiglione

Lehrstuhl für
Bioverfahrenstechnik, Technische
Universität München, Garching,
Germany

Research Article

Polymersomes for biotechnological applications: Large-scale production of nano-scale vesicles

Polymersomes have some fundamental advantages compared to their liposomal counterparts. Due to the increased stability of the polymeric membrane, polymersomes are intended to be reasonably applicable as carrier-systems and universal reaction compartments for diverse medical and biotechnological applications. Regardless of the application area, suitable methods to produce large vesicle quantities in a controlled and cost-effective manner have to be developed to put polymersome technology into action at the industrial scale. In this work, the amphiphilic triblock copolymer poly(2-methyloxazoline)₁₅-poly(dimethylsiloxane)₆₈-poly(2-methyloxazoline)₁₅ was formed into uniform polymersomes. A recently established production process, based on the use of miniaturized stirred-tank reactors at the milliliter-scale (12 mL), was successfully scaled-up to the liter-scale (1.5 L) based on solid process engineering parameters. Dynamic light scattering measurements show that using standard propeller stirrers with a dimensionless diameter $d D^{-1} \geq 0.65$ in an unbaffled stirred-tank reactor led to a narrow particle size distribution when providing a Froude number of $Fr = 6.52$ at the same time. Polymersomes with a mean diameter of 180 nm and a low polydispersity index ($PDI < 0.2$) were generated within about 1 h in one single production step. Thus, this work provides the fundamental basis for further scale-up purposes, regarding polymersome production in stirred-tank reactors at the industrial scale.

Keywords: Compartmentalization / Nanobiotechnology / Polymersomes / Process engineering / Scale-up

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1 Introduction

The encapsulation of molecules in vesicular compartments is of great interest for various biotechnological applications. In synthetic biology, compartmentalized structures can be used for the bottom-up construction of artificial cell-like systems and to mimic eukaryotic organelles or bacterial microcompartments [1]. Vesicle-based compartments can further be applied as drug

delivery systems [2, 3] and hold great potential as nano-scale reactors for enzymatic reactions [3, 4].

One possible approach to generate artificial compartments is the formation of hollow spheres by using self-assembling amphiphilic molecules. A well-known example is liposomes, which consist of a phospholipid bilayer membrane entrapping an aqueous core [5]. Due to the intrinsic softness of the phospholipid membrane, the stability of these vesicles is comparatively low and the retention of encapsulated molecules is not well controlled [6], which is a severe disadvantage for biotechnological applications. Much more stable vesicles can be generated by using amphiphilic polymers for the formation of so-called polymersomes. Most commonly, di- or triblock copolymers of the $[A]_x[B]_y$ or $[A]_x[B]_y[A]_x$ type are used for this purpose [4]. The resulting polymer membranes (3–40 nm [3]) are usually much thicker than the phospholipid membrane of liposomes

Correspondence: Dr. Kathrin Castiglione (k.castiglione@lrz.tum.de), Lehrstuhl für Bioverfahrenstechnik, Technische Universität München, Boltzmannstr. 15, 85748 Garching, Germany.

Abbreviations: PDI, polydispersity index; PDMS, poly(dimethylsiloxane); PMOXA, poly(2-methyloxazoline); PSD, particle size distribution

(3–5 nm [7]) and confer higher stability, lower permeability and significantly improved encapsulant retention [6].

An ABA triblock copolymer that has been extensively studied in the field of biotechnological applications is poly(2-methyloxazoline)-*b*-poly(dimethylsiloxane)-*b*-poly(2-methyloxazoline) (PMOXA-PDMS-PMOXA). This material has a low cytotoxicity, does not evoke inflammatory responses by biological systems and shows very low nonspecific protein binding [8, 9]. In addition, the polymer shell allows the functional integration of diverse transmembrane proteins, as reviewed recently [10]. By the integration of transport proteins, a selective mass transport between the internal space of the compartment and the surrounding environment can be established.

Due to the enormous potential of polymersomes for biotechnological applications, well-controllable, highly reproducible and scalable production methods are needed. In principle, polymersomes can be produced by a variety of methods, e.g. film rehydration [11], electroformation [12], direct dissolution of polymer powder in an aqueous phase [13], or injection of an organic solvent with dissolved polymer into an aqueous buffer phase [14]. In most cases, the resulting particle size distribution (PSD) is rather broad and the polydispersity is subsequently reduced, e.g. by repeated extrusion [15]. To avoid additional unit operations, one-step polymersome production processes are desirable, which directly produce uniform vesicles.

Narrow size distributions and controllable sizes of polymersomes have been achieved using the co-solvent method in combination with modified inkjet printers [16], microfluidic devices [17–20], and continuously working micromixers [21, 22]. These methods are highly reproducible and allow polymersome production within only one step. Whereas the theoretical possibility of pilot-scale production using micromixers exists due to the commercial availability of such devices up to the 100-L scale [21, 23, 24], the throughput of microfluidic approaches is limited to the $\mu\text{L}/\text{min}$ range [24] and scale-up is only possible by parallelization (horizontal scale-up). In addition, using specialized equipment with unknown long-term reliability and associated investment costs represent a further drawback for the realization of a horizontal scale-up strategy [25].

Recently, our group has established the first polymersome production process in miniaturized stirred-tank reactors at the milliliter-scale [26]. Using the triblock copolymer PMOXA₁₅-PDMS₆₈-PMOXA₁₅ as an exemplary amphiphile, vesicles with a *z*-average (harmonic intensity averaged particle diameter [27, 28]) of about 200 nm were generated within less than 1 h in one single production step. An S-shaped stirrer, originally developed for enzymatic biomass hydrolyses within the bioREACTOR 48 system [29, 30], achieved the best results [26]. The method is highly reproducible and a monomodal PSD was achieved. The dimensionless polydispersity index (PDI), a measure of the homogeneity of particle sizes with values between 0 and 1, was below 0.2 indicating a narrow PSD [26].

The developed production method has two significant advantages. First, the stirred-tank reactor is by far the most frequently used reactor type in chemical and biotechnological industries and the process could be transferred to existing production plants [31]. Second, a vertical scale-up is feasible since stirred-tank reactors with working volumes up to several thousand

cubic meters exist [32]. In almost all scale-up situations, complete similarity on different scales cannot be realized and the suitable scale-up criteria have to be identified. A holistic scale-up strategy can only be established by a comprehensive process characterization, leading to the identification of product quality influencing key factors [33].

In the field of particle technology, scale-up strategies are based on three basic requirements. First of all, for scale-up purposes the same material has to be used for both, the process at the model scale and at the larger scale. Using the same material with the identical properties is inevitable when transferring a certain process to greater dimensions. Hence, physical parameters influencing crucial material characteristics (e.g. temperature) have to be maintained at both scales [34]. Second, the linear scale-up of the process equipment (geometrical similarity) is needed. In the case of using stirred-tank reactors geometric similarity normally can be fulfilled without difficulty. This means that the ratios of the reactor and the stirrer dimensions (e.g. $h d^{-1}$, $d D^{-1}$, $h H^{-1}$) are constant at both scales. Finally, the relevant scale transfer criteria, which are characteristic for a given process, have to be identified and maintained. Some of the most important scale-up criteria regarding stirring tasks are the stirrer circumferential speed u_{tip} (kinematic similarity), the volumetric power input $P V^{-1}$, the maximum energy dissipation ε_{max} and dimensionless numbers such as the Newton number Ne , the Reynolds number Re and the Froude number Fr (dynamic similarity) [34].

The aim of this work was the development of a solid strategy for scaling-up the established milliliter-scale process (12 mL) to the liter-scale (1.5 L). At the large scale, uniform vesicles of comparably good quality (criteria: similar *z*-average, PDI and PSD) shall be produced as fast and efficient as at the small scale. The use of the unconventionally shaped S-stirrer at the milliliter-scale, which causes radial-, tangential- and predominantly axial flow [26], caused a remarkable challenge for the scale-up. With the ratios $d D^{-1} = 0.72$, $h H^{-1} = 0.64$ and $h d^{-1} = 1.9$ the S-stirrer has a very special geometry that is far away from most standardized stirrer geometries. Whereas the linear scale-up of the stirred-tank reactor was realized without problems (flat bottom tank with $H D^{-1} = 2.2$ at both scales), the geometric similarity cannot be maintained for the different stirrer types. Thus, the aim was not only to find a way for the efficient high-quality polymersomes production at the liter-scale. The implementation of a large-scale polymersome production process based on product quality influencing process engineering parameters by using standardized equipment, which can be an important issue for economic processes was the primary objective of this work.

2 Materials and methods

2.1 Triblock copolymer

Polymersomes were formed by the same polymer, namely PMOXA₁₅-PDMS₆₈-PMOXA₁₅, purchased from Polymersource (Dorval QC, Canada), as was used in our previous study [26]. Characterization of the polymer was carried out by Polymer-source. The central block was characterized by size exclusion chromatography. The columns were calibrated with monodisperse poly(dimethyl siloxane) and the chemical composition

Figure 1. (A) Scheme of the experimental set-up. The 20% w/v ethanolic polymer solution is continuously pumped (15 L/h) into the stirred water. It is channeled through an inlet pipe which is located directly above the stirrer. An outlet valve at the bottom of the reactor allows the sampling during the process. (B) The use of five different stirrer types was investigated. Radial flow causing Rushton Turbines (S1) as well as axial flow causing propeller stirrers with four blades (S2) or three blades (S3–S5) were driven. The almost identical stirrer types S4a and S4b only differ in the orientation of the blades, causing the downwards or upwards axial flow (cf. Table 1). One box of the background corresponds to 1 cm².

was extracted from proton NMR using CDCl₃ as solvent. The molecular mass of the side block was calculated based on the molecular mass of the center block and the chemical composition. It was found that the triblock copolymer has a molecular mass of 7600 g/mol ($M_{n, \text{PMOXA}} = 1300$ g/mol and $M_{n, \text{PDMS}} = 5000$ g/mol) and a narrow molecular mass distribution, which is given by $M_w/M_n = 1.23$ (M_w : mass-average molecular mass; M_n : number-average molecular mass).

2.2 Polymersome production at the milliliter-scale

Polymersome production at the milliliter-scale is described in detail elsewhere [26]. In brief, the polymer vesicles were produced via the ethanol method. For this purpose, 0.6 mL of a 20% w/v ethanolic polymer solution was injected into 11.4 mL stirred (S-stirrer, 4000 rpm, unbaffled reactor), double distilled and filtrated water by using a syringe. Sampling was carried out at the top of the stirred-tank reactor and a sample volume of 0.1 mL was drawn off. Details on the encapsulation efficiency can be found in the Supporting Information.

2.3 Polymersome production at the liter-scale

2.3.1 Stirred-tank reactor

Polymer vesicles were produced via the ethanol method at room temperature in an unbaffled flat bottom stirred-tank reactor (KLF 2000, Bioengineering AG, Wald, Switzerland) (diameter $D = 0.10$ m), unless noted otherwise. For a total volume V of 1.5 L (filling level $H = 0.22$ m) and a final polymer concentration c_p of 1% w/v, 0.075 L of a 20% w/v ethanolic polymer solution were continuously pumped (GUV-150, Meredos, Boven-den, Germany) into 1.425 L of stirred water (double distilled and filtrated). The polymer solution was channeled through an inlet

pipe that was located directly above the stirrer (feeding rate $\dot{V} = 15$ L/h). Immediately after finishing the feeding process, the inlet pipe was pulled out of the reactor.

Sampling was carried out by using an outlet valve at the bottom of the stirred-tank reactor and a sample volume of 1 mL was drawn off. A scheme of the experimental set-up is shown in Fig. 1A.

2.3.2 Stirrer geometries

Five different standardized stirrer types, shown in Fig. 1B, were driven clockwise. Rushton Turbines (causing radial flow in baffled reactors) as well as propeller stirrers (causing axial flow in baffled reactors) with three or four blades (blade angle α ranging from 20° to 45°) were used. Table 1 gives an overview of characteristics and dimensions of the S-stirrer at the milliliter-scale (cf. Section 2.2) and the stirrer geometries used at the liter-scale.

2.3.3 Measurement of the power input P

The characteristic power input P was measured for each stirrer type. By using a wattmeter (2100 Digital Watt Analyzer, Valhalla Scientific, San Diego, USA) connected to the motor, the power consumption was measured within 0–2000 rpm in discrete intervals. Power measurements were executed using an empty and a water-filled reactor ($V = 1.5$ L). The difference represents the net power introduced into the liquid phase.

The values of the volumetric power inputs for the investigated stirrer types at defined installation heights h_s (cf. Fig. 1B) are available within Supporting Information Tables S1–S9.

2.3.4 Calculations

Increasing agitation speed n causes increasing stirrer circumferential speed u_{tip}

$$u_{\text{tip}} = d \cdot \pi \cdot n \quad (1)$$

Table 1. Stirrer characteristics and dimensions

Stirrer type	Flow direction	d [mm]	h [mm]	$h d^{-1}$ [-]	$d D^{-1}$ [-]	$h H^{-1}$ [-]	Number blades	α , [°]
S-Stirrer ^{a)}	Radial, tangential, axial	14.4	27.5	1.910	0.71	0.671	2	90
S1 ^{b)}	Radial	40	8	0.200	0.40	0.036	6	90
S2 ^{b)}	Axial, down	48	12	0.250	0.45	0.054	4	20
S3 ^{c)}	Axial, down	54	24	0.444	0.54	0.109	3	25
S4a ^{c)}	Axial, up	65	52	0.800	0.65	0.236	3	45
S4b ^{c)}	Axial, down	65	52	0.800	0.65	0.236	3	45
S5 ^{c)}	Axial, down	90	35	0.388	0.90	0.159	3	25

^{a)}Developed in-house [29,30]

^{b)}Bioengineering, Wald, Switzerland

^{c)}Infors HT, Bottmingen, Switzerland

and leads to a rise of the volumetric power input $P V^{-1}$. The dimensionless description of the volumetric power input into the liquid phase with a certain density ρ and certain dynamic viscosity η requires the calculation of the Newton number Ne

$$Ne = \frac{P}{\rho \cdot n^3 \cdot d^5} \quad (2)$$

and the Reynolds number Re

$$Re = \frac{\rho \cdot n \cdot d^2}{\eta} \quad (3)$$

In unbaffled reactors, vortex building may occur and the acceleration of gravity g and hence the dimensionless Froude number Fr

$$Fr = \frac{n^2 \cdot d}{g} \quad (4)$$

have to be considered [34].

The kinetic energy that is introduced into the stirred liquid phase dissipates inhomogeneously and a maximum local energy dissipation $\varepsilon_{\max}$, which is an important process engineering parameter regarding particle stability, occurs in the stirrer region. The ratio of the maximum local energy dissipation to the power input per unit mass ε_{Φ} with

$$\varepsilon_{\Phi} = \frac{P}{\rho \cdot V} \quad (5)$$

is given by

$$\Phi = \frac{\varepsilon_{\max}}{\varepsilon_{\Phi}} \quad (6)$$

where Φ is a constant for a given stirrer [35]. The maximum local energy dissipation $\varepsilon_{\max}$ can be estimated by using the equation

$$\frac{\varepsilon_{\max}}{\varepsilon_{\Phi}} = \frac{4}{\left(\frac{d}{D}\right)^2 \left(\frac{h}{d}\right)^{\frac{2}{3}} 2^{0.6} (\sin \alpha)^{1.15} z_i^{\frac{2}{3}} \left(\frac{H}{D}\right)^{-\frac{2}{3}}} \quad (7)$$

This equation takes the dependence on the stirrer and reactor geometries (cf. Fig. 1A and Table 1), as well as the number of the fixed stirrers z_i and the number of the stirrer blades z into account. In unbaffled reactors, the value of $\frac{\varepsilon_{\max}}{\varepsilon_{\Phi}}$ is estimated to be four times higher [36,37].

The calculated values for u_{tip} , Ne , Re , Fr and $\varepsilon_{\max}$ for the investigated stirrer types S1–S5 (cf. Table 1) at discrete agitation speeds n and at defined installation heights h_S (cf. Fig. 1A) are available within Supporting Information Tables S1–S9.

2.4 Dynamic light scattering measurements

Samples were diluted 10-fold with double distilled and filtrated water. No sample preparation such as centrifugation, filtration or extrusion was executed. The sample (0.5 mL) was measured in 10 mm single-use polycarbonate cuvettes. The z -average, the PDI, and the intensity-based PSD were determined by using a Zetasizer NS (Malvern, Worcestershire, UK). This system is equipped with a red HeNe gas laser (633 nm) and a sensitive avalanche photodiode detector. Furthermore, this system is based on the 173° non-invasive back scatter (NIBS®) technology. Detecting the scattered light at 173° can avoid the oversight of small particles in the presence of larger particles. To come up with the varying sample composition, the laser power was automatically attenuated and the measurement position within the system was automatically moved as well. Light-scattering was performed at 25°C after equilibrating the samples for 60 s. Three measurements consisting of ten runs each (10 s per run) were recorded. Data were analyzed using the software Zetasizer 7.01. The z -average and the PDI were extracted from the cumulant analysis. The intensity-based PSD derive from the “General Purpose” non-negative-last-square (NNLS) algorithm.

2.5 Turbidity measurement

Turbidity measurements were carried out in 384 well flat bottom UV-assay plates (Brand, Wertheim, Germany). Samples (100 μ L) were measured in a microplate reader (Infinite M200, Tecan, Männedorf, Switzerland) at 280 nm. By measuring the turbidity at the beginning of the process $T(0)$ and during the process $T(t)$, the relative turbidity was calculated by the aid of the maximal turbidity $T_{\max}$ as follows:

$$\text{Rel. Turbidity}_{280 \text{ nm}} = \frac{T(t) - T(0)}{T_{\max} - T(0)} \quad (8)$$

2.6 Transmission electron microscopy

Morphological examination of the polymer vesicle dispersion was performed by using a JEM2011 (JEOL, Tokyo, Japan) transmission electron microscope (TEM) at an acceleration voltage of 120 kV. Samples were prepared as described previously [26].

Figure 2. (A) Dependence of the relative turbidity and (B) the z-average on the process time when producing polymersomes in miniaturized stirred-tank reactors via the established process [26].

3 Results

3.1 Evaluation criterion

The aim of this work was to produce uniform polymersomes of controlled size as fast and efficient as it was achieved in miniaturized stirred-tank reactors at the milliliter-scale [26]. The vesicle building process is widely understood to be a multi-step process [38, 39]. One of the most frequently encountered theories is based on the very rapid formation of small spherical micelles within the first formation step. These micelles slowly transform by fusion processes to larger intermediate structures such as worm-like or disc-like micelles [40] and finally close up to form the desired vesicles [39, 41–43]. For these reasons, to compare the efficiency of the large-scale polymersome production process with the established process at the milliliter-scale, it was chosen to consider the particle increase rate.

Measuring turbidity can be used to follow the morphological changes of block copolymer aggregates in solution. The transition from smaller to larger particles is accompanied by a remarkable increase in turbidity [39]. Figure 2A shows the increase of the relative turbidity over time, when producing polymersomes in miniaturized stirred-tank reactors via the established process [26]. The rate of particle increase and thus the efficiency of the process can be judged by the slope of the relative turbidity over time within the linear range. Besides, independently of the prevalent morphological structures, the mentioned fusion process and thus the particle increase process can also be followed by the linear increase of the z-average within the first part of the process, which is shown in Fig. 2B. Directly after polymer addition, polymer micelles are built very rapidly [31], which is represented by a z-average of about 30 nm (membrane thickness: 14 nm [26]) at the beginning of the process. Afterwards, the polymeric micelles rearrange their morphologies to build vesicles with a z-average of about 180 nm at the end of the process. When the kinetic of self-assembly is slowed down by a highly selective solvent and no free self-assembly is possible, the formation of polymersomes need significantly more time than the formation of spherical micelles. The speed of the particle increase can be equally well reflected by the linear increase of the relative

turbidity and the z-average within the beginning of the process. However, the z-average was chosen to be the most suitable parameter to show the progress of the vesicle production within the polymersome production process. To compare the different process rates, the z-average over time with its characteristic unit nanometer per second is a more descriptive indicator, compared to the relative turbidity.

3.2. Influence of the agitation speed n

Radial flow causing Rushton Turbines are widely used in biotechnology for diverse processes such as non-mammalian cell cultivations [44]. To investigate the influence of the agitation speed n , three Rushton Turbines (cf. S1 in Table 1, and Supporting Information Table S1), fixed at the stirrer shaft on top of each other ($h_{s,1} = 0.06$ m, $h_{s,2} = 0.12$ m, $h_{s,3} = 0.18$ m), were driven at 870 and 1080 rpm. Gas bubble introduction, which may reduce the efficiency of the polymersome production [26], was just not yet observed at 870 rpm. At 1080 rpm the vortex, which is built within the unbaffled reactor, just reaches the upper side of the top Rushton Turbine. Unwanted stirrer gassing, possibly causing an agitation shaft damage, would occur at higher speeds.

In Fig. 3A, the increase of the z-average within up to 1440 min (24 h) process time is shown. At 1080 rpm, the resulting initial slope of the z-average over time (0.5 nm/min) and thus the particle increase rate is significantly higher compared to the slope which results at 870 rpm (0.2 nm/min). Therefore, it becomes clear that the vesicle production process is accelerated by higher agitation speeds. Nevertheless, a narrow PSD was not achieved within the desired short process times. A low z-average of 51 nm and a very high PDI of 0.74 were the result when driving the Rushton Turbines for 60 min at 1080 rpm. Within the same time, the model process delivers a z-average of about 180 nm and a $PDI < 0.2$, which is reported to indicate a narrow size distribution [45]. Moreover, with the z-average increase of 31.0 nm/min the model process occurs about 100-times faster than the liter-scale process.

The developed polymersome production process at the milliliter-scale is based on the use of the predominantly axial

Figure 3. (A) Dependence of the z-average on the process time when driving three Rushton Turbines (S1) (cf. Table 1) at 870 and 1080 rpm. (B) Dependence of the z-average on the process time when driving the stirrer types S2–S5 (cf. Table 1) at the stirrer circumferential speed $u_{tip} = 3.02$ m/s. (C) At a constant stirrer circumferential speed $u_{tip} = 3.02$ m/s the increase of the z-average over time (slope) depends almost linearly on the dimensionless stirrer diameter $d D^{-1}$. (D) Dependence of the z-average on the process time when driving the stirrer types S4a and S5 (cf. Table 1) at a power consumption of $P V^{-1} = 12.3$ W/L. The milliliter-system serves as a reference.

flow causing S-stirrer [29, 30]. As the use of radial flow causing Rushton Turbines did not lead to acceptable results, the focus was then set on the use of axial flow causing propeller stirrers. The usefulness of five different propeller stirrers with different diameters (cf. Tables 1, and S2–S5) was investigated. Here, the stirrer geometries S4a and S4b are almost identical, but differ in the orientation of the blades, causing the downwards or upwards axial flow.

3.3 Polymersome production at constant stirrer circumferential speed u_{tip}

For the scale-up of biotechnological processes, a constant stirrer circumferential speed u_{tip} often serves as scale-up criterion. This is commonly done when the consequences of shear forces are crucial (e.g. shear sensitive mycelial cultures) or not exactly known, as is the case for polymersomes [46]. u_{tip} can easily be calculated by Eq. (1) and is equal to 3.02 m/s for the established process at the milliliter-scale (S-stirrer, 4000 rpm).

At the liter-scale, four different propeller stirrers with different geometries (cf. S2–S5 in Table 1 and Supporting Infor-

mation Tables S2–S4 and S8) were driven at $u_{tip} = 3.02$ m/s ($h_s = 0.072$ m). Looking at the resulting initial slopes of the z-average over the process time, shown in Fig. 3B, it was found that using this scale-up criterion does not lead to the desired fast process. Independent of the used stirrer type, all processes are much slower than at the milliliter-scale, which can be seen by the slower z-average increases (0.5–2.1 nm/min at the liter-scale compared to 31.0 nm/min at the milliliter-scale). Therefore, in terms of efficient polymersome production, a constant stirrer circumferential speed cannot be a reasonable scale-up criterion. However, at a constant stirrer circumferential speed u_{tip} , the increase of the z-average over time (slopes of the dotted lines in Fig. 3B) almost linearly depends on the dimensionless stirrer diameter $d D^{-1}$, as shown in Fig. 3C. Hence, at the same stirrer circumferential speed the process is sped up by larger impeller diameter.

Sometimes, in process engineering maintaining the Reynolds number Re can also serve as a scale-up strategy [33, 46]. Re can be calculated with Eq. (3) and is equal to 13 824 for the established milliliter-scale process at 20°C ($\rho_{water, 20^\circ C} = 1000$ kg/m³, $\eta_{water, 20^\circ C} = 0.001$ kg m⁻² s⁻¹). Independent of the stirrer type, at the liter-scale the agitation speeds n , causing $Re = 13,824$ are

considerably lower than the agitation speeds n , causing the same stirrer circumferential speed u_{tip} at both scales, e.g. for S4a:

$$\begin{aligned} n (Re = 13,824) &= 196 \text{ rpm} < n (u_{tip} = 3.02 \text{ m/s}) \\ &= 887 \text{ rpm.} \end{aligned}$$

Consequently, since u_{tip} was shown to be not useful for the scale-up, and since it was also shown that lower agitation speeds n will lead to a slower process (cf. Section 3.2.), an identic Reynolds number Re at both scales cannot lead to a successful scale-up.

3.4 Polymersome production at constant volumetric power input $P V^{-1}$

A constant volumetric power input $P V^{-1}$ is another frequently used scale-up strategy for biotechnological processes [47], and is equal to 12.3 W/L at the model process [26]. The use of baffles within the reactor facilitates achieving such relatively high power inputs. Many biotechnological processes are realized in baffled reactors since higher power input can be provided at a lower stirrer speed. However, at the milliliter-scale, the use of baffles was identified to be hindering in terms of high quality polymersome production [26]. Nevertheless, to check the validity of this phenomenon at the large scale, polymersome production at $P V^{-1} = 12.3 \text{ W/L}$ was investigated in a baffled reactor at first. Since stirrer S5 does not fit into a baffled reactor, stirrer S4a is the largest stirrer type, which can be used for this purpose. This stirrer type was presumed to give the most informative results, since large stirrer diameters were assumed to enable a faster polymersome production process (cf. Section 3.3).

Using stirrer type S4a (cf. Table 1 and Supporting Information Table S6) at 12.3 W/L in a baffled reactor ($h_S = 0.072 \text{ m}$) leads to a very broad PSD with high polydispersity ($PDI = 0.62$) within 1 h of process time. Moreover, the inhomogeneity of the polymersome dispersion cannot be improved by longer process times. Even after 24 h, the distribution is still very broad ($PDI = 0.59$) (Data not shown). Thus, it becomes clear that the use of baffles is not beneficial for polymersome production at the liter-scale. For this reason, the inlet pipe of the polymer solution also acting like a baffle within the reactor, was pulled out of the reactor immediately after finishing the feeding process. By providing the highest (experimental set-up depending) possible feeding rate of 15 L/h, the desired polymer concentration of 1% w/v was reached within a few seconds only, thus undesired disturbance of the flow pattern within the reactor was minimized.

Using an unbaffled reactor at the liter-scale, only the two largest stirrer types (cf. Table 1 and Supporting Information Tables S4, S5 and S9 within the supporting information) with a dimensionless diameter of $d D^{-1} \geq 0.65$ can provide the required power consumption of 12.3 W/L ($h_S = 0.05 \text{ m}$). The resulting increase of the z -average within the first 15 min of the process is shown in Fig. 3D. With initial slopes of 7.7 nm/min (stirrer S4a) and 5.5 nm/min (stirrer S5) the vesicle production process occurs much faster at a constant power consumption $P V^{-1}$ compared to the scale-up strategy $u_{tip} = \text{const.}$ (cf. Section 3.3, initial slopes: 0.5–2.1 nm/min). However, compared to the reference process at the milliliter-scale (initial slope: 31.0 nm/min), it is still significantly slower. Thus, making use of $P V^{-1} = \text{const.}$

as a scale-up criterion is not suitable for the scale-up of the PMOXA₁₅-PDMS₆₈-PMOXA₁₅ polymersome production process.

A constant Newton number Ne , which can be calculated by Eq. (2) when the power consumption is well known, can also be used as scale-up strategy [34]. At the milliliter-scale, the S-stirrer reaches an almost constant Newton number Ne of about 0.9 in unbaffled reactors [48] in the turbulent flow region. At the liter-scale, the agitation speeds n causing $Ne = 0.9$ are lower than those causing the scale-up criterion $P V^{-1} = \text{const.}$, e.g. for S4a:

$$\begin{aligned} n (Ne = 0.9) &= 1484 \text{ rpm} < n (P V^{-1} = 12.3 \text{ W/L}) \\ &= 1612 \text{ rpm.} \end{aligned}$$

Thus, since the criterion $P V^{-1} = \text{const.}$ was shown to result in a too slow process (compared to the model process), and since it was found that lower agitation speeds n slow down the polymersome production process (cf. Section 3.2.), a constant Newton number as scale-up criterion can also not lead to success.

Beside the power consumption $P V^{-1}$ and the Newton number Ne , the maximum energy dissipation $\varepsilon_{\max}$ may also play an important role in terms of process scale-up. Especially in the presence of particles, which are sensitive towards shear forces, it can play a major role for a successful scale-up. The ratio of the maximum local energy dissipation to the power input per unit mass $\Phi = \frac{\varepsilon_{\max}}{\varepsilon_{\Phi}}$ [cf. Eqs. (5) and (6)] is a measure for the inhomogeneity of the introduced kinetic energy. Φ is equal to a value of 7.4 for the S-stirrer in an unbaffled milliliter-reactor [26,48]. Hence, at the established milliliter-scale process, the prevalent maximum energy dissipation $\varepsilon_{\max}$ is about 91.0 W/kg (at 20°C with $\rho_{\text{water}, 20^\circ\text{C}} = 1000 \text{ kg/m}^3$). At the liter-scale, $\varepsilon_{\max}$ can be estimated by eqs. (5)–(7) since the agitation speed depending volumetric power input $P V^{-1}$ is known. For both investigated stirrer types, the agitation speed n , leading to the same maximum energy dissipation at the liter-scale ($\varepsilon_{\max} = 91.0 \text{ W/kg}$) is much lower than those causing the same power input $P V^{-1}$ at both scales, e.g. for S4a:

$$\begin{aligned} n (\varepsilon_{\max} = 91.0 \text{ W/kg}) &= 643 \text{ rpm} \\ &< n (P V^{-1} = 12.3 \text{ W/L}) = 1612 \text{ rpm.} \end{aligned}$$

Consequently, with the same line of arguments as discussed before, it is shown that $\varepsilon_{\max}$ is not the crucial scale-up criterion either.

3.5 Scale-up based on the dimensionless froude number Fr

Driving centric arranged stirrers in unbaffled reactors leads to the formation of a considerably high vortex within the tank, even at relatively low agitation speeds. As soon as vortex building occurs, which is the case for the established polymersome production process at the milliliter-scale, the acceleration of gravity $g = 9.81 \text{ m/s}^2$ cannot be neglected but has to be considered by the dimensionless Froude number Fr . This dimensionless number can be calculated by Eq. (4) and is equal to a value of 6.52 for the model process. In the case of vortex occurrence the Froude number and the stirrer power characteristic are interdependent [34].

Table 2. Process characteristics when driving the S-stirrer (milliliter-scale) and the stirrer types S4a, S4b and S5 (liter-scale) (cf. Table 1) at $Fr = 6.52$

Stirrer Type	n [rpm]	u_{tip} [m s ⁻¹]	Re [-]	$P V^{-1}$ [W L ⁻¹]	Ne [-]	ε_{max} [W kg ⁻¹]
S-Stirrer	4000	3.02	13,824	12.3	0.9	91
S4a	1882	6.41	132,524	16.2	0.7	230
S4b	1882	6.41	132,524	13.9	0.6	200
S5	1600	7.54	216,000	17.1	0.3	545

Figure 4. (A) Dependence of the z -average on the process time when driving the stirrer types S4a and S5 and (B) the stirrer types S4a and S4b (cf. Table 1) at a Froude number of $Fr = 6.52$. The milliliter-system serves as a reference.

Only the stirrer types S4–S5 (cf. Table 1 and Supporting Information Tables S5, S7 and S9) were able to reach the requested power consumption in the unbaffled reactor (cf. Section 3.4) thanks to their relatively large dimensionless diameter of $d D^{-1} \geq 0.65$. Thus, only these stirrer types were considered for the scale-up by the aid of $Fr = 6.52$. In Table 2, the resulting important process characteristics are summarized for the stirrer types of both scales.

In Fig. 4A, the resulting linear increase of the z -averages within the first 3 min, indicating the speed of the vesicle production process when using stirrer S4a and S5 are shown. With a slope of 20.9 nm/min (S4a) and 19.1 nm/min (S5), the z -average increase occurs similarly fast for both stirrer types. The increase of the z -average when using stirrer S4a compared to S4b is shown in Fig. 4B. The two stirrer types only differ in the orientation of their blades, causing the upwards or downwards flow direction. The almost identical slopes of the resulting two curve progressions (20.8 nm/min when using stirrer S4a, and 21.2 nm/min when using stirrer S4b) make clear that the orientation of the axial flow has no influence on the polymersome production process. However, even more important is the fact that making use of the standard stirrers S4a, S4b and S5 at $Fr = 6.52$ leads to a very fast polymersome production, occurring only slightly slower than the model process at the milliliter-scale (initial slope: 31.0 nm min⁻¹).

The curve progressions of the mean particle size (z -average) and the polydispersity (PDI) within the whole process are shown in Fig. 5A and B, respectively. Both stirrer types (S4a and S5) show results that are very similar to the model process at the milliliter-scale. Starting from an initial value of about 30 nm the z -average increases very rapidly in the beginning of the process

to reach a stable value of about 180 nm within less than 30 min. In contrast, the polydispersity is rather high in the beginning of the process ($PDI > 0.6$) and decreases very quickly to reach a low PDI of < 0.2 within half an hour. Furthermore, no later than 30 min after process begin the polymersome dispersions are of the same quality at both scales. The mean particle size and the polydispersity show similar values when using stirrer S4a (z -average = 180 nm, $PDI = 0.17$) and S5 (z -average = 181 nm, $PDI = 0.19$) at the liter-scale compared to the results when using the S-stirrer at the milliliter-scale (z -average = 177 nm, $PDI = 0.18$). In addition, the resulting PSDs are a further quality criterion of the polymersome dispersion. They are monomodal, narrow and almost congruent at both scales, as can be seen in Fig. 5C. Moreover, the TEM image in Fig. 5D shows that the desired spherical vesicles but no other undesired structures were built. Since no undesired polymer structures or aggregates were found neither by TEM measurements nor by visual examination, we conclude that the polymer is completely transformed to polymer vesicles.

Summing up, the established efficient milliliter-scale polymersome production process in miniaturized stirred-tank reactors [26] was successfully scaled up to the liter-scale, based on solid process engineering parameters. Standard process equipment and the three standard propeller stirrers S4a, S4b and S5, causing tangential and different axial flow directions (upwards or downwards) and having considerably unlike geometries (diameter, height, and blade angle), were usable for efficient polymersome production at the large scale. This is a remarkable result, since the S-stirrer used at the small-scale has a very special geometry ($d D^{-1} = 0.72$, $h H^{-1} = 0.64$ and $h d^{-1} = 1.9$) far away from standard stirrer geometries. Thus, the linear scale-up of the

Figure 5. (A) Curve progression of the z-average and (B) the PDI within the whole process. (C) Resulting PSDs after 30 min of process time. The milliliter-system serves as a reference. (D) The exemplary TEM image (sampling after 90 min) shows that the desired vesicles were built. The scale bar corresponds to 200 nm.

process equipment which normally is one of the basic requirements when developing a scale-up strategy could not be fulfilled. Nevertheless, considering the two identified crucial scale-up criteria leads to a solid scale-up of the developed polymersome production process. Firstly, the dimensionless stirrer diameter $d D^{-1}$ has to be close to that of the S-stirrer in the miniaturized stirred-tank reactor ($d D^{-1} = 0.7$, cf. Table 1), which was fulfilled with $d D^{-1} \geq 0.65$. Thus, relatively high power consumptions can be provided even in unbaffled reactors. Second, the dimensionless Froude number has to be maintained at both scales. By realizing $Fr = 6.52$ the vortex building in unbaffled reactors and consequently the influence of the acceleration of gravity g are considered. In this way, equally fast vesicle production processes can be achieved although different sized propeller stirrers are used. At these flow conditions (resulting process characteristics are summarized in Table 2), the liter-scale polymersome production process is approximately as fast as the established model process at the milliliter scale. Polymersomes can be built very efficiently within about 1 h only, showing that the successful scale-up of the established polymersome production process from the milliliter (12 mL) to the liter-scale (1.5 L) was achieved.

4 Discussion

The first generation of artificial membrane vesicles was born in the early 60s, when liposome-technology experienced the first great boom. Despite the huge efforts that were done within the ensuing twenty years, with the exception of some applications (e.g. as additive in cosmetic products), liposome technology never achieved the expected success at the industrial scale. One of the main reasons for that is the liposomal membrane stability, which is much too low for most of the intended applications [49]. In the late 90s, the membrane vesicle technology experienced a revival, when polymer vesicles made of amphiphilic block copolymers were produced for the first time [12]. These new vesicles, called polymersomes, came up with some remarkable advantages: The polymeric membrane can score with higher stability, lower permeability and therefore better retention of encapsulated guest molecules. Since then, a huge diversity of seminal polymersome applications, especially in the medical and biotechnological field has been developed [2–4].

Although numerous polymersome preparation methods were developed following the established liposome production techniques, most of them are not suitable for industrial use (e.g. film

rehydration) [49]. Beside the standard vesicle production techniques, some extraordinary one-step polymersome production methods, making use of inkjet printers [16], microfluidic devices [17–20] or continuous working micromixers [21, 22] were developed, leading to an excellent vesicle size control. Superfocus Interdigital Micro Mixers (SFIMM) have been claimed to be usable at the production scale (100 L) and polymersome production at flow rates up to 0.58 L/h was shown e.g. by using a Caterpillar Micro Mixer (CPMM) or a Slit Interdigital Micro Mixer (SIMM-V2) [21]. In contrast, the throughput of microfluidic approaches is often limited to the μL range [24] enabling only horizontal scale-up strategies. In any case, the use of specialized equipment with unknown long-term reliability represents a substantial drawback. Moreover, independent of the achieved advancements the need to establish a scalable production process for polymersomes has been voiced within the last years [50, 51].

The analogy in between polymersomes and liposomes is obvious relating to almost any topic discussed to date about self-assembled vesicles. Consequently, it may seem logical that the challenge of transferring polymersome technology from the academic bench to the industrial scale is similar to what it was for liposome-technology about thirty years ago. Firstly, a good control of vesicle quality is important. Additionally, for an ideal process at the industrial scale, a minimum number of process steps and equipment is highly desirable [52]. However, without employing further unit operations, almost all currently available polymersome production methods lead to broad particle size distributions. The resulting polydispersity can be reduced by sonication [53], which requires a very high energy input [49] and is inappropriate for a scale-up. Sequential and multiple extrusion through several polycarbonate membranes with defined pore size can also be carried out to narrow the size distribution [53, 54]. Continuous high pressure extrusion is possible at the liter-scale [55, 56]. However, high pressure (> 100 bar) is needed and membrane clogging can cause technical limitations and product loss [49]. High shear homogenization enabled liposome production up to several hundred kilogram product per batch [57] and can even be carried out in a continuous process [58]. Nevertheless, a pressure up to 1000 bar is needed, leading to mechanical and abrasive stress of the equipment [59].

Liposomes can be produced in stirred-tank reactors at a large scale [49, 57–60]. They are produced most frequently by the ethanol injection method, leading to broad PSDs [59]. For this reason a homogenizer, increasing the technical requirements, is often used afterwards [57, 58]. The feasibility of industrial scale liposome production was shown, but the crucial process parameters for realizing the scale-up were not discussed or mentioned [57]. Within another study, the feasibility of a scale-up was shown by simply increasing the batch volume without identifying the process influencing key factors or the relevant scale-transfer criteria [60]. Besides, the needed process time to reach a certain liposome quality, which is essential when bearing product-costs in mind was not discussed [45]. However, although liposome production is possible within stirred-tank reactors at the industrial scale, to our knowledge, no studies about large-scale polymersome production in stirred-tank reactors are published to date. Since amphiphilic polymers show slower self-assembly behavior compared to amphiphilic lipids [61], and since polymersomes show considerably higher mechanical stability (e.g.

towards shear forces within a stirred-tank reactor) than liposomes [6], a certain liposome production process cannot simply be transferred one-to-one to get a successful polymersome production process. The vesicle quality influencing process parameters are not necessarily the same. This aspect becomes impressively clear when considering liposome production at a large scale published in 2014 by Charcosset et al. Here, stirring at 300 rpm was sufficient to achieve good vesicle quality [45], whereas even the agitation speed of 870 rpm was far too slow for polymersome production within our study. Moreover, no influence of the stirring rate on liposome size and size distribution was found by Kremer et al. in 1977 [49, 62].

The stirred-tank reactor has become the most frequently industrially used reactor within the last two centuries, resulting in the availability of standardized reactors and diverse standardized stirrer geometries. Available from the milliliter to the cubic meter-scale, stirred-tank reactors are the perfect candidates for vertical scale-up strategies. Within this work, this was manifested by the successful process engineering based scale-up of the formerly developed polymersome production process from the milliliter- to the liter-scale. For this purpose the same polymer was used at both scales, since the use of the same material with identical properties is a basic prerequisite for all scale-up strategies [34]. PMOX₁₅-PDMS₆₈-PDMS₁₅, known for its low cytotoxicity, and low nonspecific protein binding was chosen due to the possibility of functional transmembrane protein integration in membranes made of this polymer. However, the transferability of our considerations to other polymer systems is of great interest and is thus subject of our future studies. Since amphiphilic block copolymers of different chain lengths are reported to build polymersomes when vigorous stirring is applied [61] and since different block copolymers are able to form polymersomes via the ethanol method, we feel confident that our considerations are of great importance for further polymer systems. Other polymers will surely show different self-assembly kinetics and changed behavior towards, for instance, shear forces within the reactor. Nonetheless, the shown approach of scaling-up polymersome production in stirred-tank reactors can be a generally usable guideline. Thus, realizing a very simple and robust one-step process design at the liter-scale, where polymersomes can be produced very efficiently and in a controlled manner is a real boost towards polymersome technology polymersome technology.

5 Concluding remarks

The successful scale-up of the formerly developed efficient PMOX₁₅-PDMS₆₈-PMOX₁₅ vesicle production process in miniaturized stirred-tank reactors [26] to the liter-scale, based on process engineering parameters was demonstrated. The most frequently used reactor type, the stirred-tank reactor, equipped with cost-efficient standard process equipment was applied successfully. It was shown that uniform polymer vesicles with a mean diameter of about 180 nm and low polydispersity ($\text{PDI} < 0.2$) could be produced within 1 h. For this purpose, axial flow causing propeller stirrers are to be used in an unbaffled reactor. The scale-up of the established process from the milliliter (12 mL)

to the liter-scale (1.5 L) based on the process parameter stirrer circumferential speed u_{tip} , volumetric power input $P V^{-1}$, maximal energy dissipation ε_{max} , and based on the dimensionless Reynolds and Newton number Re , Ne did not succeed. In contrast, the use of propeller stirrers with a dimensionless diameter $d D^{-1} \geq 0.65$ at a constant Froude number $Fr = 6.52$ led to success. Thus, the successful scale-up of the milliliter-scale polymersome production process [26] by factor 125 was demonstrated. Furthermore, by considering the identified scale-up criteria, a further solid scale-up of the established efficient polymersome production process to the hundred liter-scale can be assured.

Practical application

Polymersomes have a considerably higher membrane stability than liposomes. Thanks to the increased stability, a huge diversity of potential polymersome applications, especially as carrier-systems in the medical field and as universal reaction compartments in the biotechnological field, were developed. Nevertheless, to make the polymersome-technology competitive at the industrial-scale, suitable methods to provide large vesicle quantities in a controlled and cost-effective manner have to be implemented. In this work, the successful scale-up of a formerly developed efficient polymersome production process in miniaturized stirred-tank reactors to the liter-scale is demonstrated. The use of standard propeller stirrers in an unbaffled stirred-tank reactor is sufficient for an efficient process, when the identified scale-up criteria are considered. The ratio of the stirrer diameter to the reactor diameter $d D^{-1}$ and the dimensionless Froude number Fr were found to be the key factors. Thus, uniform polymersomes with low polydispersity were achieved within about 1 h.

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Nomenclature

c_p	[% w/v]	polymer concentration
d	[m]	stirrer diameter
D	[m]	reactor diameter
g	[m/s ²]	acceleration of gravity

h	[m]	stirrer height
h_S	[m]	installation height of the stirrer
Fr	[-]	Froude number
H	[m]	filling level
n	[s ⁻¹]	agitation speed
Ne	[-]	Newton number
P	[W]	power
Re	[-]	Reynolds number
u_{tip}	[m/s ¹]	stirrer circumferential speed
V	[L]	reaction volume
$\dot{V}$	[L/h ¹]	feeding rate
z_i	[-]	number of stirrers fitted at the agitator shaft

Greek symbols

α	[°]	blade angle
ε_{max}	[W/kg]	maximum local energy dissipation
ε_Φ	[W/kg]	power input per unit mass
η	[kg m s ⁻¹]	dynamic viscosity
ρ	[kg/m ³]	density
Φ	[-]	ratio of maximum local energy dissipation to power input per unit mass

6 References

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