Journal of Applied Polymer Science

Efficient production of uniform nanometer-sized polymer vesicles in stirred-tank reactors

Sarah Theresa Poschenrieder, Sabine Gabriele Wagner, Kathrin Castiglione
Institute of Biochemical Engineering, Technische Universität München, Boltzmannstraße 15, Garching, D-85748, Germany
Correspondence to: K. Castiglione (E-mail: k.castiglione@lrz.tum.de)

ABSTRACT: Polymer vesicles, so-called polymersomes, gain more and more attention as potential carriers for medical and biotechnological applications. To put the production of these nanocompartments into action at an industrial scale, an efficient and scalable process has to be established. Moreover, being able to control the resulting particle size distribution (PSD) is vital. In this work, the amphiphilic triblock copolymer poly(2-methyloxazoline)$_{15}$–poly(dimethylsiloxane)$_{68}$–poly(2-methyloxazoline)$_{15}$ is formed into polymersomes in miniaturized stirred-tank reactors. Varying flow conditions have a huge impact on the resulting PSD. Dynamic light scattering measurements show that driving a S-shaped stirrer at 4000 rpm in unbaffled reactors leads to a monomodal PSD with a low polydispersity index (PDI<0.2). Vesicles with a mean diameter of 200 nm are achieved within less than 1 h in a single production step. The robustness of the established process is shown by producing uniform polymersomes at different temperatures and varying pH and buffer molarities. © 2015 Wiley Periodicals, Inc. J. Appl. Polym. Sci. **2016**, *133*, 43274.

KEYWORDS: copolymers; membranes; nanostructured polymers; self-assembly

Received 17 September 2015; accepted 25 November 2015
DOI: 10.1002/app.43274

INTRODUCTION

In recent years, the preparation of polymeric hollow structures at the nanoscale has found increasing interest. Polymer vesicles, so called polymersomes,[1] are made of self-assembling block copolymers with simple linear architecture that are arranged into well-organized layers.[2] Under certain conditions the colloidal assembly of the amphiphilic copolymer, including hydrophilic and hydrophobic segments, leads to the intended vesicular nanometer-sized compartments.[2–6] Because of their ability to encapsulate guest molecules into their interior, these vesicular structures can be used for a wide field of applications. Some important ones span from imaging of tissues *in vivo* using near-infrared emissive polymersomes[7] to anti-cancer drug delivery.[8,9] By loading polymersomes with enzymes they can also be turned into biocatalytically active nanoreactors.[10–12] For the application in biotechnology as well as for medical or industrial purposes a feasible control of the resulting vesicle size distribution is required. The distribution does not only depend on the nature of the polymer[5,6,13,14] and its molecular mass but can also be influenced by preparation methods and preparation conditions.[13,15,16]

So far polymer vesicles have been typically produced under inchoate laboratory conditions. Supposedly, the most simple vesicle-formation technique is the dropwise addition of a polymer solution to a vigorously stirred aqueous solution.[15,17] This barely reproducible procedure leads to vesicle dispersions with rather broad size distributions,[17,18] where both undesired micelles and vesicles coexist. To narrow the distribution, a multistep preparation procedure is inevitable. Several subsequent extrusion-steps through polycarbonate membranes with defined pore size are often necessary.[5,15,17] The main drawback of this method is the limited treatable vesicle dispersion volume.[19] High pressures up to 10.5 MPa are needed for vesicle extrusion at a large scale.[19–21] As micelles cannot be removed by extrusion, an additional time-consuming size exclusion chromatography is needed.

In the recent past, efforts have been made to develop a one-step polymersome preparation process where vesicle size can be controlled. Creative problem-solving approaches have been investigated such as using microfluidic devices[22] and modified commercial inkjet printers.[23] In both cases, the possibility of preparing polymersomes of narrow size distribution in a single step was demonstrated. Nevertheless, for industrial polymersome applications the development of techniques that control vesicle uniformity is essential[24] but not sufficient. Enabling an easy scale-up of the developed process is just as highly important.[12,25] Therefore, it was the major goal of this study to develop an efficient, reproducible, and scalable production process where vesicles of monomodal, narrow particle size distribution can be formed in a single production step.

For this study the amphiphilic triblock copolymer poly(2-methyl-oxazoline)$_{15}$–poly(dimethylsiloxane)$_{68}$–poly(2-methyloxazoline)$_{15}$ (PMOXA$_{15}$–PDMS$_{68}$–PMOXA$_{15}$) was chosen. Because of its good biocompatibility[26] and the low nonspecific protein-binding properties of the PMOXA-block[27] this polymer can be used for polymersome production in the medical and biotechnological field. Furthermore, it was shown that PMOXA–PDMS–PMOXA can be used to form nanoreactors. Several membrane proteins could successfully be integrated in polymer membranes formed by polymers of different block lengths.[28–32]

The stirred-tank reactor is still the most important production reactor in chemical and biotechnological industries. Having that fact in mind, polymersomes were formed in miniaturized stirred-tank reactors in this work. Performing vesicle formation on a low-volume scale (12 mL) and using a reaction block where up to 48 reactors can be used in parallel, enabled polymer-saving and very efficient investigations on polymersome production under diverse process conditions. Moreover, completely different flow conditions were generated by using four diverse stirrer geometries that were driven at varying agitation speeds in baffled and unbaffled reactors.

All investigations were done with the focus on producing polymersomes of monomodal and narrow particle size distribution (PSD). The polydispersity index (*PDI*) of the investigated polymersome dispersions, measured by dynamic light scattering, was chosen to be the most important quality criterion. This dimensionless number, ranging from 0 to 1, indicates the width of the investigated size distribution. The lower the *PDI*, the narrower the PSD. Monomodal, narrow particle size distributions are represented by low polydispersity indices ($PDI<0.2$). The mean diameter of the polymersome dispersion is given by the primary and most stable parameter of dynamic light scattering, the *z-average*. The *z-average* is the harmonic intensity averaged particle diameter.[33,34]

EXPERIMENTAL

Triblock Copolymer

PMOXA$_{15}$–PDMS$_{68}$–PMOXA$_{15}$ was purchased from Polymersource (Dorval QC, Canada). It has a molecular mass of 7600 g mol^{-1} ($M_{n,\ \mathrm{PMOXA}}$=1300 g mol^{-1}, $M_{n,\ \mathrm{PDMS}}$=5000 g mol^{-1}) and the degrees of polymerization are N(PMOXA)=15 and N(PDMS)=68. Its narrow molecular mass distribution is given by M_w/M_n=1.23 (M_w: mass-average molecular mass; M_n: number-average molecular mass).

Miniaturized Stirred-Tank Reactor System

The bioREACTOR 48 (2mag, Munich, Germany) was used for polymersome production. Up to 48 miniaturized stirred-tank reactors can operate simultaneously. The single-use reactors die-casted from poly-styrene (2mag AG, Munich, Germany) have a working volume of 8–15 mL. They are available with four baffles or unbaffled. Temperature control of the reaction medium is realized by a heat exchanger integrated in the reaction block. A reflux cooler realizes cooling of the headspace to reduce volume loss by evaporation. The cover of the reaction block is accessible for the tips of a liquid-handling system that enables automation of intermittent feeding of the ethanolic polymer solution.[35,36]

Vesicle Preparation via Direct Dispersion Method

Unless noted otherwise, 120 mg PMOXA$_{15}$–PDMS$_{68}$–PMOXA$_{15}$ powder (stored at −20°C) were dispersed in 12 mL of PBS (137 m*M* NaCl, 2.7 m*M* KCl, 12 m*M* PO_4^{3-}, *pH* 7.4) leading to a final concentration of 1% (w/v). Using baffled and unbaffled milliliter reactors and four different stirrer geometries the dispersion was stirred at 1000–4000 rpm and at 25°C for up to 48 h. All results and relating standard deviations derive from a three-fold determination.

Vesicle Preparation via Ethanol Method

Unless noted otherwise, a 20% (w/v) polymer solution was prepared by solving the polymer in ethanol (99.8%). For a final polymer concentration of 1% (w/v), 0.6 mL of the polymer solution were continuously or intermittently added into 11.4 mL of the aqueous solution. For this purpose, a commercial dosage pump (Reglo Analog MS 4-4/12, Ismatec, Idex Health & Science, Wertheim, Germany) or a liquid handler (Freedom EVO, Tecan, Männedorf, Switzerland) was used. Feeding rates ranging from 1.0 to 6.8 mL h^{-1} or from 0.25 to 10.00 mL h^{-1}, respectively, were realized. All results and relating standard deviations derive from a three-fold determination.

Dynamic Light Scattering Measurements

To avoid the occurrence of polymer aggregates that disturb the measurements, all samples were centrifuged for 6 min at 13,000 rpm (Mikro 20, Hettich, Tuttlingen, Germany). The supernatant was diluted 10-fold with the appropriate solute. Filtration or extrusion was not executed.

The *z-average*, the *PDI* and the intensity-based PSD were determined via dynamic light scattering measurements. For this purpose 0.5 mL of the prepared sample were filled into 10 mm single-use polycarbonate cells. All measurements were carried out with a Zetasizer NS (Malvern, Worcestershire, UK). This system is equipped with a red HeNe gas laser (633 nm) and a sensitive avalanche photodiode detector. To achieve an acceptable count rate the laser power was automatically attenuated and the measurement position within the system was automatically moved as well. To perform light-scattering at 25°C all samples were equilibrated for 60 s. Three measurements consisting of 10 runs each (10 s per run) were recorded.

Static Light Scattering Measurements

The molecular mass of the polymer vesicles prepared by the established ethanol method was determined via static light scattering measurements. All measurements were carried out as commissioned work by ALV (Langen, Germany). A commercial goniometer (ALV, Langen, Germany) equipped with a green frequency-doubled Nd-YAG laser (532 nm) was used. To fulfil the quality criterion of static light scattering measurements all samples were extruded three times through polycarbonate membranes with 0.2 µm pore diameter (Millipore, Billerica, MA, USA). Polymersome dispersions with polymer concentration of 2.0, 1.67, 1.0, and 0.5 mg mL^{-1} were analyzed at scattering angles between 20° and 150° (3 runs per scattering angle) and at 25°C. Data were extrapolated in a Berry plot to determine the average molecular mass of a single polymer vesicle. According to Nardin *et al.* (2000) the refractive index increment dn/dc of PMOXA–PDMS–PMOXA vesicles in water was defined to be 0.188 mL g^{-1}.[5]

Transmission Electron Microscopy

The polymersome dispersion was prepared in double-distilled water and diluted hundredfold. Afterwards, 2 μL of the sample were deposited on a plasma-treated carbon coated copper grid and were negatively stained with 2.5% uranyl acetate solution. Morphological examination of the polymersomes was performed using a JEM 100 CX (JEOL, Tokyo, Japan) operating at 100 kV acceleration voltage.

Cryo-Transmission Electron Microscopy

The vesicle dispersion was prepared in double-distilled water and diluted hundredfold. Afterwards, 2 μL of the sample were adsorbed for 30 s on a plasma-treated carbon coated copper grid, blotted with filter paper and frozen into liquid ethane (−178°C). Electron micrographs of the samples were recorded at an accelerating voltage of 120 kV using a JEM 2010 (JEOL, Tokyo, Japan).

STIRRER GEOMETRIES

Figure 1(a) shows the four stirrer geometries that were used to generate different flow conditions. The so-called gas-inducing stirrer (2mag, Munich, Germany), the paddle-shaped stirrer, the H- and the S-stirrer (all stirrers were developed in-house) can be driven in the reaction block with up to 48 miniaturized stirred-tank reactors (2mag, Munich, Germany).[35–44]

The cylindrical single-use reactors (height: 86 mm, inner diameter: 20 mm, working volume: 8–15 mL) are available with four evenly spread baffles (height: 59 mm, width: 2 mm) or unbaffled (2mag, Munich, Germany). By using a magnetic inductive drive, the stirrers can freely rotate on individual axes [Figure 1(b)] at controllable stirrer speeds from 100 rpm to their characteristic maximal agitation speed (Table I).

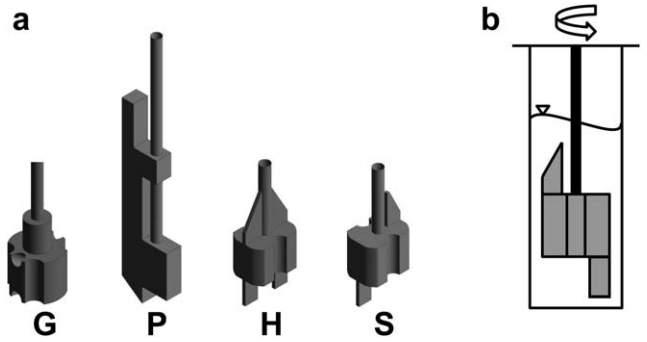

Figure 1. (a) Stirrers with four different geometries can be driven in the miniaturized stirred-tank reactors. The gas-inducing stirrer (G), the paddle-shaped stirrer (P), the H-stirrer (H), and the S-stirrer (S) are available. (b) By using a magnetic inductive drive, the stirrers can freely rotate on individual axes in the cylindrical reactors (height: 86 mm, inner diameter: 20 mm, working volume: 8–15 mL). Here, an unbaffled reactor is shown.

The gas-inducing impeller introduces gas bubbles into the medium. By sucking in the medium from the bottom a strong axial flow and high turbulences are generated when gas is simultaneously induced through a hollow shaft.[37,44] In case of using the one-sided paddle stirrer, a predominantly tangential flow path in the reactor can be observed. Here, a rotating lamella is formed which spreads out along the reactor wall. Power is distributed uniformly into the reaction medium.[40] The H- and the S-stirrer were developed primarily for mixing purposes of solids-bearing suspensions. Rotation of the H-stirrer causes mostly tangential but no axial flow. The S-shaped impeller, which is geometrically similar to the H-stirrer, causes an efficient mixing on the upper and lower side of the impeller. This stirrer type generates radial, axial and tangential flow.[41–43]

Table I. Key Data of the Four Stirrer Types

	Gas-inducing stirrer	Paddle stirrer	H-stirrer	S-stirrer
Working volume (mL)	8–15	8–12	10–14	10–14
Diameter d (mm)	14.5	16.2	14.4	14.4
Height h (mm)	8.0	57.0	29.0	27.5
Ratio of stirrer diameter to inner reactor diameter $\frac{d}{D}$	0.73	0.81	0.72	0.72
Ratio of stirrer height to reactor fill level $\frac{h}{H}$ (12 mL filling volume in unstirred and unbaffled reactors)	0.19	1.33	0.69	0.64
Maximum agitation speedin unbaffled reactors (rpm)	4000	2000	3000	4000
Ratio of maximal energy dissipation to mean power input $\varepsilon_{max}/P \cdot V^{-1}$	10[a]	6[b]	-[c]	7.4[b]
Flow Pattern	Strongly axial	Predominantly tangential	Predominantly tangential	Axial, radial, tangential
Compatible with baffled reactors	Yes	No	Yes	Yes

[a] In baffled reactors.
[b] In unbaffled reactors.
[c] Data not available.

Table I gives an overview of the most important characteristics of the four different stirrer types.

RESULTS

Similar to the production methods of lipid vesicles (liposomes), polymer vesicles can be produced via various methods. Generally, the preparation methods can be divided into solvent-free techniques and techniques making use of organic solvents.[17] In this study, the direct dispersion method (solvent-free) as well as the ethanol method (solvent-based) were used for polymersome production.

Since stress on particles occurs in the velocity field of stirred-tank reactors, the stirrer- and reactor type have crucial influence on particle behavior.[45] Therefore, the influence of different flow conditions on the vesicle production process was investigated for both, the direct dispersion and the ethanol method.

Direct Dispersion Method

Solvent-free vesicle production techniques are particularly important in the field of drug delivery or biotechnological applications where the presence of solvent traces may cause toxic effects. Certainly, the most simple solvent-free production method is the direct dispersion method. Therefore, $PMOXA_{15}$–$PDMS_{68}$–$PMOXA_{15}$ polymer powder was directly dispersed [1% (w/v)] in aqueous buffer [phosphate buffered saline (PBS), *pH* 7.4] at 25°C.

Completely different flow conditions were generated by driving the four impeller types [Figure 1(a)] at varying stirrer speeds in baffled and unbaffled reactors. In baffled reactors, stirrer speeds between 1000 and 2000 rpm were investigated. The only exception was the paddle-shaped stirrer, which is incompatible with this reactor type because of its geometry. In unbaffled reactors, the maximum agitation speed was dependent on the stirrer type as summarized in Table I. The resulting polymersome dispersions were analyzed after 4, 24, and 48 h process time.

Influence of Reactor Type and Agitation Speed. In general, using baffled reactors enables faster mixing of the reaction medium because of higher turbulences in the reactor. Therefore, using baffles should facilitate dispersing the solid polymer powder. Nevertheless, a lower polydispersity was always obtained when using reactors without baffles. Figure 2(a) exemplarily shows the obtained *PDI*s after 24 h when driving the four stirrer types at 2000 rpm in both reactor types. The respective PSDs when making use of the S-stirrer are shown in Figure 2(b).

A broad particle size distribution indicated by a rather high polydispersity (*PDI*=0.45) is the result of using this stirrer geometry in baffled reactors. In contrast, a clearly narrower PSD indicated by a lower polydispersity (*PDI*=0.23) was achieved in unbaffled reactors under the same conditions. Furthermore, the use of baffles led to the occurrence of some larger morphologies in the micrometer range. This also is indicated by a *z-average* of 267 nm in baffled reactors compared to 191 nm in unbaffled reactors (data not shown).

Summing up, regardless of the impeller type, the agitation speed and the process time, narrower PSDs were achieved in unbaffled reactors. Hence, to produce polymersomes, reactors without baffles should be used. Furthermore, independently of the used stirrer geometry, it was ascertained that higher stirrer speeds lead to lower *PDI*s [Figure 2(c)]. Therefore, providing low energy input by driving the impellers at low agitation speed does not lead to monomodal PSDs. In Figure 2(d) the resulting PSDs when driving the S-stirrer in reactors without baffles are shown. A monomodal size distribution with low polydispersity (*PDI*=0.14, *z-average*=193 nm) was achieved at 4000 rpm. In contrast, driving the impeller at 1000 rpm resulted in a very broad size distribution (*PDI*=0.56, *z-average*=347 nm).

Since driving the four different stirrer types, which induce completely different flow conditions, at the same agitation speed and in the same reactor type led to very uneven PSDs (Figure 3), it can be postulated that the prevalent flow pattern has an essential effect on the resulting vesicle dispersion quality. Moreover, it becomes clear that solely controlling agitation speed is not sufficient to control the resulting vesicle size distribution. Thus, a closer investigation of the stirrer characteristics in conjunction with the vesicle production process was required.

Influence of Stirrer Type. The gas-inducing stirrer provides good and fast mixing because of the strongly axial flow. Therefore, this impeller type was used to efficiently disperse the polymer powder in the aqueous phase. Though, because of the gas input, foam formation was observed. Solid polymer was found in abundance within the foam above the reaction medium and therefore could not be turned into polymer vesicles. Furthermore, the gas-inducing stirrer exhibits a high ratio of the maximal energy dissipation ε_{max} to the mean volumetric power input $P \cdot V^{-1}$. Having a value of 10 in baffled reactors[39] this stirrer type induces high shear forces which may destroy the vesicle membrane. For these reasons, using the gas-inducing stirrer for vesicle production purposes is not reasonable.

In contrast, the paddle-shaped stirrer, which predominantly induces tangential flow, was developed for biotechnological processes where microorganisms that are sensitive to shearing are to be used. With $\varepsilon_{max}/P \cdot V^{-1}=6$ it uniformly distributes the power in the reaction medium.[40] Therefore, this impeller type was assumed to be a good choice for the production of polymersomes that are expected to be sensitive towards shear forces. Within 24 h a monomodal PSD with low polydispersity (*PDI*=0.17) was achieved at 2000 rpm in unbaffled reactors [Figure 2(a)]. Nevertheless, the maximum agitation speed of this stirrer type is limited to 2000 rpm. As higher stirrer speeds were shown to affect lower *PDI*s [Figure 2(c)], the paddle-shaped stirrer was not the best choice either.

Polymersome dispersions with even narrower PSDs were achieved when using the H-stirrer and S-stirrer at their maximal possible agitation speed (3000 rpm and 4000 rpm) in unbaffled reactors. Monomodal, narrow size distributions with *PDI*s of only 0.13 and 0.14, respectively, were achieved within 24 h. The S-stirrer was found to be the best impeller for polymersome production in the miniaturized stirred-tank reactors because of the fact that the results obtained with this impeller had a slightly higher reproducibility than with the H-stirrer (data not shown).

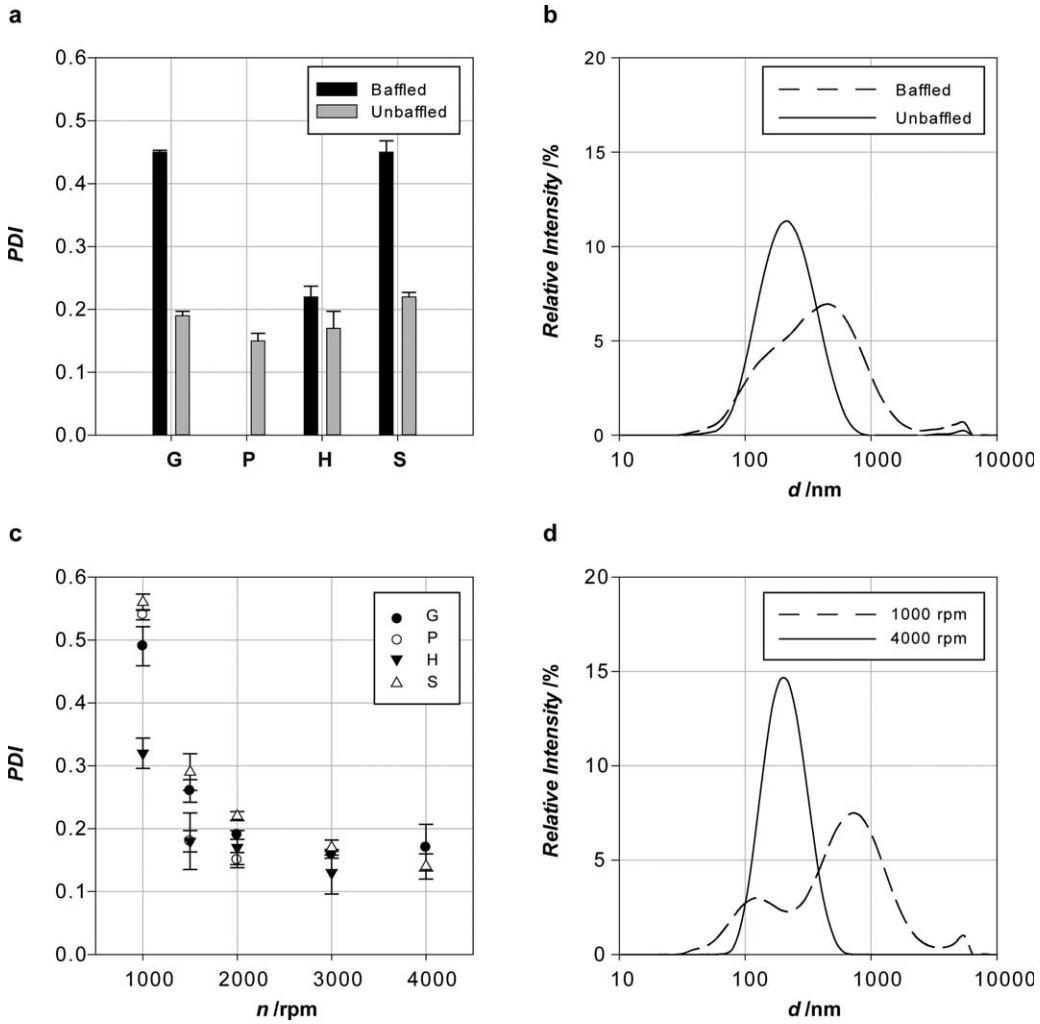

Figure 2. Direct dispersion method: (a) and (c) show the polydispersity indexes (PDIs) after 24 h process time when using the gas-inducing stirrer (G), the paddle-shaped stirrer (P) (can only be driven in unbaffled reactors), the H-stirrer (H), and the S-stirrer (S). In (b) and (d) the intensity-based particle size distributions (PSDs) when using the S-stirrer are shown. (a) Dependence of the PDI on the reactor type (baffled or unbaffled) when driving the stirrers at 2000 rpm. (b) Dependence of the PSDs on the reactor type (baffled or unbaffled) when driving the S-stirrer at 2000 rpm. (c) Dependence of the PDI on the agitation speed when using unbaffled reactors. (d) Dependence of the PSDs on the agitation speed when driving the S-stirrer in unbaffled reactors.

The S-stirrer generates axial, radial, and tangential flow and therefore provides very good mixing even in unbaffled reactors.[42] Because of its characteristic geometry (only two blades), the volumetric power input of this stirrer type is lower compared to the H-stirrer. It is about 12 W L^{-1} at its maximal agitation speed. With $\varepsilon_{max}/P \cdot V^{-1}=7.4$[43] the ratio of maximal energy dissipation to volumetric power input is only slightly above that of the low-shear paddle-shaped stirrer. Using this stirrer type at the maximum agitation speed led to narrow size distributions (*PDI*=0.17) within a very short time of only 4 h. For comparison, when driving the paddle-shaped stirrer, the same *PDI* was achieved 20 h later.

To sum up, in a single production step uniform polymersomes with a mean diameter of about 200 nm are rapidly producible via the direct dispersion method by using the S-stirrer at 4000 rpm in unbaffled reactors. Elongation of the process time up to 24 h and 48 h enables a slightly lower polydispersity (*PDI*=0.14 or rather 0.13).

Polymer Conversion. The results have shown that narrow PSDs can be achieved in miniaturized stirred-tank reactors. Nevertheless, despite enabling long process times, a complete conversion of the $PMOXA_{15}$–$PDMS_{68}$–$PMOXA_{15}$ powder to polymersomes could not be achieved. In any case 25–50% of the polymer remained in the form of undesired polymer residues (data not shown). Zhang and Eisenberg, who investigated the formation of polymersomes consisting of polystyrene-b-poly(acrylic acid) (PS-PAA) diblock-copolymers, postulated that the high fractions of the insoluble hydrophobic block of the amphiphilic block copolymer are the reason for that behavior.[46] Hence, direct dispersion of solid polymer powder is not suitable for efficient production of $PMOXA_{15}$–$PDMS_{68}$–$PMOXA_{15}$ vesicles. Previous dissolving of the block-copolymer in a common solvent was proposed to prepare vesicle dispersions with complete conversion.

Ethanol Method

To avoid the side effect of polymer residues when producing polymersomes by the method of direct dispersion, a solvent-

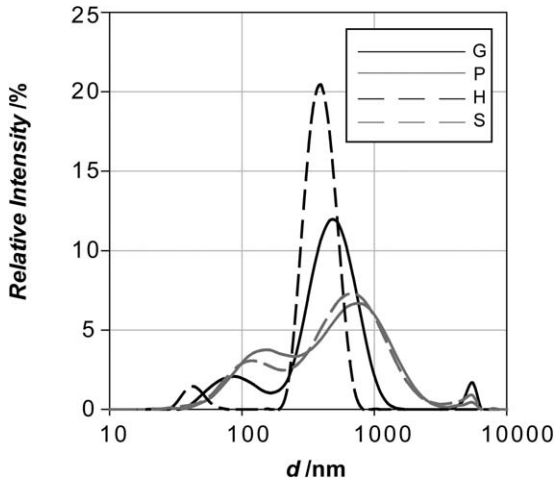

Figure 3. Direct dispersion method: The intensity-based particle size distributions (PSDs) after 4 h process time when using the gas-inducing stirrer (G), the paddle-shaped stirrer (P), the H-stirrer (H), and the S-stirrer (S) at 1000 rpm in unbaffled reactors are shown.

based technique was investigated. Ethanol was chosen to be the most suitable solvent. Because of its low toxicity, the already mentioned disadvantage of remaining solvent traces in the vesicle membrane was kept on a low level. Besides, an enormous advantage regarding the aspired realization of an industrial-scale vesicle production process also comes along with this method. The dosage of a liquid polymer solution can be realized much easier than the dosage of the cohesive solid polymer powder.

Based on the results obtained with the direct dispersion method, all further investigations were performed using the S-shaped stirrer in unbaffled reactors.

Efficient One-Step Polymersome Production. In order to enable a scalable process with defined and reproducible process conditions, the ethanolic polymer-solution was either continuously channeled directly into the reaction medium (PBS, *pH* 7.4, 25°C) using a conventional dosage pump, or an intermittent feeding was realized using a liquid handler. Here, by dosing 10 µL at each dosage step at varying dosage frequencies variable feeding rates were obtained.

High polymer concentrations in the feed provide the desired low ethanol residues in the final vesicle dispersions. In this context, it has to be mentioned that rising polymer concentration in the feed results in rising fluid viscosity (data not shown). As viscosity highly influences the feeding process, the polymer concentration was varied within the range of 5–50% (w/v). A polymer concentration of 20% (w/v) was found to offer a good compromise between realizing a low ethanol concentration in the final vesicle dispersion [5% (v/v) at 1% (w/v) final polymer concentration] and a fluid viscosity that enables a reliable dosage process. A 20% (w/v) polymer solution has a dynamic viscosity of 6.2 mPa s at 22°C.

In Figure 4 the achieved *PDI*s when feeding the polymer solution at feeding rates in the range of 1.0–6.8 mL h^{-1} using the pump and the liquid handler are shown. Significant dependence of the resulting polydispersity on the investigated feeding rates was not observed. Using the liquid handler led to slightly lower *PDI*s than feeding the polymer solution via the dosage pump. In any case narrow, monomodal PSDs and mean polymersome diameters of about 200 nm were achieved within 4 h. Thus, the possibility of using common dosage pumps for controllable polymer solution feeding was demonstrated. Nevertheless, for the reason of a higher degree of automation, the liquid handler was used for further investigations.

Having manufacturing costs in mind, process time is an important parameter when developing an efficient polymersome production process. Figure 5(a) clearly demonstrates that the elongation of the process time up to 24 h results in a decreasing polydispersity. A *PDI* of only 0.11 was achieved.

It is proposed that the continuous impact of moderate shear forces induced by the stirrer is a reason for decreasing *PDI*s. The longer the process time the more vesicle membranes can be destroyed by shearing forces. Since the particle size distribution predominantly is narrowed down from its right side [Figure 5(b)], larger vesicles are obviously more fragile than small ones. This behavior is not only reflected in a decreasing *PDI* but also in decreasing mean diameters. The *z-average* decreases from 220 nm after 1 h to 184 nm after 24 h.

It is worth mentioning that a process time longer than 4 h led to a small amount of undesired tiny polymer flocs. It seems likely that vesicle membrane segments of destroyed polymersomes are not able to re-assemble to new polymer vesicles but built undesired aggregating morphologies. It therefore is highly reasonable to shorten the process time as much as possible. By implementing a short process, low energy requirements and complete conversion of polymer to polymersomes can be achieved all-in-one.

Since feeding the polymer solution may be time consuming, the influence of different feeding rates was studied closer in the

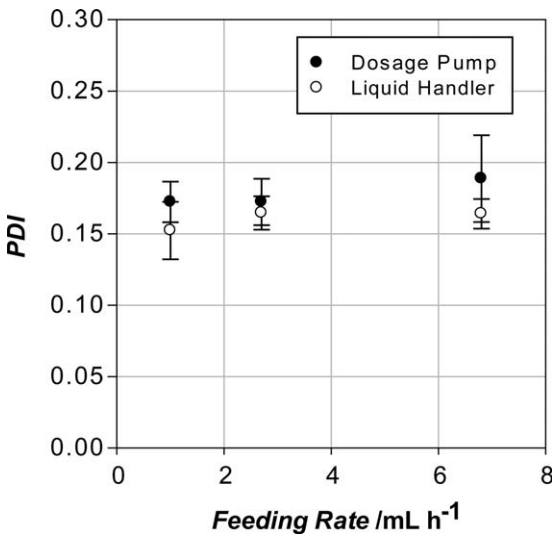

Figure 4. Ethanol method: The dependence of the polydispersity indexes (PDIs) after 4 h process time on different feeding rates realized by using the dosage pump and the liquid handler when making use of the S-stirrer at 3000 rpm in unbaffled reactors is shown.

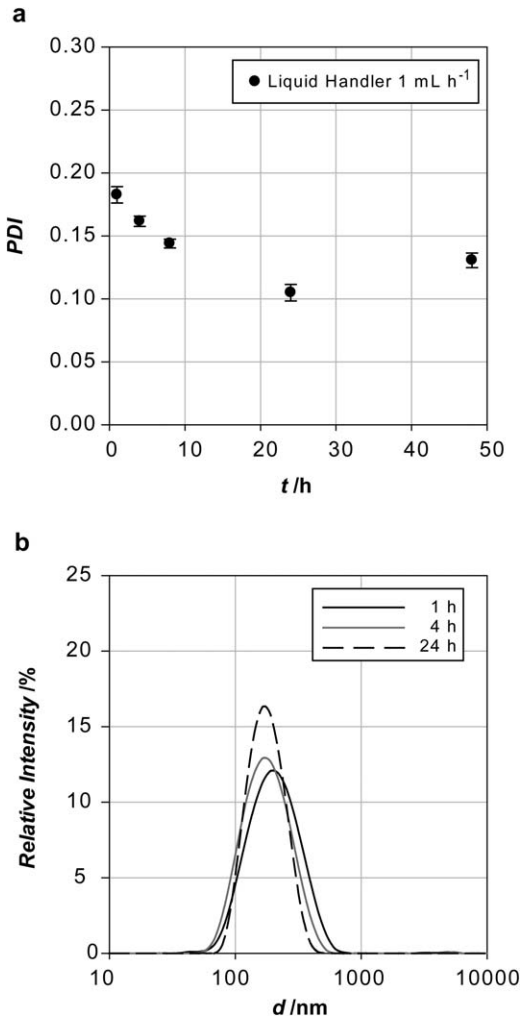

Figure 5. Ethanol method: (a) The polydispersity indexes (PDIs) and (b) the intensity-based particle size distributions (PSDs) when using the S-stirrer at 4000 rpm in unbaffled reactors are shown. (a) Dependence of the PDI on the process time when realizing 1 mL h^{-1} feeding rate by using the liquid handler. (b) Dependence of the respective PSD on the process time.

course of the process. Using the liquid handler, feeding rates from 0.25 to 10 mL h^{-1} were realized. As soon as the final polymer concentration was reached, the feed was stopped and the process was investigated for another 4 h. Figure 6 shows that the established polymersome production process is quite resistant in terms of different feeding rates. Uniform polymersomes of about 200 nm in mean diameter and *PDI*s lower than 0.2 were achieved within only 1 h when adding the polymer solution at 0.5–10 mL h^{-1}. Only the lowest investigated feeding rate of 0.25 mL h^{-1} led to polymersome dispersions with a bimodal PSD and undesired micelles (not shown), which is reflected in *PDI*s>0.2. High feeding rates result in shorter process times which are greatly desirable for economic reasons.

Robustness of the Established Production Process under Different Conditions. To scrutinize the established polymersome production process, polymer vesicles were produced under diverse conditions. For this purpose, the final polymer concentration, the process temperature, the *pH*, and the buffer molarity were varied.

With the aim to achieve an efficient polymersome production process, including high quality *and* high quantity, high final polymersome concentrations are in general desirable. Therefore, the final polymer concentration was varied within 0.5–5.0% (w/v). Investigations whether high polymer concentrations lead to a high quantity of uniform polymersomes with narrow PSD were performed. Figure 7(a) shows that this was not the case. The polydispersity of the resulting vesicle dispersion increased with higher polymer concentrations. 5% (w/v) final polymer concentration results in a bimodal PSD and the formation of undesired micelles (data not shown). Therefore, $PDMS_{15}$–$PMOXA_{68}$–$PDMS_{15}$ vesicles should be produced with 0.5–1.0% (w/v) final polymer concentration.

The possibility of producing polymersomes at different temperatures is very important particularly if biotechnological processes ought to be realized. Here, participating enzymes may exhibit completely different temperature stabilities. Therefore, the polymer vesicle production should be feasible in a wide range of temperatures. Hence, polymersomes were produced at 8°C, 25°C, and 40°C. In Figure 7(b) it is shown that vesicles of low polydispersity (*PDI*<0.2) can be achieved within only 0.5 h at 25°C and 40°C. The process time has to be extended to 1 h when producing polymersomes at 8°C. Probably the reaction kinetics of the polymer self-assembling process is delayed at low temperatures.

The feasibility of building vesicles at varying *pH* and molarity is similarly important for biotechnological and medical applications. The *pH* of the used buffer was varied within *pH* 5 and *pH* 8 [Figure 7(c)], whereas the molarity was adjusted to 0–100 m*M* [Figure 7(d)]. In any case uniform vesicles with *PDI*<0.2 were achieved within only 0.5–1.0 h.

Summing up, the convenience of the established polymersome production process for a wide field of applications can be assumed.

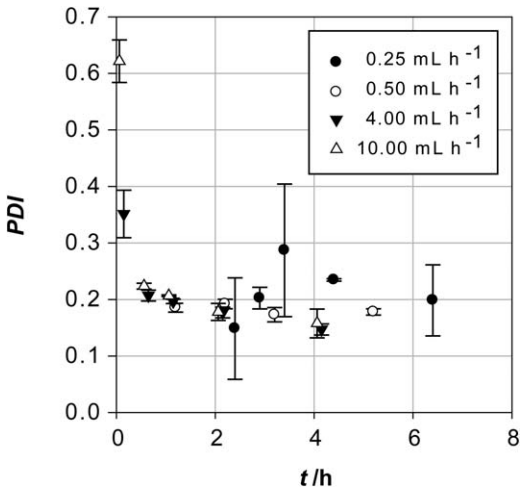

Figure 6. Ethanol method: The dependence of the polydispersity indexes (PDIs) on process time when driving the S-stirrer at 4000 rpm in unbaffled reactors and when realizing 0.25–10.0 mL h^{-1} feeding rate by using the liquid handler is shown.

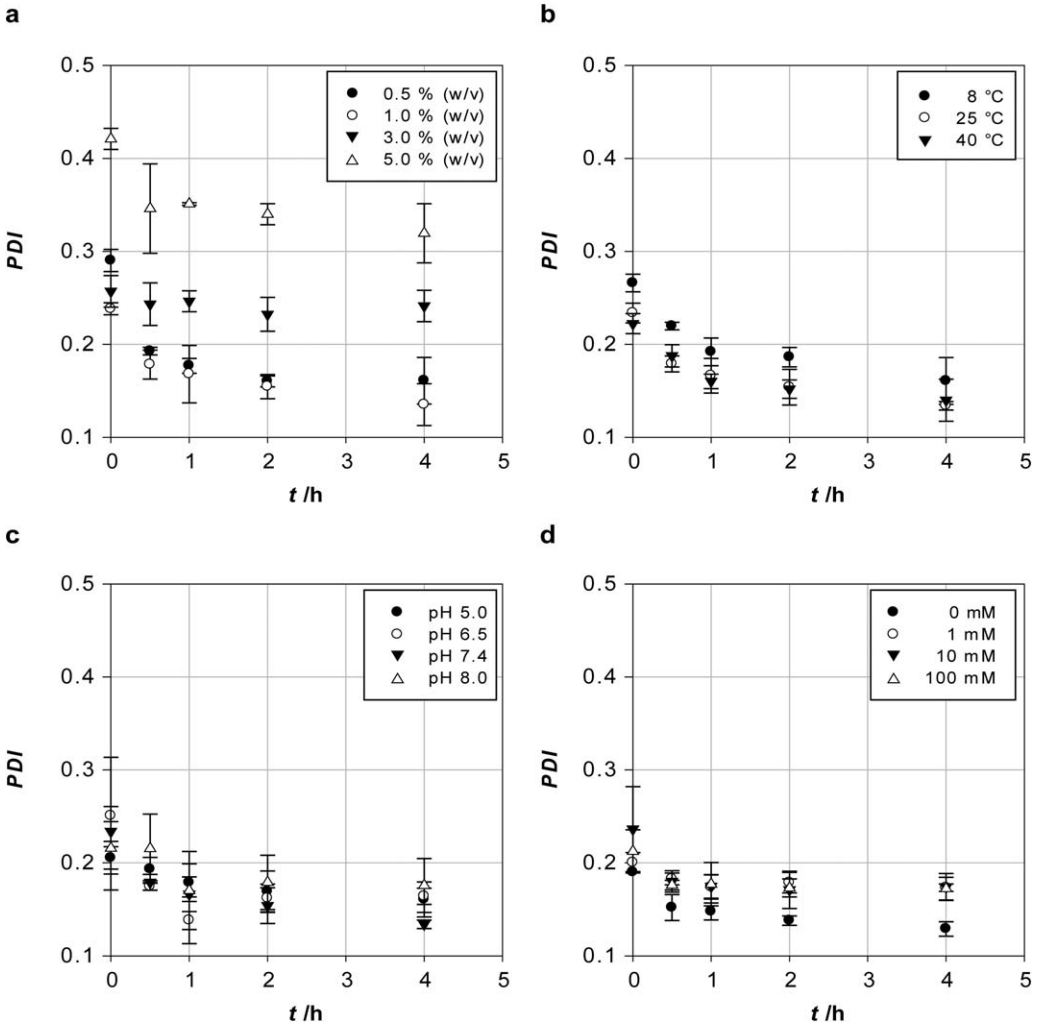

Figure 7. Ethanol method: The polydispersity indexes (PDIs) when using the S-stirrer at 4000 rpm in unbaffled reactors and realizing a feeding rate of 1 mL h^{-1} by using the liquid handler are shown in the course of the process. Dependence on (a) the final polymer concentration, (b) the process temperature, (c) the pH, and (d) the buffer molarity.

Characterization of the Polymer Vesicles. In addition to the use of dynamic light scattering measurements, polymersomes produced by the established efficient single-step production process were also analyzed by static light scattering. Using this technology permits the determination of the average molecular mass of a single vesicle. Investigating a polymersome dispersion with a narrow particle size distribution (*PDI*=0.15, *z-average*=166 nm), it was found to be 3.266×10^8 g mol^{-1}. The aggregation number of a single polymersome N_{agg} that indicates the number of polymer chains forming one vesicle was determined to be 43,000.

Imaging techniques clearly show that using the established polymersome production process, polymer vesicles are formed exclusively. No worm micelles or other undesired morphologies were observed when making use of transmission electron microscopy (TEM) [Figure 8(a,b)]. The membrane thickness of these polymersomes was determined by cryo-TEM. An even membrane of about 14 nm in thickness [Figure 8(c)] was apparent. At 4°C and at room temperature these polymersomes are stable in size for at least one year.

DISCUSSION

Within the last years, a huge diversity of seminal polymersome applications especially in the medical and biotechnological field has been developed.[7–12] Nevertheless, regardless of the field of application, polymer vesicles were mostly produced under inchoate process conditions at the laboratory scale. For this reason, the need for establishing a scalable polymersome production process where vesicle size can be controlled was requested earlier.[12,25]

To achieve uniform polymersomes, two extraordinary one-step vesicle production techniques were established in the group of Stephan Förster. In 2005, Hauschild *et al.* demonstrated that polymersomes with a narrow size distribution can be formed using modified common inkjet printers. By "printing" an ethanolic poly(2-vinyl-pyridine-ethylenglycol) (P2VP-PEG) solution into a stirred aqueous solution a very good control of vesicle size and shape as well as high reproducibility were achieved.[23] Five years later, the members of the same group showed that a microfluidic device can be used to control the size of P2VP-

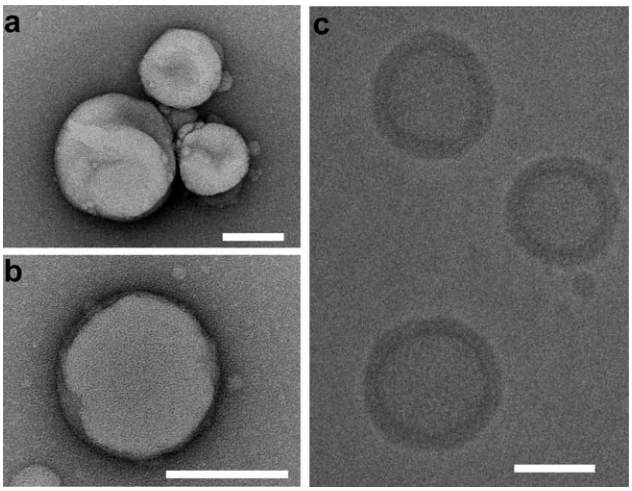

Figure 8. Ethanol method: (a,b) TEM and (c) cryo-TEM micrographs of vesicles prepared from $PMOXA_{15}$–$PDMS_{68}$–$PMOXA_{15}$ by the established polymersome production method. The scale bar corresponds to 100 nm.

PEG based polymersomes. By varying the volumetric flow ratio of two water streams and a single polymer solution stream in a crosswise microchannel system, monomodal, well-defined size distributions and the adjustment of vesicle size over a wide range (40 nm to 2 μm) were realized.[22] With a final polymer concentration of <0.01% (w/v) the polymersome gain was significantly lower when using the microfluidic device compared to making use of the inject printer, where polymer concentrations up to 0.65% (w/v) were reached.[22,23] Whereas very good vesicle size control and high reproducibility were shown for both techniques, the scalability of these two systems is restricted to a horizontal scale-up by parallelization (scale-out). Because the small dimensions of microfluidic devices make them more susceptible for undesired effects like fouling or clogging, their long-time performance has to be investigated on a case by case basis in the context of the respective process. From this it follows that the perceived risk associated with the replacement of existing classical reactors by other technologies represents a big hurdle for horizontal scale-up strategies and a vertical scale-up is usually preferred .[47,48]

The stirred-tank reactor has become the most frequently industrially used reactor within the last two centuries. Since 1855, when Thomson first investigated the power input concerning stirring tasks, impellers, and stirred-tank reactors have continuously been developed and characterized.[49] Much research has already been done, resulting in the availability of standardized reactors and various standardized impeller types. Moreover, since stirred-tank reactors are available from the milliliter-scale to the cubic meter-scale, they are the perfect candidates for vertical scale-up purposes.

So far, solely the use of $PMOXA_{15}$–$PDMS_{68}$–$PMOXA_{15}$ was investigated. Since the vesicle formation phenomena from several amphiphilic block-copolymers were found to run through similar pathways,[50] it can be supposed that the established process can also be used to produce uniform polymersomes from different polymers.

CONCLUSIONS

It was demonstrated that $PMOXA_{15}$–$PDMS_{68}$–$PMOXA_{15}$ vesicles could efficiently be formed in miniaturized stirred-tank reactors. Hereby, the prevalent flow pattern has a huge impact on the resulting vesicle size distribution. Using unbaffled reactors and driving the S-shaped stirrer, which induces radial, axial, and tangential flow, at 4000 rpm (12 W L^{-1}) enables a reproducible production of uniform polymersomes. Controllable feeding of an ethanolic polymer solution into the stirred aqueous phase was provided by applying either a common dosage pump or a pipetting robot. Monomodal and narrow vesicle size distributions with low polydispersity ($PDI<0.2$) were achieved in a single production step within less than 1 h. Furthermore, it was shown that by applying the established process, a 1% (w/v) vesicle dispersion with low polydispersity can be achieved at a wide range of temperature (8–40°C), *pH* (*pH* 5–8) and buffer molarity (0–100 m*M*).

With respect to the desired industrial applications of polymer vesicles, a scale-up of the polymersome production process is required. Engineering characteristics of the miniaturized stirred-tank reactors are very similar to industrially used large-scale stirred-tank reactors. Besides, important stirrer characteristics such as mixing time, volumetric power input, and maximal energy dissipation are well known for the established process. Moreover, using the miniaturized stirred-tank reactors the successful scale-up and scale-down of diverse biotechnological processes have already been shown.[35,51,52] A robust vertical scale-up of the established polymersome production process to the liter-scale can therefore be assured. Thus, an important step toward industrial usability of the polymersome technology is taken.

CONTRIBUTION OF AUTHOR

All listed authors, namely S. T. Poschenrieder, S. G. Wagner and K. Castiglione, meet the requested three author criteria. S. T. Poschenrieder and S. G. Wagner performed the experimental work and the interpretation of data. All authors designed the experiments and interpreted the results. S. T. Poschenrieder and K. Castiglione wrote the manuscript. All authors contributed to research design, critically revised the manuscript and approved it before submission.

ACKNOWLEDGMENTS

The support of this work by Prof. Dirk Weuster-Botz and the possibility of using the outstanding technical facilities at his Institute of Biochemical Engineering in Garching (Technische Universität München) are gratefully acknowledged. The authors are grateful to Dr. Marianne Hanzlik (Department of Chemistry, Technische Universität München) for carrying out the TEM and cryo-TEM measurements. The TUM Graduate School is acknowledged as well. This research project is funded by the German Federal Ministry of Education and Research (project funding reference number 031A178).

REFERENCES

1. Discher, D. E.; Ahmed, F. *Annu. Rev. Biomed. Eng.* **2006**, *8*, 323.
2. Nardin, C.; Widmer, J.; Winterhalter, M.; Meier, W. *Eur. Phys. J. E* **2001**, *4*, 403.

3. Zhang, L. F.; Eisenberg, A. *Science* **1995**, *268*, 1728.
4. Jenekhe, S. A.; Chen, X. L. *Science* **1998**, *279*, 1903.
5. Nardin, C.; Hirt, T.; Leukel, J.; Meier, W. *Langmuir* **2000**, *16*, 1035.
6. Soo, P. L.; Eisenberg, A. *J. Polym. Sci. Polym. Phys.* **2004**, *42*, 923.
7. Ghoroghchian, P. P.; Frail, P. R.; Susumu, K.; Blessington, D.; Brannan, A. K.; Bates, F. S.; Chance, B.; Hammer, D. A.; Therien, M. *J. Proc. Natl. Acad. Sci. U.S.A.* **2005**, *102*, 2922.
8. Meng, F. H.; Zhong, Z. Y.; Feijen, J. *Biomacromolecules* **2009**, *10*, 197.
9. Cheng, Z. L.; Tsourkas, A. *Langmuir* **2008**, *24*, 8169.
10. Winterhalter, M.; Hilty, C.; Bezrukov, S. M.; Nardin, C.; Meier, W.; Fournier, D. *Talanta* **2001**, *55*, 965.
11. De Vocht, C.; Ranquin, A.; Willaert, R.; Van Ginderachter, J. A.; Vanhaecke, T.; Rogiers, V.; Versees, W.; Van Gelder, P.; Steyaert, J. *J. Control. Release* **2009**, *137*, 246.
12. Nallani, M.; Benito, S.; Onaca, O.; Graff, A.; Lindemann, M.; Winterhalter, M.; Meier, W.; Schwaneberg, U. *J. Biotechnol.* **2006**, *123*, 50.
13. Discher, B. M. *Science* **1999**, *284*, 1143.
14. Discher, D. E.; Eisenberg, A. *Science* **2002**, *297*, 967.
15. Nardin, C.; Meier, W. G. *Chimia* **2001**, *55*, 142.
16. Antonietti, M.; Förster, S. *Adv. Mater.* **2003**, *15*, 1323.
17. Kita-Tokarczyk, K.; Grumelard, J.; Haefele, T.; Meier, W. *Polymer* **2005**, *46*, 3540.
18. Nardin, C.; Winterhalter, M.; Meier, W. *Langmuir* **2000**, *16*, 7708.
19. Rameez, S.; Bamba, I.; Palmer, A. F. *Langmuir* **2010**, *26*, 5279.
20. Schneider, T.; Sachse, A.; Rossling, G.; Brandl, M. *Drug. Dev. Ind. Pharm.* **1994**, *20*, 2787.
21. Schneider, M.; Sachse, M.; Rossling, A. G. *Int. J. Pharm.* **1995**, *117*, 1.
22. Thiele, J.; Steinhauser, D.; Pfohl, T.; Förster, S. *Langmuir* **2010**, *26*, 6860.
23. Hauschild, S.; Lipprandt, U.; Rumplecker, A.; Borchert, U.; Rank, A.; Schubert, R. *Small* **2005**, *1*, 1177.
24. Brown, L.; McArthur, S. L.; Wright, P. C.; Lewis, A.; Battaglia, G. *Lab. Chip* **2010**, *10*, 1922.
25. Van Rijn, P.; Tutus, M.; Kathrein, C.; Zhu, L.; Wessling, M.; Schwaneberg, U.; Boker, A. *Chem. Soc. Rev.* **2013**, *42*, 6578.
26. Broz, P.; Benito, S. M.; Saw, C.; Burger, P.; Heider, H.; Pfisterer, M.; Marsch, S.; Meier, W.; Hunziker, P. *J. Control. Release* **2005**, *102*, 475.
27. Woodle, M. C.; Engbers, C. M.; Zalipsky, S. *Bioconjug. Chem.* **1994**, *5*, 493.
28. Wong, D.; Jeon, T. J.; Schmidt, J. *Nanotechnology* **2006**, *17*, 3710.
29. Nardin, C.; Thoeni, S.; Widmer, J.; Winterhalter, M.; Meier, W. *Chem. Commun.* **2000**, 1433.
30. Graff, A.; Fraysse-Ailhas, C.; Palivan, C. G.; Grzelakowski, M.; Friedrich, T.; Vebert, C.; Gescheidt, G.; Meier, W. *Macromol. Chem. Phys.* **2010**, *211*, 229.
31. Kumar, M.; Grzelakowski, M.; Zilles, J.; Clark, M.; Meier, W. *Natl. Acad. Sci. U.S.A.* **2007**, *104*, 20719.
32. Ranquin, A.; Versees, W.; Meier, W.; Steyaert, J.; Van Gelder, P. *Nano Lett.* **2005**, *5*, 2220.
33. Koppel, D. E. *J. Chem. Phys.* **1972**, *57*, 4814.
34. Thomas, J. C. *J. Colloid Interface Sci.* **1987**, *117*, 187.
35. Kusterer, A.; Krause, C.; Kaufmann, K.; Arnold, M.; Weuster-Botz, D. *Bioprocess Biosyst. Eng.* **2008**, *31*, 207.
36. Weuster-Botz, D.; Puskeiler, R.; Kusterer, A.; Kaufmann, K.; John, G. T.; Arnold, M. *Bioprocess Biosyst. Eng.* **2005**, *28*, 109.
37. Puskeiler, R.; Kaufmann, K.; Weuster-Botz, D. *Biotechnol. Bioeng.* **2005**, *89*, 512.
38. Hortsch, R.; Weuster-Botz, D. *Adv. Appl. Microbiol.* **2010**, *73*, 61.
39. Hortsch, R.; Weuster-Botz, D. *Biotechnol. Prog.* **2010**, *26*, 595.
40. Hortsch, R.; Stratmann, A.; Weuster-Botz, D. *Biotechnol. Bioeng.* **2010**, *106*, 443.
41. Riedlberger, P.; Weuster-Botz, D. *Bioresour. Technol.* **2012**, *106106*, 138.
42. Riedlberger, P.; Brüning, S.; Weuster-Botz, D. *Bioprocess Biosyst. Eng.* **2013**, *36*, 927.
43. Riedlberger, P. Miniaturisierte Rührreaktoren zur enzymatischen Hydrolyse von suspendierten Pflanzenmaterialien. Ph.D. Thesis, Technische Universität München, June, **2012**.
44. Puskeiler, R.; Weuster-Botz, D. *Chem. Ing. Tech.* **2004**, *76*, 1815.
45. Henzler, H. J. In Advances in Biochemical Engineering/Biotechnology; Scheper, T., Ed.; Springer-Verlag: Berlin, Heidelberg, New York, **2000**; Vol. *67*, Particle stress in bioreactors, p 35.
46. Zhang, L. F.; Eisenberg, A. *Polym. Adv. Technol.* **1998**, *9*, 677.
47. Dudukovic, M. P. *Chem. Ing. Sci.* **2010**, *65*, 3.
48. Dudukovic, M. P. *Ind. Eng. Chem. Res.* **2007**, *46*, 8674.
49. Thomson, J. *Proc. Roy. Soc. Lond.* **1854−1855**, *7*, 509.
50. Du, J. Z.; O'Reilly, R. K. *Soft Matter* **2009**, *5*, 3544.
51. Knorr, B.; Schlieker, H.; Hohmann, H. P.; Weuster-Botz, D. *Biochem. Eng. J.* **2007**, *33*, 263.
52. Hoefel, T.; Faust, G.; Reinecke, L.; Rudinger, N.; Weuster-Botz, D. *Biotechnol. J.* **2012**, *7*, 1277.