Effect of Detergents on Morphology, Size Distribution, and Concentration of Copolymer-Based Polymersomes

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Title: *Effect of detergents on morphology, size distribution and concentration of copolymer-based polymersomes*

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Abstract

Polymersomes made of amphiphilic diblock copolymers are generally regarded as having higher physical and chemical stability than liposomes composed of phospholipids. This enhanced stability arises from the higher molecular weight of polymer constituents. Despite their increased stability, polymer bilayers are solubilized by detergents in a similar manner to lipid bilayers. In this work, we evaluated the stability of poly(ethylene glycol)-block-poly(ϵ -caprolactone) (PEG-PCL) based polymersomes exposed to three different detergents: N-Octyl- β -D-Glucopyranoside (OG), Lauryldimethylamine N-oxide (LDAO), and Triton X-100 (TX-100). Changes in morphology, particle size distribution, and concentrations of the polymersomes were evaluated during the titration of the detergents into the polymersome solutions. Furthermore, we discussed the effect of detergent features on solubilization of the polymeric bilayer and compared it to the results reported in the literature for liposomes and polymersomes. This information can be used for tuning the properties of PEG-PCL polymersomes for use in applications such as drug delivery or protein reconstitution studies.

Introduction

Amphiphilic diblock and triblock copolymer macromolecules, comprising of hydrophilic and hydrophobic blocks, are known to assemble into supramolecular structures such as micelles, polymer vesicles (polymersomes), rod-like micelles, or planar membranes.^{1,2} There is a strong interest to use these supramolecular assemblies in various research and industrial purposes as they closely mimic their biological equivalents, amphiphilic phospholipids and their self-assemblies (liposomes). Targeted and controlled release of an encapsulated drug payload is the most commonly studied medical application, but polymersomes are also used to study the reconstitution of transmembrane proteins within the membrane layer of the assembly for the preparation of selective separation devices and sensors^{1,3-9}. Polymersomes are a promising nanoscale assembly for targeted therapy and treatment of severe disorders, including cancer², due to their improved stability in comparison to micellar structures¹⁰ and liposomes¹. The superior chemical and physical stability of polymersomes over liposomes is due to the higher molecular weight (MW) of amphiphilic block copolymer chains, which form a thicker and more rigid bilayer^{1,11} when they self-assemble into vesicles.

Another type of amphiphilic molecule – detergents – can be introduced to the liposome or polymersome solution to change the properties of the self-assemblies; such as morphology, bilayer permeability, and particle size distribution^{9,12,13}. Despite the enhanced stability of polymersomes, higher membrane viscosity, and a larger bending modulus (as compared to liposomes¹⁴⁻¹⁷), polymersomes can also be solubilized by detergents. The detergent molecules are inserted into the bilayer structure, which causes a change in the amphiphile packing, rearrangement of the bilayer curvature, and ultimately, at a high enough detergent concentration, complete disassembly of the vesicles^{9,14,18,19}.

Several bilayer solubilization mechanisms have been described, and while they all have minor variations, the general consensus is that the solubilization is a three stage process^{19–22}. In the first stage, the detergents migrate into the bilayer, resulting in an increase in vesicle size. In the second stage, when the saturation point of detergents in the bilayer is achieved, the transition from vesicular to mixed micellar structures is initiated. In the third stage of solubilization, a complete transition of the vesicles to mixed micelles, consisting of amphiphiles and detergents, takes place and further addition of detergent only leads to the reduction of the phospholipid/polymer concentration within the mixed micelles^{9,19,20,22–24}.

Multiple studies investigating the mechanism of liposome solubilization have shown that the concentration of detergents required to solubilize liposome vesicles is dependent on two factors, including (1) the composition of the phospholipids used to construct the liposomal lipid bilayer, and (2) the type of detergent used for solubilization^{9,14,27–29,18,20–26}. In contrast to published studies regarding solubilization of liposomes, there is a limited number of publications that focus on the explanation of the solubilization effect on the block copolymer-based vesicle systems^{30,31}. Moreover, the studies are often limited to optical density (OD) measurements and dynamic light scattering (DLS) analysis²⁶, with visualization made on giant unilamellar vesicles^{24,30,32}. DLS is known to have significant drawbacks for proper analysis of polydisperse solutions, as occurs with vesicles mixed with detergents^{33–38}. Therefore, DLS analysis as a stand-alone method only partially describes the processes of vesicle solubilization and does not provide any information on particle concentrations or changes in vesicle morphology. As a result, conclusions based solely on DLS measurements are often inaccurate and vague. Very few studies complement OD and DLS analysis of nanostructures with cryogenic transmission electron microscopy (cryoTEM), which allows the visualization of the changing morphologies of the self-assemblies^{9,21,27,39}. However, when the

concentrations of the studied particles drop, it becomes more difficult to visualize the system properly, as very few of the particles will be retained on the TEM grid. Additionally, cryoTEM provides only qualitative and not quantitative information about the particle size distribution and concentrations, thus, only partially explaining the solubilization mechanism⁹. In the most detailed study on detergent solubilization of polymeric bilayers, Pata *et al.* investigated the solubilization of polymeric membranes made of poly(ethylene glycol)-*block*-poly(ethylene ethylene) (PEG-PEE) and poly(ethylene glycol)-*block*-poly(butadiene) (PEG-PB) block copolymers with Triton X-100 (TX-100)³⁰. The authors based their conclusions on OD and DLS measurements that were combined with visualized solubilization of giant unilamellar vesicles. As a result, the morphological transformations occurring in the nanostructures, the changes in particle concentration, and the nanoparticle size distribution analysis were neglected, as was the quantitative overview on the solubilization process. Furthermore, the study was limited to one detergent type (TX-100), thus there is still ample opportunity to broaden the understanding of the effect of detergent type on the process of solubilization of polymer-based bilayers. Finally, the mentioned studies were made on PEG-PEE and PEG-PB based systems and it can be expected that block copolymer bilayers of different compositions will not behave in the same manner upon introduction to different detergent types, similar to membranes composed of different phospholipids. This could, theoretically, allow one to modify the resulting bilayer membrane and polymeric nanostructure properties for a specific application by combining the right copolymer and detergent type¹³.

In the presented work, we investigated the detailed effects of the three detergents on a poly(ethylene glycol)-*block*-poly(ϵ -caprolactone) (PEG-PCL) based polymersome system. PEG-PCL consists of a non-toxic PEG section and an in-vivo biodegradable PCL part^{40–42}. They can be easily synthesized in large scale, with the use of non-toxic catalysts such as

calcium⁴³ or enzymes⁴⁴, and self-assembled structures can be prepared via scalable methods, such as direct hydration^{2,40}. This makes PEG-PCL based systems attractive for industrial applications such as drug delivery^{40,45}. Furthermore, PEG-PCL products are already approved as drug delivery systems by health care authorities, such as the U.S. Food and Drug Administration (FDA)^{2,46}. Multiple studies have investigated PEG-PCL systems for conjugation with proteins (e.g. glycoproteins or antibodies), cell binding (targeting studies), and cell viability^{40,47–49}, however there is no study focusing on the PEG-PCL bilayer solubilization. To the best of our knowledge, this is the first study to investigate the detergent-induced vesicle solubilization of a polymersome system that combines OD analysis, DLS, tunable resistive pulse sensing (TRPS), and cryoTEM. Due to the joint application of the above-mentioned analytical methods, the effect of solubilization was characterized, not only qualitatively (by optical density and average particle size), but also quantitatively (by TRPS measurements providing particle size distribution and particle concentrations in the solution). Further understanding of the process has been provided by the precise morphology analysis using cryoTEM. This study explains, in detail, the mechanism of solubilization of PEG-PCL polymersomes induced by three different detergents.

Experimental Section

Materials

The three detergents used - N-Octyl- β -D-Glucopyranoside (OG), Lauryldimethylamine N-oxide (LDAO) and Triton X-100 (TX-100) - in the study are commonly employed in bilayer membrane research for solubilization, or the extraction and reconstitution of transmembrane proteins^{23,27,50,51}. The detergents have different chemical structures, MWs, and critical micelle concentrations (CMC) in pure H₂O and those are presented in Figure 1.

Figure 1. Chemical structures of the detergents used in the study, A) – N-Octyl- β -D-Glucopyranoside (MW = 292 g/mol, CMC $\approx$ 20.0 mM),; B) – Lauryldimethylamine N-oxide (MW = 229 g/mol, CMC $\approx$ 2.0 mM) ; C) – TX-100 ($n=10$) (MW = 625 g/mol CMC $\approx$ 0.24 mM).

N-Lauryldimethylamine N-oxide (30 % aqueous solution) was obtained from Sigma Aldrich, Denmark and used as received. N-Octyl- β -D-Glucopyranoside ($\geq$ 99 %) was obtained from Anatrace, USA and used as 30% aqueous solution. TX-100 ($\geq$ 99.8 %) was obtained from Sigma Aldrich, Denmark and used as 20% aqueous solution. Phosphate buffered saline (PBS) was prepared by dissolving the salts (8 mg/mL NaCl, 0.2 mg/mL KCl, 1.44 mg/mL Na₂HPO₄, 0.24 g KH₂PO₄) in Milli-Q water, pH was adjusted to 7.4 with HCl. All chemicals for preparation of PBS were analytical grade and were obtained from Merck KGaA, Germany. Poly(ethylene glycol) methyl ether (Mn~550) (PEGME) was obtained from Merck KGaA, Germany. Poly(ethylene glycol)₄₅-*block*-poly(ϵ -caprolactone)₄₄ (PEG45-PCL44) (PDI 1.2)

was purchased from Advanced Polymer Materials, Canada. Proton nuclear magnetic resonance (H-NMR), size-exclusion liquid chromatography (SEC) analyses for the diblock copolymer are presented in the Supporting Info in the Figures S1 and S2, respectively. The results of differential scanning calorimetry (DSC) are presented in the Figures S3 and S4.

Preparation of Polymersomes

Polymersomes were prepared via direct hydration as previously reported in Sui *et al*, in PBS (pH 7.4, 136 mM NaCl, 2.6 mM KCl)⁴⁰. In brief, 1 g of PEG-PCL was mixed with 10 g of PEGME, to promote the hydration of copolymer, as PEGME is both soluble in the polymer as well as in water. The mixture was heated to 60°C and stirred at 300 rpm for 30 minutes. Subsequently, the temperature was lowered to 40°C, and 100 mL of PBS buffer was added. The obtained solution was stored at room temperature and filtered through a 0.2 µm polyethersulfone (PES) filter (Sarstedt, Germany) prior to use.

Characterization of Polymersomes and the Solubilization Process

Stability of the Particles in the Detergent Solution - Optical Density

OD measurements were made with a VarioskanTM microplate reader (Thermo Fischer Scientific, MA, USA) to determine the vesicle-solubilizing concentrations for each detergent. To perform the titration experiment, 200 µL of filtered polymersome solution was mixed with detergent to obtain the desired concentration in the well of a polystyrene flat-bottom, clear microplate (Greiner Bio-One, Austria). Kinetic OD measurements at a wavelength of 400 nm were made at specified time points at 20°C. All samples were prepared in triplicate. We observed no further changes in OD after 90 minutes of detergent incubation and therefore chose 2 hours for the detergent incubation time in the analyzed samples.

Polydispersity Index and Average Hydrodynamic Diameter - Dynamic Light Scattering

To prepare the samples for DLS measurements, filtered polymersome solution was mixed with detergents to reach the desired concentrations and was stirred for 2 hours at 750 rpm at room temperature. Average hydrodynamic diameter (Z-Avg) and polydispersity index (PDI) measurements were made by a DLS instrument, the Zetasizer Nano (Malvern, UK). All measurements were made in batch mode at 20°C using poly(methyl methacrylate) (PMMA) cuvettes (Sarstedt, Germany). The DLS measured samples were further analyzed by cryoTEM visualization and TRPS measurements.

Morphology - Cryogenic Transmission Electron Microscopy

Samples for visualization were vitrified on a glow-discharged lacey formvar film enforced by silicon monoxide coating and supported by a copper mesh grid (Ted Pella Inc., USA). The samples were vitrified in liquid ethane with use of VitrobotTM Mark IV (FEI, USA) and subsequently mounted in a Gatan cryoholder (FEI, USA). The images were acquired in cryogenic mode using a Tecnai G2 20 TWIN 200 kV TEM equipped with a FEI High-Sensitive 4k x 4k Eagle camera.

Size distribution and particle concentrations - Tunable Resistive Pulse Sensing

Virgin sample and three samples corresponding to each stage of solubilization, based on the results from OD, DLS, and cryoTEM analyses, for each of the detergents were chosen for TRPS analysis. Size distribution and particle concentrations measurement were analysed with a TRPS qNano Gold system (Izon Science, New Zealand), equipped with a NP150 polyurethane nanopore (Izon Science, New Zealand) and by following the standard protocol

recommended by the manufacturer ⁵². The analytical window for TRPS measurements was optimized for the particles with diameter between 50 nm and 550 nm. To perform the TRPS measurements, the samples were diluted 50 times with PBS buffer and analyzed under the voltage of 0.96 V and a pressure of 13 millibar. Data analysis was performed using the data capture and analysis software, Izon Control Suite V3.4 (Izon Science, New Zealand).

Results & Discussion

Characterization of Polymersome Solution

The polymersome solution, without detergent addition, was analyzed with DLS, cryoTEM, and TRPS as a standard for comparison to solutions with the three chosen detergents throughout the rest of this study. The results of the DLS measurements of the polymersome solution before and after filtration through a 0.2 μm polyethersulfone (PES) filter are listed in Table 1. The cryoTEM images of the same solutions before and after filtration are shown in Figure 2.

Table 1. Dynamic light scattering results of unfiltered and filtered (0.2 μm PES) PEG-PCL solution, presenting the PDI and hydrodynamic radiuses (D_h) of the two particle populations measured.

			Peak 1	Peak 2
	PDI	Z-Avg	D_h (nm) / Intensity (%)	D_h (nm) / Intensity (%)
Unfiltered	0.45 ± 0.02	196 ± 2 nm	285 ± 220 nm / 92%	3200 ± 1140 nm / 8%
Filtered via 0.2 μm PES	0.25 ± 0.01	160 ± 2 nm	192 ± 60 nm / 79%	63 ± 15 nm / 21%

Figure 2. CryoTEM micrographs of unfiltered PEG-PCL polymersome solution (left) and filtered solution via 0.2 μ m PES filter (right), scale bar: 500 nm.

CryoTEM visualization revealed that the unfiltered PEG-PCL polymersome solution consisted of vesicles with a membrane thickness varying between 15-20 nm (majority unilamellar, though some multilamellar vesicles were present). Additionally, the samples contained single micelles, rod-like micelles, and micrometer-sized multilamellar vesicles. Filtration removes the latter, however, rod-like micelles are still present in the filtered solution, together with sub-micron vesicles and single micelles. Likewise, the PDI of the filtered solution was 0.25 and the Z-Avg was 160 nm, compared to a PDI of 0.45 and Z-Avg of 196 nm for the unfiltered sample. Through TRPS measurements, the concentration of the particles in the filtered PEG-PCL solution was measured to be in the range of $2\text{E}+13$ particles/mL, with a mean physical diameter of 115 nm. A histogram of the particle distribution for the filtered solution is presented in Figure 3. Due to pore blockage from the presence of the micrometer-sized vesicles in the unfiltered PEG-PCL polymersome sample, it was not possible to measure particle distribution with TRPS. Throughout the rest of the article we refer to filtered PEG-PCL polymersome solution as the virgin sample.

Figure 3. Particle size distribution histograms obtained from TRPS measurements: A) Starting material of the polymersome solution; B) Solutions with *N*-Octyl- β -D-Glucopyranoside (OG); C) Solutions with Lauryldimethylamine *N*-oxide (LDAO); D) Solutions with Triton X-100 (TX-100). The particle concentration drops as the detergent concentration is increased. TRPS has a limited resolution window and for the chosen settings particles ≤ 50 nm in diameter were not detectable.

Three Stage Solubilization of PEG-PCL Polymersomes in Mixtures with Detergents

OG is a glucopyranoside and one of the most commonly applied nonionic detergent used for the solubilization of integral membrane proteins and their reconstitution into proteoliposomes, due to its high CMC value and non-denaturing properties^{23,50,51,53,54}. LDAO is a zwitterionic amine oxide-based detergent with a C12 alkyl tail. It is widely used for solubilization of bacterial membranes for the isolation of integral membrane proteins^{53–56}. TX-100 is a nonionic detergent often used for transmembrane protein reconstitution and it is known to penetrate the

247 lipid membrane at the onset of solubilization without affecting the membrane bilayer structure
248 ^{31,39}. The selected detergents were chosen based on their frequent use in membrane protein
249 solubilization and reconstitution studies. OD measurements of the PEG-PCL polymersome
250 solutions with titrated detergents are presented in Figure 4.

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Figure 4. Results of the optical density (O.D.) measurements of the PEG-PCL polymersome upon introduction of the detergents: A) *N*-Octyl- β -D-Glucopyranoside (OG); B) Lauryldimethylamine N-oxide (LDAO); C) Triton X-100 (TX-100). Concentrations are provided in mM as well as fractions of the critical micelle concentration (CMC).

The OD measurements were obtained to define the detergent concentrations at which the transition between the solubilization stages of PEG-PCL polymersomes occur for each detergent ²¹. As shown in Figure 4A, at low concentrations of OG (1.7 mM – 8.5 mM) the normalized OD increased by 0.1 (for 120 minutes contact time). In contrast, upon introduction of LDAO and TX-100, normalized OD increased by 0.4 and 0.3 (see Figure 4B and 4C), respectively. The increase in turbidity for all of the investigated samples upon exposure to low detergent concentrations can be explained by the increase of the vesicle diameter. This is in alignment with the first stage of bilayer solubilization. At the low concentrations of detergents, so-called subsolubilizing concentrations, an increase of the vesicle diameter occurs due to the penetration of the detergent molecules into the bilayer ^{25,28,57}. The subsequent observed drop in OD at increasing detergent concentrations (starting from 10.2 mM, 1.1 mM, and 2.4 mM, for OG, LDAO, and TX-100, respectively) indicates initiation of the second stage of solubilization, where disintegration of the vesicles occurs due to their bilayer becoming saturated with detergent molecules. Full solubilization (stage 3) occurred at 23.4 mM for OG, 8.7 mM for LDAO, and 6.4 mM for TX-100, when the normalized OD reached 0.13, 0.22, and 0.43 for OG, LDAO and TX-100 respectively. This observed drop in OD is related to the third stage of solubilization – formation of smaller single micelles with higher detergent-to-copolymer ratio. The limited drop in OD for TX-100 compared to OG and LDAO can be linked to the higher MW of TX-100 and incomplete bilayer penetration by the detergent ²¹. Nonetheless, TX-100 solutions are known to have increased turbidity in comparison to LDAO and OG, due to its lower cloud point in aqueous solution ⁹. Moreover, Figure 4 shows that between the second and third stage of solubilization, OD measurements are more time dependent for all detergents, since disintegration of the vesicles is a kinetic process.

Effect of N-Octyl- β -D-Glucopyranoside on Polymersome Morphology

After determining the changes in OD of the polymersome solutions with the three detergents DLS measurements were made as a first step to explain the morphological changes happening upon detergent titration. Namely, the average diameter of particles (Z-Avg) and the polydispersity index (PDI) were measured. The results of the DLS measurements for the samples with different OG concentrations are presented in Figure 5A.

Figure 5. DLS measured average hydrodynamic diameter (Z-Avg) and polydispersity index (PDI) of particles upon introduction of the detergents: A) N-Octyl- β -D-Glucopyranoside (OG); B) Lauryldimethylamine N-oxide (LDAO); C) Triton X-100 (TX-100). The Z-Avg and PDI drops sharply when the detergent concentration capable of solubilizing the majority of the vesicles is reached.

At concentrations of OG between 1.7 mM to 13.5 mM, the Z-Avg remains unchanged at roughly 200 nm, indicating that the vesicles were stable within this range. However, cryoTEM micrographs (presented in Figure 6) show that from 5.1 mM OG, the number of rod-like micelles and single micelles was significantly higher than for the virgin sample. Interestingly, at a concentration of 5.1 mM OG the presence of rod-like micelles was the most prominent morphology, in comparison to the other samples containing OG. This explains the increased OD of the solutions (see Figure 4A), as the enlarged vesicles with detergent-saturated bilayers are present with single micelles and rod-like micelles in the sample^{19,21}. The significant increase of the population of rod-like micelles is barely detectable by DLS, as Z-Avg remain unchanged and PDI only slightly increases, due to the large vesicles scattering light with a higher intensity than the smaller particles present in the solution. At 13.5 mM OG, a detergent concentration in the middle of the second stage of solubilization, the total particle concentration measured by TRPS dropped from $2\text{E}+13$ particles / mL to $8\text{E}+12$ particles / mL (for the particles above 50 nm). Moreover, the number of the particles in the range of 100 - 200 nm in physical diameter, corresponding to vesicles, dropped by half (see Figure 3A), from $3\text{E}+12$ particles / mL to $1.5\text{E}+12$ particles / mL.

After reaching concentrations of 20.1 mM OG, Z-Avg drops to 167 ± 17 nm and PDI increases to 0.43 ± 0.03 , indicating an increased number of single micelles in the solution and a decreased number of vesicles. As shown in cryoTEM micrographs (Figure 6), mostly single-micelles and rod-like micelles were present in the solution, with a few vesicles visible with radiuses below 100 nm.

At concentrations reaching 23.4 mM OG, the bilayer of the vesicles was completely solubilized. The Z-Avg dropped to 50 ± 2 nm and the PDI was reduced to 0.10 ± 0.01 . This is also confirmed by TRPS measurements, as the measured total particle concentration dropped to 2.04×10^{12} (see Figure 3A). No pore formation in the vesicle bilayer, typically observed for liposomes^{21,25}, was observed with cryoTEM at any of the OG concentrations.

Figure 6. Cryogenic Transmission Electron Microscopy micrographs of vesicle samples with different concentrations of N-Octyl-β-D-Glucopyranoside OG, scale bar: 500 nm.

Effect of Lauryldimethylamine N-oxide on Polymersome Morphology

The Z-Avg and PDI of polymersome solutions exposed to increasing LDAO concentrations are presented in Figure 5B. At concentrations up to 2.2 mM LDAO, the Z-Avg and PDI remained unaffected, averaging at sizes of 210-230 nm with a PDI of 0.40-0.44. These numbers indicate stability of the polymersomes in the presence of the detergent as they show a slight

increase in size, but are still similar to the results from the virgin solution (see Table 1 and Figure 2). These changes also correlate to the increase in OD, up to concentrations of 1.1 mM LDAO (see Figure 4B). The cryoTEM micrographs of the samples with 0.2 mM and 1.1 mM LDAO exhibit enlarged vesicles (Figure 7) confirming the DLS and OD readings.

Figure 7. Cryogenic Transmission Electron Microscopy micrographs of vesicle samples with different concentrations of) Lauryldimethylamine N-oxide (LDAO), scale bar: 500 nm.

However, for the sample with 2.2 mM LDAO, the OD decreased in comparison to the samples with lower LDAO concentrations. This is due to the decreased vesicle concentration and size which is observable on the cryoTEM micrograph (Figure 7). The DLS measurement is not sensitive to these changes, as PDI and Z-Avg remain unchanged at LDAO concentrations up to 2.2 mM.

At 4.4 mM LDAO, the Z-Avg decreased sharply to 157 ± 37 nm and PDI increased to 0.52 ± 0.02 . Additionally, the error of the measurements was also increased at this concentration (see Figure 5B). These results indicate that the average size of the particles decreased, while differences in the particle populations became more prominent. The cryoTEM micrograph of the sample with 4.4 mM LDAO, shows the presence of predominantly unilamellar vesicles. These vesicles were smaller in size in comparison to the polymersome samples with 0.2 mM and 1.1 mM LDAO (Figure 7). The size of the vesicles in the polymersomes sample with 4.4 mM LDAO was comparable to the sample containing 2.2 mM LDAO, however, the population of rod-like micelles was decreased. They were most likely solubilized to single micelles, which were present in the solution with the vesicles. These morphological findings correlate to the OD measurements, as the polymersome sample with 4.4 mM LDAO experienced a sharp drop in OD (see Figure 4B). The drop in concentration of vesicles at 4.4 mM LDAO was quantified with TRPS (see Figure 3B), as the total concentration of particles dropped from $2\text{E}+13$ particles / mL in the virgin solution, to $8.65\text{E}+12$ particles / mL. Furthermore, the concentration of particles between 100-200 nm was reduced by half, which is identical to the effect observed at 13.5 mM of OG, which is the concentration where stage 3 of the solubilization process occurs (see Figure 3A).

In contrast to OG, the rod-like micelle morphology tends to decay at increased LDAO concentrations (above 4.4 mM), with most of the particles being single micelles. Even though the two detergents have similar MW – 229 g/mol for LDAO and 292 g/mol for OG, their critical micelle concentration is different. LDAO is characterized by a 10-fold lower CMC value (~ 2 mM in pure H_2O) compared to OG (~ 20 mM in pure H_2O). Thus, LDAO molecules arrange into micelles at lower concentrations than OG, and in combination with PEG-PCL chains, are more prone to form single micelles than long rods, thereby solubilizing the bilayer at lower

concentrations than OG. At 6.5 mM LDAO the PDI dropped to 0.24 ± 0.12 , and Z-Avg to 54 ± 16 nm. While the error of the PDI readings was more pronounced, these DLS results correlate with the observed drop in concentration of polymersomes and an increased concentration of single micelles – also confirmed by cryoTEM imaging (Figure 7). Complete solubilization was reached at 8.7 mM LDAO, where the measured PDI was 0.16 ± 0.01 and Z-Avg reached 44 ± 1 nm. CryoTEM confirmed that the sample mostly contained single micelles and sub-200 nm length rod-like micelles, while the TRPS measurement showed a reduction in total particle concentration to 1.69×10^{12} particles / mL. Similar to OG, – no pore formation was observed within the vesicle bilayer was observed on cryoTEM micrographs at any point during the vesicle solubilization (see Figure 7).

Effect of Triton X-100 on Polymersome Morphology

The PDI and Z-Avg measured by DLS for polymersomes mixed with TX-100 are presented in Figure 5C. TX-100 did not induce significant changes to the Z-Avg, at concentrations up to 3.2 mM TX-100. However, the cryoTEM micrograph of the sample with 0.4 mM TX-100 (presented in Figure 8), showed larger vesicles in comparison to the virgin solution (see Figure 2), which was also observed as an increase in the OD measurements (see Figure 4C).

Figure 8. Cryogenic Transmission Electron Microscopy micrographs of vesicle samples with different concentrations of Triton X-100 (TX-100), scale bar: 500 nm.

The PDI did increase at concentrations up to 3.2 mM of TX-100. This relates to the morphological changes observed with cryoTEM. Upon the addition of TX-100, long rod-like micelles were formed with lengths exceeding 2 μm . Their population and lengths increased until a concentration of 4.0 mM of TX-100 was reached, where most of the structures were solubilized into single micelles (see Figure 8). When the concentration reached 4.0 mM TX-100, it could be elucidated from DLS measurements that a complete vesicle solubilization took place, since the Z-Avg dropped to 48 ± 1 nm and the PDI dropped to 0.25 ± 0.02 . Some vesicles were still visible on the cryoTEM micrographs at 4.0 mM TX-100, though their population was greatly reduced. While a further increase in the detergent concentration resulted in a continued decrease of OD (see Figure 4C), the Z-Avg and PDI remained constant. A PDI below 0.2 was not reached even at the highest tested concentration of 6.4 mM TX-100, as in

the case of OG and LDAO, when complete solubilization was achieved. This could indicate that a small portion of the insolubilized vesicles was still present. Nevertheless, cryoTEM micrographs of the 4.8 mM TX-100 sample, revealed unusual aggregates with a morphology between large micelles and rod-like micelles (see Figure 8). The results of TRPS measurements (presented in Figure 3D) showed that the total particle concentration was $1.60\text{E}+13$ particles / mL, $1.49\text{E}+13$ particles / mL and $6.78\text{E}+12$ particles / mL for 0.4 mM, 1.6 mM, and 4.8 mM TX-100, respectively. A sharp decrease in the concentration of particles in size range of 100-200 nm, in comparison to the virgin solution, was recorded only for the sample with 4.8 mM TX-100. Nevertheless, the particle concentrations did not drop to the levels observed for the samples with OG and LDAO (Figure 3B and 3C). The concentration of 100 nm particles at 4.8 mM TX-100 was twice as high as the highest concentrations of OG (23.4 mM) and LDAO (8.7 mM) with $1\text{E}+12$ particles / mL for TX-100, in comparison to $5\text{E}+11$ particles / mL for both OG and LDAO. We speculate that this discrepancy in particle concentration is due to the presence of the amorphous aggregates formed when TX-100 was used for the polymersome solubilization. The phenomenon of formation of amorphous aggregates can be explained by the significantly higher MW of TX-100 (615 g/mol) in comparison to OG and LDAO, and the difference in the structure of the hydrophilic and hydrophobic moieties of the detergents. TX-100 consists of a long hydrophilic chain of PEG and a hydrophobic alkylphenyl group, in comparison to LDAO and OG, which both have hydrophobic chains and a hydrophilic groups. LDAO has the smallest hydrophilic region composed of amine oxide and OG has a large glucose headgroup (see Figure 1). TX-100 has the lowest CMC of the three tested detergents, however it was found to be the least effective, solubilizing the bilayer at CMC fractions reaching 2000 % even though TX-100 has previously been described, in early work on liposome solubilization, to reside within the group of ‘fast-solubilizing’ detergents^{21,26,58}. TX-100 instead slowly and partially solubilizes the bilayer, which leads to the formation of larger

aggregates and mixed micelles. The breakdown of the bilayer should result in the partial destabilization of the vesicles and consequently to the leakage of vesicles, but no vesicles with an open bilayer were observed with cryoTEM.

Comparison of the Observed Solubilization Process to the Literature

The three stages of solubilization were defined and studied for PEG-PCL polymersomes exposed to increasing concentrations of the three investigated detergents until complete solubilization and breakdown of the vesicles was confirmed. Across the whole studied concentration spectras of the investigated detergents, opened pores were not observed within the vesicle bilayer. The formation of pores was expected at the solubilizing concentrations since such phenomena is known to occur in liposomes and is considered a prerequisite for the vesicle solubilization^{21,25}. It can be speculated that the absence of pore formation is a result of a significant mismatch in the detergent length in comparison to the diblock chain. Even for the largest detergent molecule in this study – TX-100 – the mass of the PEG45-PCL44 chain (average MW = 7000 g/mol) is more than 10 times larger. In comparison, for commonly used vesicle-forming phospholipids, MW ranges between 700 and 800 g/mol⁵⁹, which is significantly closer to the MW of the detergents. Additionally, polymers have certain characteristics that are absent in the lipid constituents. Polymers are not perfectly pure systems, even when the PDI is low. The block copolymer used in this study was characterized with a PDI of 1.2. Nevertheless, in contrast to pure lipid mixtures, a polymer mixture will always consists of polymer chains with a variety of block lengths⁶⁰. These polymers with non-uniform chain lengths build the vesicle bilayer, and due to the membrane liquidity and lateral diffusion, they will be able to reshuffle within the layer. Thus, enabling chain configurations to alter within the bilayer and modifying the packing of the chains. In fact, molecular dynamics modelling has indicated that block copolymer membranes are capable of adjusting the

thickness of the bilayer when facing membrane constituents with a hydrophobic mismatch. As a result, polymer chains not only move within the bilayer, but they are also able to compress in the vicinity of the molecule causing the hydrophobic mismatch – such as membrane proteins^{7,8,61,62}. Similar behavior can be expected when detergents, with much shorter hydrophobic chains than the polymer molecules, are incorporated into the bilayer.

Due to the MW of the PEG-PCL diblock copolymer, a fully-extended, hydrophilic PEG block will always be longer than a hydrophilic head of a lipid molecule, and the hydrophilic constituent of the detergent. Due to the significantly longer hydrophilic region of the detergent, it should be more difficult for the detergent to penetrate through the hydrophilic moiety of the amphiphilic polymer in comparison to its lipid counterpart^{1,8,11,19,63}. Thus, a higher stability of the bilayer towards detergent attack would be expected. Nevertheless, in this work we have found that the concentrations required to solubilize PEG-PCL based polymersomes can be significantly lower than those reported for liposomes, such as a reported concentration of 80 mM OG required to solubilize 15 mg/mL of egg lecithin vesicles⁶⁴, or egg sphingomyelin (SM)/cholesterol vesicles that were reported to withstand concentrations as high as 5 mM of TX-100²⁴. 6.4 mM was the highest TX-100 concentration used in this study, which is less than a half of the concentration reported by Nallani et al. to solubilize 10 mg/mL of egg-phosphatidylcholine (PC) lipid vesicles (16 mM TX-100), where the analysis was based on DLS and OD measurements⁶⁵. The bilayer stability towards detergent is very material dependent²², as other liposomal formulations have been reported to be completely solubilized at concentrations as low as 0.3 mM or 0.4 mM TX-100, for SM and 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine based systems respectively²⁴. The analyzed PEG-PCL system is more prone to a detergent attack than previously described polymersomes of PMOXA-PDMS (5 mg/mL), which were reported to fully withstand concentrations of 16 mM of TX-100³¹.

Higher sensitivity to the detergent presence, may be caused by the less flexible hydrophobic building block of the copolymer (PCL) in comparison to PDMS⁶⁶. Flexible PDMS chains may be expected to accommodate detergent molecules easier than stiffer PCL, resulting in higher stability of the bilayer towards detergent attack⁸. Obviously, concentration of the particles will be an essential factor affecting the detergent concentration at which solubilization occurs. The mentioned studies do not include particle concentration measurements, thus the quantitative comparison of the results is limited.

Summary and Conclusions

This work presents the first analysis of detergents' effect on PEG-PCL based polymersomes. It is the first thorough analysis combining OD and DLS measurements with detailed qualitative cryoTEM analysis and quantitative TRPS particle concentration measurements. We have demonstrated the effect of an increasing concentration of three detergents: OG, LDAO, and TX-100 on PEG-PCL polymersomes. The titration of these detergents into the polymersome solutions was carried out to the point at which no polymersomes were observed on cryoTEM micrographs. It has been found that despite the higher MW of polymers in comparison to lipid molecules (thus expected higher stability of polymersomes in general), significantly lower concentrations of detergents can disrupt the vesicular bilayer of PEG-PCL, than was previously reported for liposomes. The solubilization mechanism of polymersomes is comparable to that of liposomes, however the expected pore formation within the bilayer was not observed for the PEG-PCL polymersomes at any stage of the solubilization with the tested detergents. The behavior of the solubilization also differs depending on the detergent used. Even though LDAO and OG have ten and one hundred fold higher CMC than TX-100, respectively, it was shown in this work that they are capable of reducing the concentration of polymersomes at lower CMC

fractions. This effect is caused by the significantly higher MW and difference in structural properties of TX-100 in comparison to LDAO and OG.

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Supporting Information

Figure S1. H-NMR spectrum of PEG-PCL diblock and chemical structure.

Figure S2. Size-exclusion liquid chromatography chromatogram of the PEG-PCL diblock.

Figure S3. Differential scanning calorimetry curve of PEG-PCL diblock.

Figure S4. Differential scanning calorimetry curve, representing T_g region of the PEG-PCL diblock.

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