Extending the self-assembly of coiled-coil hybrids

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RAPID PREPARATION OF POLYMERSOMES BY A WATER ADDITION – SOLVENT EVAPORATION METHOD

In this Chapter a water addition – solvent evaporation method to produce polymersomes is demonstrated for the first time. The process involves solubilizing an amphiphilic block copolymer in an appropriate volatile organic solvent (THF), mixing it with an aqueous solution, and then evaporating the organic solvent under reduced. The parameters that influence the formation and size of the polymersomes are easily controlled, thus polymersomes are formed, tuned, and stabilized in less than five minutes. The method was demonstrated with a series of peptidic block copolymers, where the hydrophilic block is a charged designed peptide, and the hydrophobic block is poly(γ-benzyl L-glutamate) with varying degrees of polymerization (35 – 100 monomers), and the polymersome formation was monitored and confirmed with dynamic light scattering, optical microscopy, and transmission electron microscopy. The scope of the technique was also probed with noncovalent peptidic polymer complexes, and fully synthetic charged and non-charged coil-coil block copolymers of varying length. The method was found to be very robust with regards to salt concentration and initial mixing, and the polymersome size could be precisely adjusted, with the same block copolymer forming polymersomes ranging from ~ 200 nm to ~ 2 μm in diameter. Given the simplicity and versatility of the water addition – solvent evaporation method it is a very appealing new tool for polymersome preparation.

INTRODUCTION

Vesicles formed from lipids have been prepared since the early 1960s.[1,2] During these decades many techniques have been developed to prepare lipid vesicles, such as film rehydration, bulk rehydration, electroformation, solvent injection, aqueous phase injection, emulsion based methods (solvent evaporation or dialysis), and detergent dialysis. There are many variations on these methods, and also techniques to modify preformed vesicles such as vortexing, freeze-thawing, extrusion, or sonication have been applied.[3] Each of these methods results in lipid vesicles with different properties, and a remarkable range of properties are accessible: diameters from 25 nm to 100 μm, unilamellar or multilamellar vesicles, vesicles with relatively narrow or broad size distributions, vesicles encapsulating varying amounts of water soluble compounds, or incorporating material within the membrane, and vesicles with or without (residual) organic solvent. As a result, the physical properties required by a specific application often dictate which method of

vesicle formation should be applied. For example, for tumor targeting and retention purposes 100 nm vesicles are favored,[4] while for the study of membrane mimics giant vesicles are preferred, and the same method cannot be used to make these different types of vesicles. In addition to the final vesicle characteristics, each technique has associated advantages/disadvantages such as the use of organic solvents, high energy input, availability of specific equipment, or the time required for preparation. These aspects also frequently direct the choice of vesicle preparation method. For instance, when biomolecules are involved physiological ionic strength buffer is often required, and sonication or organic solvents cannot be used.

While lipid-based vesicles are the most extensively studied, other amphiphiles can assemble into vesicles as well. Examples are block copolymers and modified cyclodextrins. Polymer vesicles are particularly interesting as there is a profusion of choice regarding block composition and length, which results in a large variety of potential vesicle properties. Research into polymer vesicles, referred to herein as 'polymersomes', has been carried out since 1995,[5] and the remarkable vesicle properties arising from the choice of polymers have only been partially revealed. In order to allow the potential of polymersomes to be more fully exposed, the variety of preparation techniques that allows one to access different polymersomes needs to be extended. Not all methods used to assemble lipids into vesicles can be transferred directly to polymers due to their different physical/chemical properties, for example many block copolymers cannot be directly hydrated as lipids can. Hence techniques used for lipids are tailored to polymers, or new procedures are developed. The earliest method to prepare polymersomes, and currently the most commonly used, is to dissolve the polymer in an organic solvent and slowly add water, followed by dialysis to remove the organic solvent.[5-7] Other methods are solvent injection followed by dialysis,[8] bulk and film rehydration,[7] direct hydration,[9] electroformation,[10,11] dewetting from a template,[12] and a microfluidic double emulsion technique.[13]

Here a new method is presented, the 'water addition – solvent evaporation' method, to prepare polymersomes. The method is inspired by a rapid liposome preparation technique and is adapted to meet the requirements of block copolymers.[14] The advantages of this method are that the polymersome sizes can be readily and precisely tuned over a wide range; it is a robust method that can be applied to many different types of block copolymers; and finally it is very practical, as the process is complete in less than five minutes, uses standard laboratory equipment, and does not require high energy input.

RESULTS AND DISCUSSION

The new technique for preparing polymersomes consists of three steps: (I) the block copolymer is dissolved in THF, (II) the aqueous phase is added all at once and gently mixed, (III) the THF is removed by rotary evaporation under reduced pressure.

The method is initially demonstrated with the amphiphilic rod-rod block copolymer $PBLG_{50}$-K, in which PBLG is poly(γ-benzyl L-glutamate), and K is a charged peptide with 22 amino acid residues. Using the standard conditions (PBS, one minute vortexing, evaporation at 20 °C) $PBLG_{50}$-K self-assembled into large structures with an average hydrodynamic diameter of ~ 1200 nm, and a low size distribution (PDI 0.06) (Figure 1a). The particles were spherical and when the water soluble dye rhodamine B was added to the buffer it was observed by fluorescence microscopy to be encapsulated within the structures (Figure 1b), confirming that the block copolymer assembled into polymersomes. With transmission electron microscopy (TEM) it was established that polymersomes were the predominant structure, with the only other morphology being a very small quantity of spherical micelles. The average diameter of the polymersomes as measured by optical microscopy and dynamic light scattering were in agreement with one another, while the sizes of the dried polymersomes observed with electron microscopy were ~ 50 % smaller, likely due to deflation while drying.

The polymersome formation process and the extent to which $PBLG_{50}$-K polymersome sizes could be tuned were investigated, proceeding through the three parts of the method.

(I) Dissolving the polymer in THF

The water addition – solvent evaporation method was modified from a rapid liposome preparation technique.[14] For this method the lipids are also dissolved in an organic solvent, the aqueous phase quickly added, and the organic solvent removed by rotary evaporation under reduced pressure. However, the organic solvent used in the rapid liposome method is chloroform, which is immiscible with water, and liposomes form during evaporation. Many block copolymers, including the PBLG-peptide series used in this study, are not soluble in chloroform because of the hydrophilicity of the corona forming block. THF is a polar organic solvent that dissolves a wider range of polymer blocks. As it also has a low boiling point (66 °C), it is suitable to use in a solvent evaporation method. Unlike chloroform, THF is miscible with water; hence the block copolymers are exposed to a high water content upon addition of the aqueous phase, and aggregation induced by the hydrophobic block is expected before solvent evaporation.[18] Therefore the block copolymer specific adaptations mean that the current method has a different mechanism of vesicle formation to the rapid preparation of liposomes and is therefore quite dissimilar.

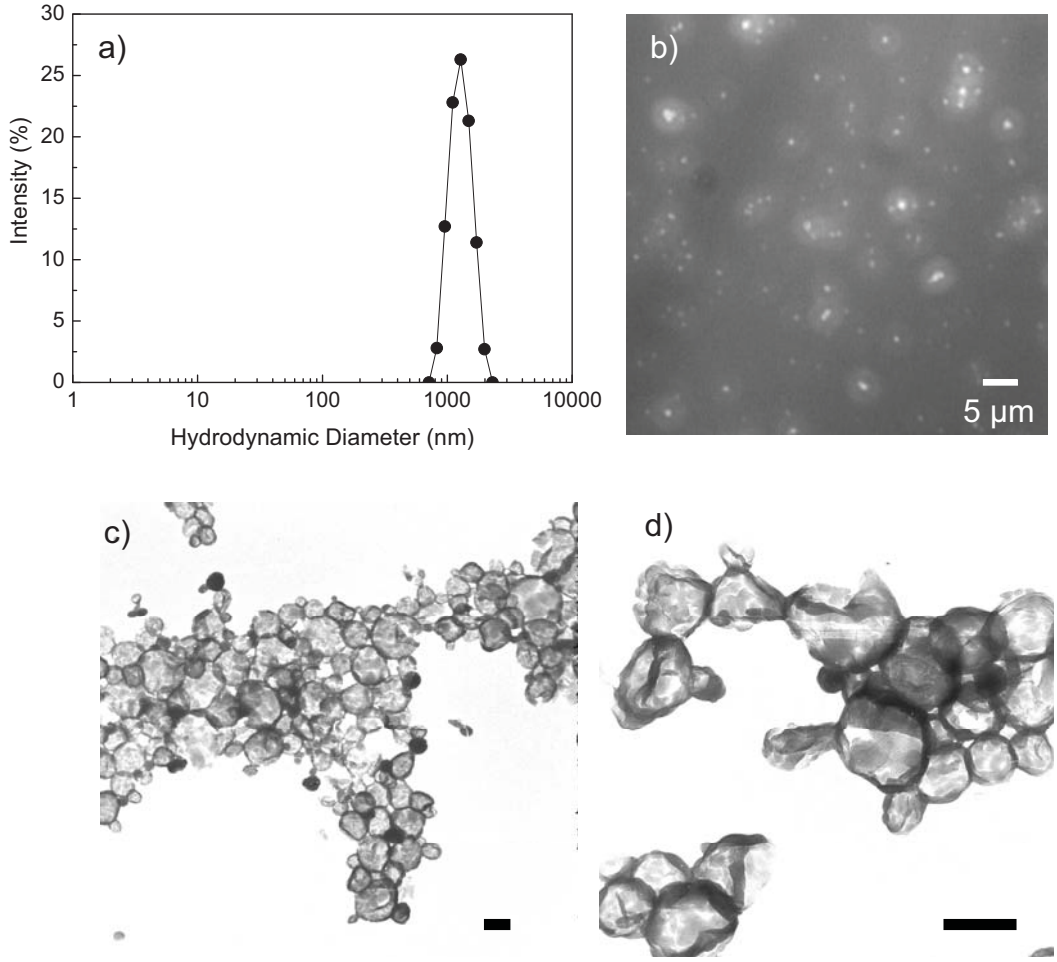

Figure 1. a) DLS intensity distribution for $PBLG_{50}$-K polymersomes prepared with the standard conditions. b) Fluorescence microscope image of $PBLG_{50}$-K polymersomes with encapsulated rhodamine B. c,d) TEM images of PTA stained $PBLG_{50}$-K polymersomes. Scale bars = 500 nm. Conditions: 2 mL THF, 0.02 μmol $PBLG_{50}$-K, 3 mL PBS, vortexing 1 minute at 200 rpm, THF evaporated for 2 minutes at 20 °C.

(II) Addition of aqueous buffer

To demonstrate the self-assembly process, $PBLG_{50}$-K samples were monitored with dynamic light scattering (DLS) and optical microscopy throughout the polymersome formation procedure. $PBLG_{50}$-K has a hydrodynamic diameter (D_h) of 8 nm in THF, in accordance with the theoretical value for molecularly dispersed block copolymer.[19] Upon addition of PBS large particles with a narrow size distribution (PDI ~ 0.06) were observed in the THF/PBS mixture (Figure 2a), which contained a polar interior (Figure 2b), suggesting that the block copolymer already assembles into polymersomes in the mixed solvent phase.

Additionally, it was observed by DLS and optical microscopy that the aggregate size continued to increase after the initial rapid structure formation (Figure 2c, d). This sustained size increase could be caused by three phenomena, operating either alone or in

tandem: ripening, fusion, or aggregation (see Figure A1). In general, polymersomes in mixed solvent systems increase in size in response to water addition in order to reduce the interfacial free energy between the core and solvent in the corona (if the core is still mobile).[20] This often occurs by fusion of smaller polymersomes, with low growth rates when the water content is high, as is the case in this system.[21,22]

In an attempt to observe the transition from molecularly dispersed block copolymer to polymersomes of ~ 1 μm, DLS was carried out as PBS was added incrementally to the THF solution (Figure 2c inset).[23] At a critical water content of ~ 5 v/v % PBS to THF the solvent quality of the mixture is sufficiently poor for the hydrophobic PBLG block that it aggregates, leading to microphase separation, and nanostructure formation. For PBLG-K (or PBLG-E, *vide infra*) block copolymers with shorter PBLG blocks, the onset of PBLG induced aggregation occurs at higher PBS/THF ratios. As more PBS is added the solvent becomes increasingly poor for the PBLG block and the structures continue to increase in size.[24] The trend of increasing particle size upon PBS addition is reliable, but the exact values for D_h should not be over interpreted due to the juxtaposition of the aforementioned kinetic growth effect, and because the size of the jumps in water content can affect the water volume at which structural transitions occur.[6,22]

During polymersome preparation, the introduction of the aqueous phase is generally the most critical step in the process, being the point at which precipitation of the block copolymer is likely to occur. In the present case polymersomes form upon PBS addition, and the manner in which samples are treated prior to THF removal may be expected to influence the final structures. Under the standard conditions the aqueous phase is added to the organic phase and then gently vortexed for one minute at a speed such that the phases are mixed but not agitated. Simply leaving the samples for one minute without mixing also resulted in well-defined final polymersomes, with similar sizes to those prepared with the mixing step (average D_h ~ 1000 nm, PDI 0.01, Figure 3). The THF can also be evaporated directly after addition of the aqueous phase (Figure 3). The inevitable mixing upon addition of the aqueous phase and during solvent evaporation is therefore adequate to yield polymersomes with a narrow size range. However, if the THF solution and PBS were mixed with high energy input by vortexing the flask for 1 minute at 2000 rpm such that the solution is strongly agitated, the material precipitated. Therefore, within practical limits, the structure formation is relatively insensitive to the way in which the organic and aqueous phases are combined, and no special care is required at this stage. Although the mixing step is not necessary with this block copolymer, the polymersome size distribution was found to be slightly less reproducible without the mixing step for a different block copolymer series (*vide infra*), therefore it was included to standardize the procedure.

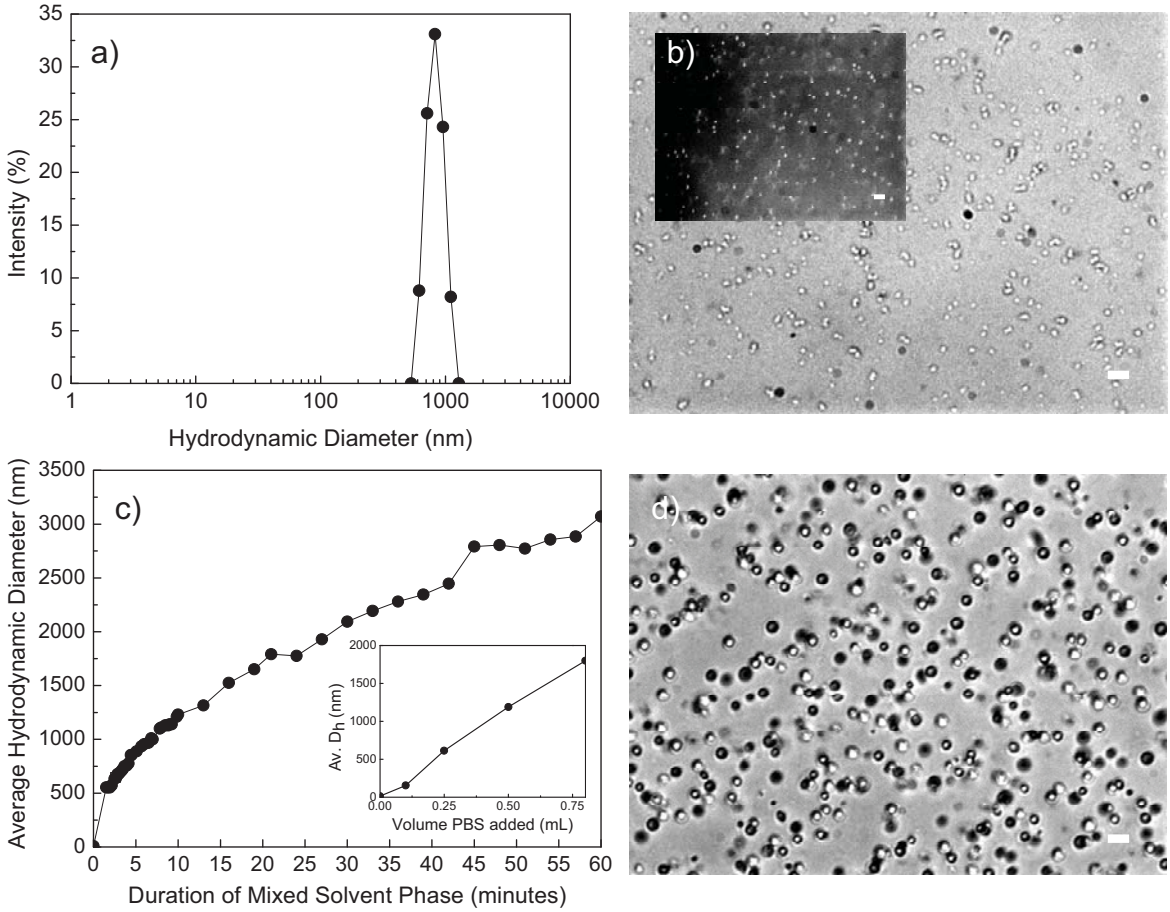

Figure 2. Particle characteristics in 2:3 v/v THF:PBS prior to THF evaporation: a) D_h distribution of $PBLG_{50}K$ polymersomes measured ~ 5 minutes after addition of the PBS. b) Optical bright field and fluorescence (inset) microscope images of $PBLG_{50}$-K polymersomes ~ 2 minutes after addition of PBS. Scale bar = 5 μm. c) The change in average D_h of $PBLG_{50}K$ assemblies with time after addition of PBS. Note that the initial points are subruns from DLS. Inset, average D_h during incremental PBS addition. d) Optical microscope image of $PBLG_{50}$-K particles ~ 30 minutes after addition of PBS. Scale bar = 5 μm.

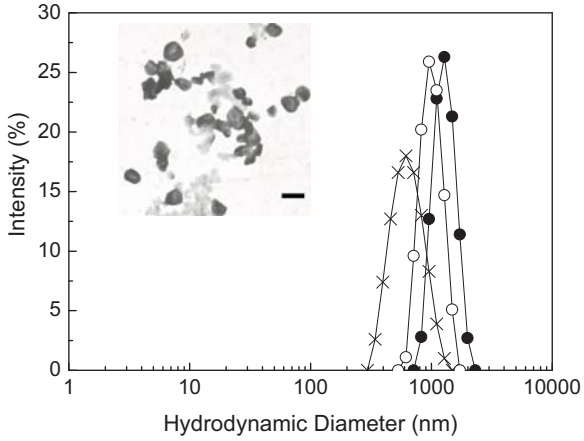

Figure 3. DLS intensity distributions for $PBLG_{50}$-K polymersomes prepared with 1 minute of vortexing at 200 rpm prior to THF evaporation (●), 1 minute without mixing (○), and immediate evaporation (×). Inset: PTA stained $PBLG_{50}$-K polymersomes prepared with immediate evaporation. Scale bar = 500 nm.

(III) Evaporation of the organic solvent

Many block copolymers cannot be hydrated from the bulk phase, thereby necessitating dispersal in an organic solvent followed by introduction of the aqueous phase. Since it is not possible to remove all of the organic solvent, all polymersomes prepared using solvent displacement techniques contain residual organic solvent. The advantages of removing the THF by rotary evaporation under reduced pressure are that it is very rapid, and the polymersome sizes can be adjusted and frozen during the same step (*vide infra*). The disadvantage is that more THF is likely to remain compared to removal by dialysis, which is the typical method for extracting the organic solvent. The samples were evaporated for 30 seconds after the boiling of THF subsided, with 97% of the THF removed at 20 °C, as determined by NMR spectroscopy, and insignificant volumes of water evaporated. After the majority of the THF has been evaporated the particle size does not change for at least four months at 4 °C, presumably because the PBLG blocks are frozen in the poor solvent, and the polymersomes are metastable kinetically trapped non-equilibrium structures.

During evaporation of the THF the composition changes within the interior of the polymersomes. To illustrate this, a small amount of the fluorescent dye rhodamine B was added to samples after the PBS and THF had been mixed, but before solvent evaporation. The polymersome membrane is impermeable to rhodamine B, as the interior of the polymersomes remains dark when observed by fluorescence spectroscopy (Figure 4a). However, after solvent evaporation for two minutes at 20 °C the interior of the polymersomes contains the fluorescent dye (Figure 4b). This means that the aggregates are permeable to rhodamine B during THF evaporation, and it is likely that they are also permeable to THF/PBS.

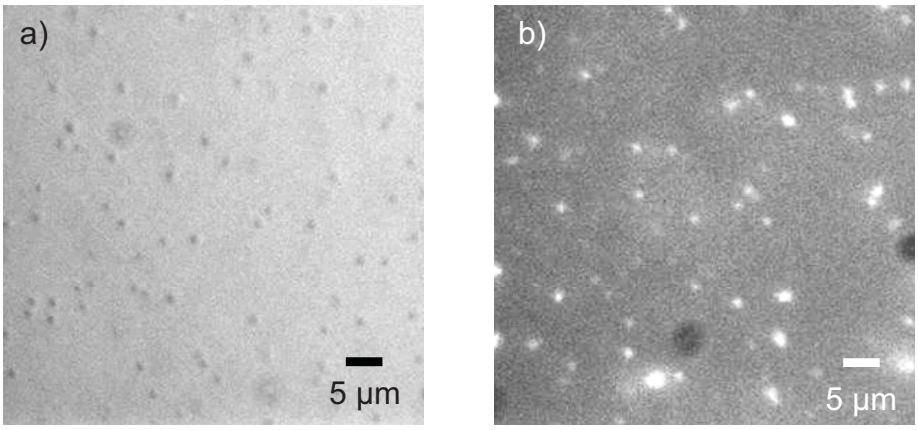

Figure 4. a) Fluorescence microscopy image of $PBLG_{50}$-K polymersomes in the THF/PBS mixture after the addition of rhodamine B. The rhodamine B is outside the polymersomes. b) Fluorescence microscopy image of $PBLG_{50}$-K polymersomes in PBS after THF evaporation, showing that the rhodamine B is inside the polymersomes. Scale bars = 5 µm.

The size of the polymersomes in PBS can be controlled by the time dependent particle size increase in the mixed solvent phase. Figure 5 shows the resultant sizes of the

polymersomes for which THF was evaporated at different times after addition of the aqueous phase. As the duration of the mixed solvent phase is extended and the particles increase in size, the polymersome diameters after THF removal also become larger. The sizes of the polymersomes in PBS are therefore dependent on the particle size in the mixed THF/PBS phase, which indicates that there is some degree of continuity between the particles pre- and post-THF evaporation, i.e. there is not complete disassembly and reassembly of the structures during the evaporation process. The largest polymersomes that could be formed from $PBLG_{50}$-K in this way at 20 ºC were ~ 2 μm. If the period of mixed solvent was extended further the polymersomes were not dispersed homogenously throughout the solution, but some formed a fine layer at the top of the PBS.

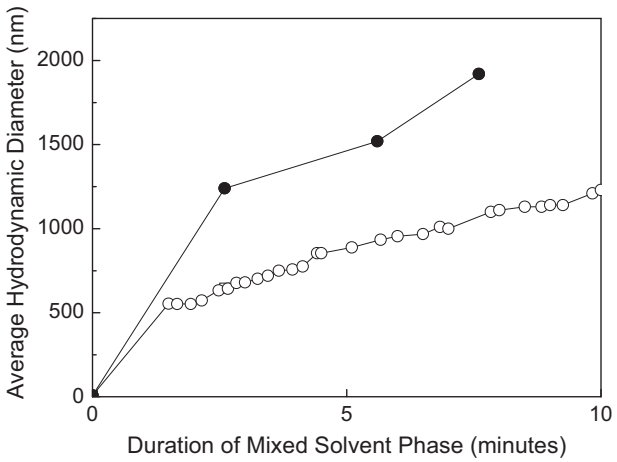

Figure 5. Average hydrodynamic diameter of the particles in 2:3 v/v THF:PBS (○), and in PBS after removal of the THF at 20 °C (●).

While the final sizes can be increased by allowing for growth of the particles before THF removal, a more dramatic change in polymersome size can be obtained by evaporating the THF at different temperatures. After one minute in the mixed solvent phase the THF was evaporated from the samples at 5 °C, 20 °C, 37 °C, and 70 °C. At each temperature evaporation was continued for 30 seconds after the boiling of THF subsided. The samples were analyzed with TEM and optical microscopy, confirming that polymersomes are the exclusive morphology at each temperature (Figure 6a). When the THF is evaporated from samples at 5 °C some of the polymersomes segregate to the top of the PBS, as was seen for extended time delays before evaporation. Removal of the THF at 20 °C yields polymersomes with an average D_h of ~ 1200 nm, as described above. Increasing the temperature to 37 °C and 70 °C led to decreased polymersome diameters of ~ 650 nm and 450 nm respectively (Figure 6b). Thus, using this rapid and simple method, polymersomes are formed upon addition of water, and these rearrange to stable polymersomes during the evaporation process with the final size able to be precisely controlled over the entire range from < 500 nm to > 2 μm by the temperature at which the THF is removed.[25]

Usually polymersome sizes increase when they are prepared at higher temperatures because the corona is more hydrated, hence the solvent-core interactions are greater and the vesicles reduce their curvature (i.e. grow) to minimize the core-solvent surface area.[26] In this case the trend is the opposite, probably because of the properties of the complex corona. The corona, peptide K, is a coiled-coil forming peptide that gradually changes conformation from α-helical to random coil as the temperature increases (Figure A2). This changing secondary structure increases the effective area per corona block, which may be the reason for the decrease in aggregation number.

When a period of particle growth in THF/PBS (at room temperature) was followed by THF evaporation at elevated temperatures, the growth in the mixed solvent phase again resulted in larger polymersomes in PBS, as was the case with evaporation at 20 ºC (Figure 6c). However, the polymersome sizes in PBS were predominantly determined by the temperature during THF evaporation rather than the particle size in THF/PBS. The samples prepared at 70 °C contain very few large polymersomes (Figure 6a), illustrating that the brief time of solvent evaporation at elevated temperature is sufficient for the molecular rearrangements that result in large scale structural changes.

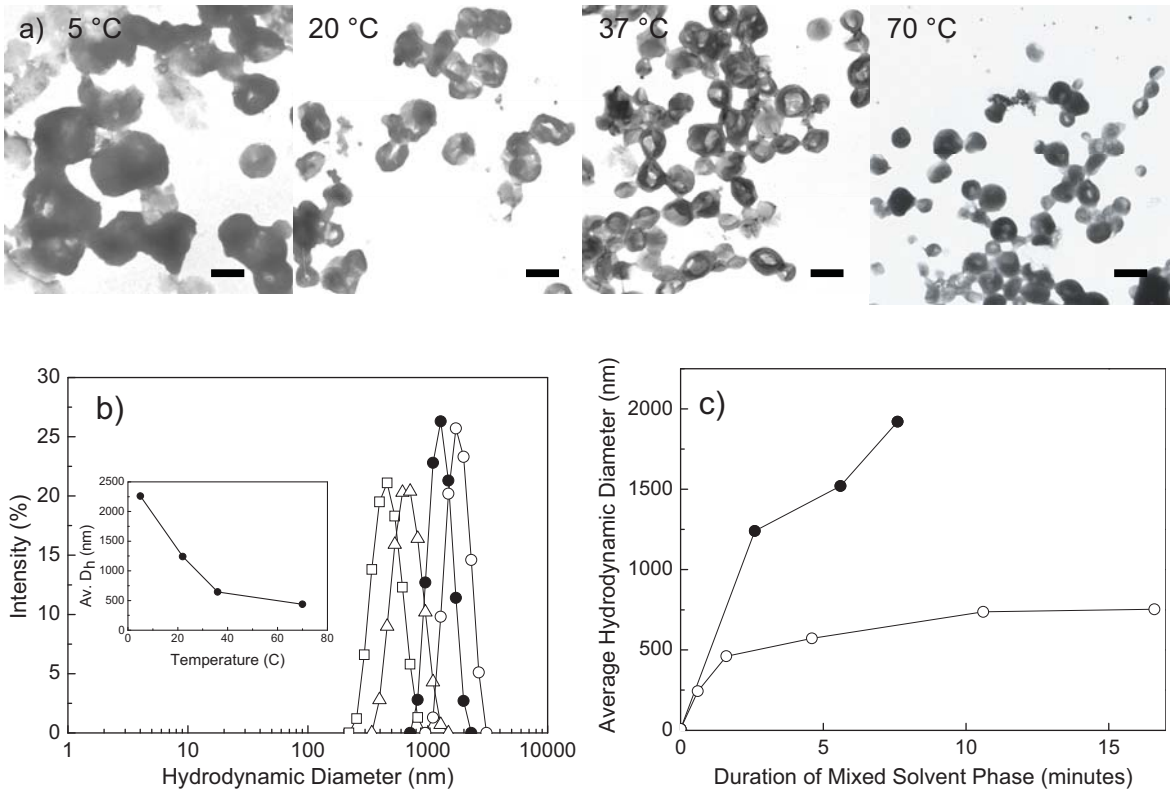

Figure 6. a) PTA stained TEM images of polymersomes prepared at different temperatures. Scale bars = 500 nm. b) The effect of the temperature at which the THF is evaporated on the D_h distributions of $PBLG_{50}$-K vesicles. Inset, average D_h as a function of temperature. c) The average D_h of $PBLG_{50}K$ as a function of the time between PBS addition and THF evaporation at 20 °C (●), and 70° C (○).

Osmolarity of aqueous phase

In all of the experiments described so far the aqueous phase was phosphate buffered saline of physiological ionic strength (50 mM phosphate, 100 mM KCl). At pH 7.0 the hydrophilic block of $PBLG_{50}$-K has a net charge of +3 (resulting from six positively charged lysine side chains and three negatively charged glutamic acid side chains), and the salt in the PBS is expected to affect the repulsion between charged head groups, and hence the self-assembly of the block copolymer. In order to investigate the sensitivity of polymersome size to salt content in the aqueous phase, polymersomes were prepared using aqueous solutions of a range of ionic strengths (Figure 7). In water the polymersome sizes were ~ $1/6^{th}$ the size of those prepared in physiological strength PBS. This is presumably because the absence of charge screening of the peptide K corona increases the repulsion between the charged peptides, resulting in a decrease of the aggregation number.[20] Above 0.5 mM buffer an abrupt increase in the polymersome size was observed, and at 5 mM buffer the particles had essentially the same size as with physiological ionic strength. To put this into perspective, at 5 mM buffer there is a (homogenous) charge density throughout the buffer of 0.03 charges/nm^3, in comparison to the charge density on peptide K of ~ 1 charge/nm^3, indicating a strong adsorption of counterions inside the corona layer.[27]

Without the charge screening the corona repulsion 'freezes' the structures at ~ 200nm: the larger polymersomes that were accessible in PBS by using low temperatures of THF evaporation, or by allowing for a period of growth prior to removing the THF are not accessible due to the strong charge repulsion between corona blocks. Also the conditions which reduce the polymersome size in PBS, such as using an elevated temperatures of THF evaporation or omitting the mixing step do not decrease the particle size in water because the repulsion within the corona overwhelms these effects. Hence the effect of salt on the polymersome sizes dominates the effect of temperature, which in turn dominates the duration of the mixed solvent phase.

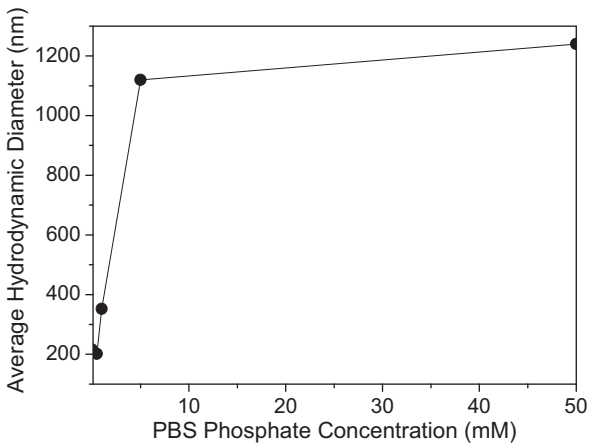

Figure 7. Average hydrodynamic diameter of $PBLG_{50}$-K vesicles as a function of ionic strength of the aqueous phase.

Regarding the salt content of the aqueous phase, this method to prepare polymersomes appears to be robust in comparison to the common slow water addition method followed by dialysis that was first published by Eisenberg and co-workers,[5] which for $PBLG_{50}$-K only worked when using water as the aqueous phase (Figure 8). When buffer was used (5 mM or 50 mM PBS), large undefined aggregates formed, presumably because the long period of stirring that this method required combined with the reduced electrostatic repulsion between sheets at higher ionic strength led to the collapse of hydrated structures.

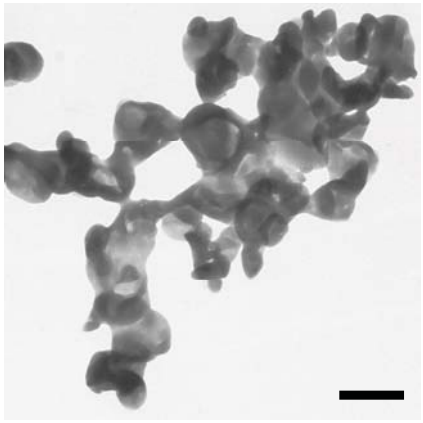

Figure 8. PTA stained TEM image of $PBLG_{50}$-K polymersomes prepared in water by the common slow water addition followed by dialysis method. Scale bar = 500 nm.

Mechanism of polymersome formation

The mechanism of polymersome formation using this method is likely to be similar to the gradual water injection method followed by dialysis, in which there is a structural evolution as water is added that is driven by changes in interfacial tension, core stretching, and repulsion between corona blocks. The most common route is structures of decreasing interfacial curvature, going from spherical micelles to rod-like micelles, lamellar sheets, which close to form polymersomes, and subsequent polymersome growth. Due to the rod-like blocks of $PBLG_{50}$-K the stability of planar interfaces is expected to be quite pronounced,[28] which may alter the route to polymersomes.[29] In the gradual water addition method water is injected very slowly to a solution of the block copolymer in an organic solvent, typically at a rate of ~ 0.2 wt% per minute, to allow for rearrangement of the polymer chains. The water content is increased in this way until the polymersomes are in frozen equilibrium (~ 10-50 wt%), and the organic solvent is then removed by dialysis.[6,16] In this water addition – solvent evaporation procedure the entire aqueous phase is added in less than 2 seconds, inducing rapid morphological reorganization. The structures are then adjusted and frozen by removing the organic solvent under reduced pressure. The reproducibility of the diameters and PDI of the polymersomes that this rapid water addition method yields, and the fact that polymersomes are the exclusive morphology, shows that the limited time available for large scale chain mobility is nevertheless sufficient for well-defined ordering of $PBLG_{50}$-K.

The properties of the polymersomes that form always depend on the characteristics of the block copolymer – and the range of possible block copolymers is what makes polymersomes so interesting. In general the sizes of polymersomes can be varied while the hydrophobic block is mobile. For $PBLG_{50}$-K the charged and structured corona block infers potential to regulate the polymersome sizes over a wide size range. The parameters that have been varied to precisely dictate the final polymersome size are the ratio of THF to PBS, the duration of the mixed solvent period, the temperature at which the THF is evaporated, and the salt content in the aqueous phase.

Different block copolymers

In order to test the scope of this simple technique it was also applied to range of different block copolymers, all of which yielded polymersomes. $PBLG_{35}$-K self-assembled into polymersomes of similar sizes and PDI to those composed of $PBLG_{50}$-K, but with less robust membranes, as seen by degree of collapse upon drying in TEM images (Figure 9a). The PBLG-K block copolymers were designed such that in aqueous solution the hydrophilic block, the designed peptide K, forms a noncovalent coiled-coil complex with peptide E, which has a complementary amino acid sequence.[30] E and K form a parallel dimer (denoted E/K), hence the binding of PBLG-K with E via coiled-coil folding results in a linear supramolecular diblock with the M_w of the corona twice that of PBLG-K.[31] In THF K and E do not fold into the coiled-coil conformation because the polarity is too low.[32] When the water-addition solvent-evaporation procedure was applied to an equimolar mixture of $PBLG_{35}$-K and E the resulting polymersomes were ~ 1/3rd the size of PBLG-K polymersomes (D_h ~ 400 nm, PDI 0.09, Figure 8b). The smaller polymersome diameters are expected because the larger area of the corona block in the polymeric complex increases the curvature of the corona-core interface.

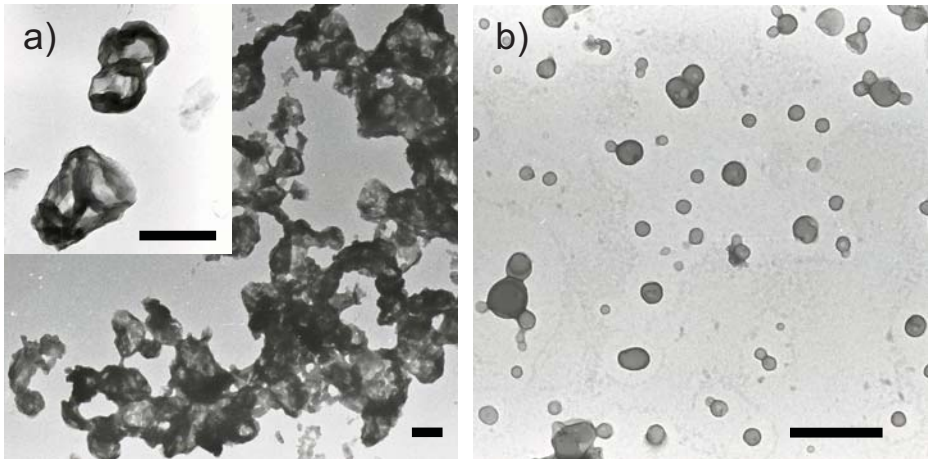

Figure 9. PTA stained TEM images of a) $PBLG_{35}$K polymersomes (inset showing extent of collapse), and b) polymersomes prepared with both $PBLG_{35}$K and E dissolved in the THF. Scale bars = 500 nm.

The method was also applied to a related series of block copolymers, $PBLG_n$-E. Whereas peptide K has a net charge of 3+, peptide E is negatively charged (3-) in PBS. Several $PBLG_n$-E block copolymers were used, with n varying from 37 to 100, and it was shown that all samples formed polymersomes (Figure 10). For $PBLG_{36}$-E the effect of each parameter on the final polymersome sizes was investigated in some detail, and all trends were the same as those observed for $PBLG_{50}$-K. The major difference between the self-assembly of the PBLG-K and PBLG-E block copolymers was that the sizes of the polymersomes formed from the PBLG-E series were significantly smaller than for the PBLG-K block copolymers (see Figure 11 for an example). The electrostatic interactions between corona chains therefore have a strong influence on which polymersome size is energetically favorable, as was described in detail for $PBLG_{50}$-K.

Thus far polymersomes have been prepared with negatively charged (E), positively charged (K), or neutral coronas (E/K). The ability of E and K to form a coiled coil during the solvent evaporation method allows the possibility of preparing polymersomes with different surface functionalities, tailored to particular applications.

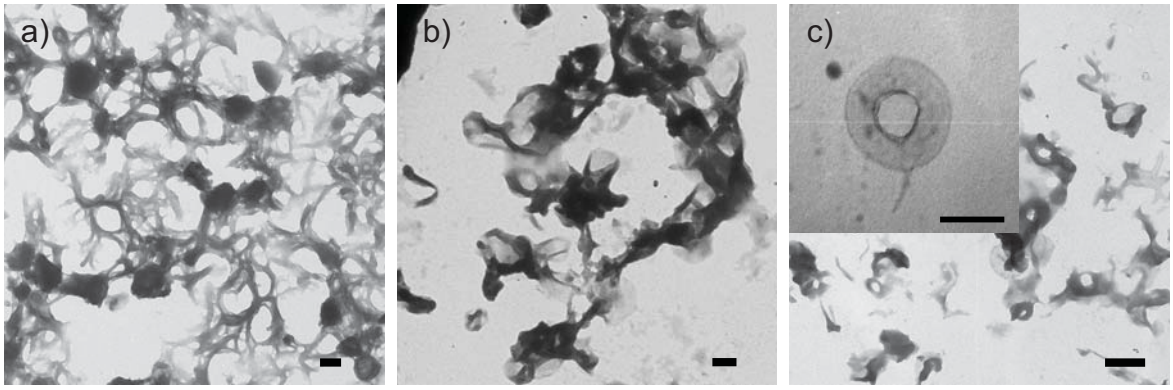

Figure 10. OsO4 stained TEM images of polymersomes prepared using a) $PBLG_{37}$-E, b) $PBLG_{80}$-E, and c) $PBLG_{100}$-E. OsO_4 stains hydrophobic substructures. Scale bars = 200 nm.

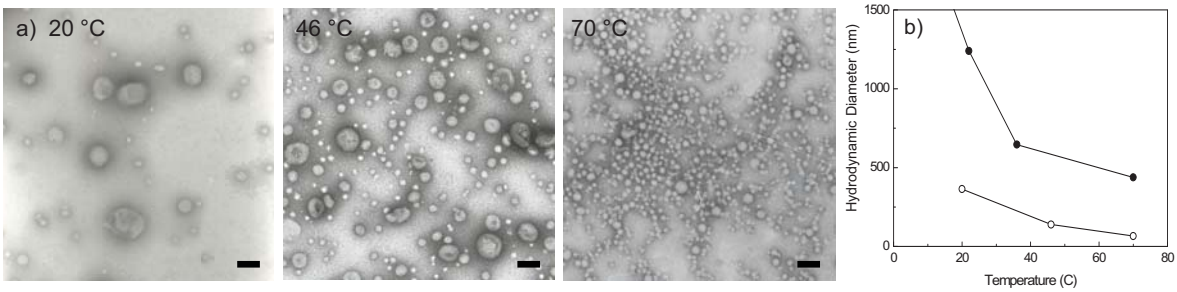

Figure 11. a) PTA stained TEM images of $PBLG_{37}$-E structures prepared at the temperatures indicated. Scale bars = 200 nm. b) The average D_h of $PBLG_{37}$-E structures (○) in comparison to $PBLG_{50}$-K polymersomes (●) as a function of the temperature at which the THF is evaporated.

The technique was additionally applied to traditional synthetic coil-coil block copolymers to further investigate its scope. PS_{106}-PAA_{17} is a polystyrene-*b*-poly(acrylic acid) with block proportions typical of those that have been demonstrated to pack into 'crew cut' polymersomes by the gradual water addition/dialysis method.[20] Using the water addition –

solvent evaporation technique PS_{106}-PAA_{17} formed well defined polymersomes with an average D_h of ~ 150 nm in water. In physiological strength PBS the polymersome sizes are larger, ~ 650 nm (Figure 12a, b), which is due to reduced repulsion between the ionic corona chains, as was also observed for the PBLG-K and PBLG-E series. The method was also employed with non-charged block copolymers that have been shown to form giant polymersomes by direct hydration. Two different poly(ethylene oxide)-*b*-polybutadiene block copolymers (PEO_{26}-PBD_{46} and PEO_{80}-PBD_{125}) formed polymersomes with average D_h values ranging from 180 nm to 350 nm in water and PBS (Figure 12c,d, Table 1), with the membrane thickness clearly depending on the M_n of the block copolymer.

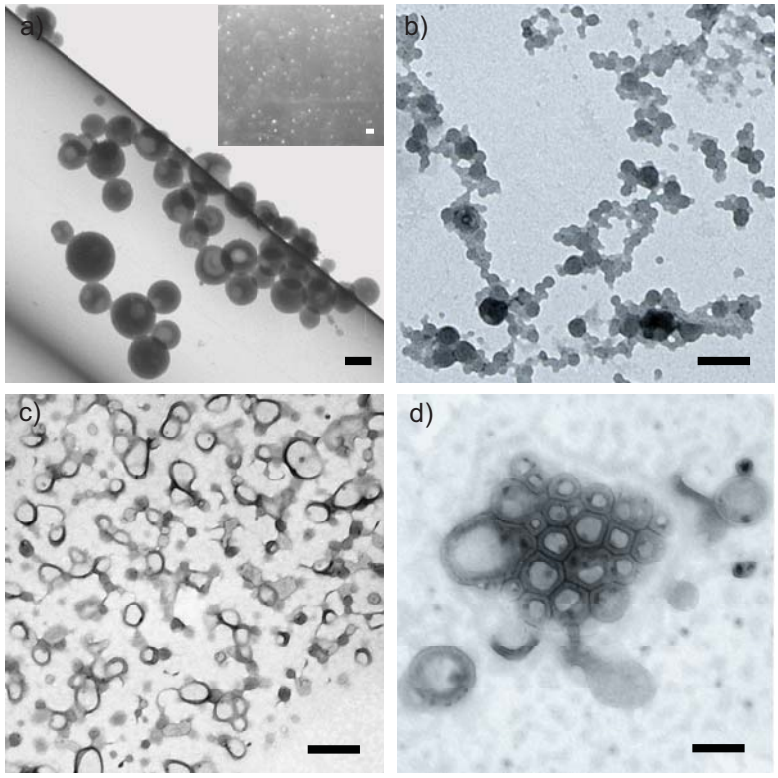

Figure 12. OsO4 stained TEM images of polymersomes prepared from a) PS_{106}-PAA_{17} in PBS (inset, optical microscope image), b) PS_{106}-PAA_{17} in H_2O, c) PEO_{26}-PBD_{46} in PBS, d) PEO_{80}-PBD_{125} in PBS. Scale bars = 200 nm.

Table 1. Different block copolymers to which the water addition – solvent evaporation (WA-SE) method was applied, together with the average hydrodynamic diameters of the polymersomes, and the average sizes using other polymersome preparation methods.

Sample	D_h using WA-SE method, nm		Other methods	D_h
	PBS[a]	Water[b]		
$PBLG_{50}$-K	1200	210	sonication[c]	undefined
$PBLG_{35}$-K	1173	165	sonication[c]	223 nm
$PBLG_{35}$-K/E	398		sonication[c]	152 nm
$PBLG_{37}$-E	192	168	sonication[c]	250 nm
$PBLG_{37}$-E/K	167		sonication[c]	170 nm
$PBLG_{37}$-E/K-PEG	130		sonication[c]	105 nm
$PBLG_{80}$-E	133	140	sonication[c]	385 nm
$PBLG_{100}$-E	149	132	sonication[c]	undefined
PS_{106}-PAA_{17}	600	150	-	-
PEO_{26}-PBD_{46}	200	350	Film hydration[33]	Microns
PEO_{80}-PBD_{125}	180	230	Film hydration[33]	Microns

[a] Conditions: 0.02 mg mL^{-1} block copolymer, 2 mL THF, 3 mL 50 mM PBS, pH 7.0, 20 °C THF evaporation.

[b] Conditions: 0.02 mg mL^{-1} block copolymer, 2 mL THF, 3 mL H_2O, 20 °C THF evaporation.

[c] In PBS, see Chapter 4.

CONCLUSIONS

In this Chapter a rapid, simple, robust, and tunable process for preparing polymersomes is demonstrated. Block copolymers that are molecularly dispersed in the organic solvent THF assemble into polymersomes upon quick addition of an aqueous phase. The fast addition of the aqueous phase does not negatively influence the final polymersome properties. The structures are frozen by evaporation of the THF under reduced pressure. The method itself is very robust, with the additional ability to adjust the polymersome sizes during water addition/solvent evaporation depending on the characteristics of the block copolymer in question. The potential of this method to tune the polymersome sizes is demonstrated with $PBLG_{50}$-K. Because $PBLG_{50}$-K has a charged and structured corona the temperature at which the THF is removed and the salt content of the aqueous phase determine the size of the polymersomes. By using different preparation parameters the same block copolymer assembles into polymersomes with the average size precisely specified within the 200 nm to 2 μm range. The approach is widely applicable as polymersomes were prepared from many types of block copolymers: peptide based rod-rod block copolymers having a range of hydrophobic block lengths, and with positively or negatively charged coronas, noncovalent polymer complexes, and synthetic charged and non-charged coil-coil polymers. The procedure is readily accessible as it requires only standard laboratory equipment, and takes less than five minutes, and a range of vesicle properties are readily accessible by varying the block copolymer/s used or simple procedural parameters.

EXPERIMENTAL SECTION

Materials

Peptides and peptide block copolymers were prepared and characterized as described in Chapter 4. The synthetic coil-coil block copolymers P697-StAA, P7252B-EOCL, P4753-BDEO, and P9055-BdEO were obtained from Polymer Source, Inc.. The molecular characteristics of the peptides and block copolymers are given in Table 1. Phosphate buffered saline, PBS: 30 mM $K_2HPO_4.3H_2O$, 19 mM KH_2PO_4, 100 mM KCl, pH 7.0. Rhodamine B was obtained from Fluka, and butylated hydroxytoluene stabilized tetrahydrofuran was from Biosolve.

Table 1. Molecular Characteristics of the Compounds used in this Study

Name	structure	M_n (g/mol)	PDI[3]
E	Ac-G(E I A A L E K)$_3$-NH_2	2380.6[1]	
$PBLG_{37}$-K	$PBLG_{37}$-G(K I A A L K E)$_3$-NH_2	10135[2,3]	1.3
$PBLG_{50}$-K	$PBLG_{50}$-G(K I A A L K E)$_3$-NH_2	13279[2,3]	1.5
$PBLG_{36}$-E	$PBLG_{36}$-G(E I A A L E K)$_3$-NH_2	10230[2,3]	1.1
$PBLG_{55}$-E	$PBLG_{55}$-G(E I A A L E K)$_3$-NH_2	14396[2,3]	1.3
$PBLG_{80}$-E	$PBLG_{80}$-G(E I A A L E K)$_3$-NH_2	19877[2,3]	1.4
$PBLG_{100}$-E	$PBLG_{100}$-G(E I A A L E K)$_3$-NH_2	24262[2,3]	1.4
$PBLG_{250}$-E	$PBLG_{250}$-G(E I A A L E K)$_3$-NH_2	57148[2,3]	1.7
PS_{106}-PAA_{17}		12240[4]	1.11[4]
PEO_{26}-PBD_{46}		3800[4]	1.04[4]
PEO_{80}-PBD_{125}		10400[4]	1.1[4]
PEO_{45}-PCL_{105}		15500[4]	1.4[4]

[1] Obtained from MALDI-TOF MS. [2] Based on a comparison of ^{1}H-NMR peaks. [3] GPC calibrated with polystyrene standards. [4] As specified by Polymer Source, Inc.. PBLG: poly(γ-benzyl L-glutamate), amino acids in the designed peptides are represented by their one letter codes, Ac: acetyl.

Preparation of polymersome suspensions

The majority of the polymersome suspensions were prepared as follows, with variations to this method described in the text. The block copolymer (0.02 μmol, or 0.01 μmol each of the PBLG-peptide block copolymer and the complementary peptide) was dissolved in 2 mL tetrahydrofuran (THF) in a 50 mL round bottomed flask. 3 mL of PBS was added in less than 2 seconds and vortexed for one minute at 200 rpm. The THF was evaporated using a rotary evaporator (40 rpm, water bath at 20 °C), yielding the polymersome suspensions. The final concentration of block copolymer was 6.7 μM.

For encapsulation experiments 2 μM rhodamine B in PBS was used. To test the permeability of the polymersomes 100 μL of 20 μM rhodamine B in PBS was added after vortexing.

For comparison with the current rapid preparation of polymersomes, samples were also prepared with the commonly used slow addition of the aqueous phase.[5,16] The block copolymer (0.02 μmol) was dissolved in 2 mL THF in a 50 mL round bottomed flask. 3 mL of PBS (5 mM and 50 mM) or water was added dropwise at a rate of 0.2 wt% (4 μL) per minute using a syringe pump (NE-300, just infusion, Prosense .B.V), while stirring at 700 rpm. The samples only contained well-defined aggregates (DLS) when there was no salt in the aqueous phase, and these were dialyzed against water at room temperature for at least 24 hours with at least 5 changes of buffer using dialysis tubing with a molecular weight cut-off of 1000 g/mol.

Characterization of polymersome suspensions

Circular Dichroism

Circular Dichroism spectra were obtained as detailed in the experimental section of Chapter 2, except that the temperature dependent spectra were measured in 5 °C intervals.

Dynamic Light Scattering

Dynamic light scattering was conducted as detailed in Chapter 3. To calculate the hydrodynamic diameter of objects in THF and mixtures of THF and water/PBS, viscosities and refractive indices of were used from reference 17.

Transmission Electron Microscopy

Transmission electron microscopy was conducted as detailed in the experimental section of Chapter 5.

Optical Microscopy

Differential interference contrast (DIC) and fluorescence optical micrographs were recorded with a Zeiss axiovert-200 inverted microscope equipped with a 63 x objective long-range working lens. The images were recorded with a black and white CCD camera (AxioCam MRm) connected to image-recording and -processing software (Axiovision 4.4).

Nuclear Magnetic Resonance

In order to quantify the amount of THF remaining in the PBS after the standard evaporation time ^{1}H-NMR spectra were recorded on a Bruker DPX300 spectrometer at room temperature. The proton resonance of water was used for calibration.

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18. The method was tested with chloroform/methanol, DCM, and THF. Using $CHCl_3$/MeOH and DCM with 50 or 5 mM PBS or milli-Q water resulted in precipitation. With the water immiscible organic solvents monomodal size distributions with reasonably low PDI were observed in some instances with DLS, but macro aggregates were always visible.
19. $PBLG_{50}$-K is α-helical in THF, as determined by circular dichroism. The length of an average $PBLG_{50}$-K cylinder with complete α-helical secondary structure is 11 nm. The effective 'spherical' diameter of rods can be obtained by: L/ln(L/D), where L is the length of the rod, and D is the diameter. For PBLG50K this gives ~ 6.5 nm. For small objects the hydrodynamic diameter can be considerably larger than the actual object, hence a hydrodynamic diameter of 8 nm is in the expected range for an object with an effective diameter of 6.5 nm.
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23. Electron microscopy images of the intermediate structures, or of the particles prior to THF evaporation were not reliable due to evaporation of the THF during grid preparation, which could change the morphology of the structures.
24. Adding THF to decrease the H_2O:THF ratio decreases the hydrodynamic diameter.
25. Polymersomes form upon water addition to the THF solution, and their size is adjusted by the temperature at which the THF is evaporated. Note that when rhodamine B is added to polymersomes in the mixed solvent phase and then the THF evaporated at different temperatures all final polymersomes, be they larger or smaller than the polymersomes in the mixed solvent contain rhodamine B, hence during both size incease and size decrease processes the membrane in permeable.
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APPENDICES

Addition of aqueous buffer

When PBS is added to the solution of $PBLG_{50}$-K in THF, polymersomes self-assemble, which are observed to increase in time with DLS and optical microscopy (Figure A1). This effect could be due to polymersome ripening, fusion, or aggregation, or a combination of the three. DLS is not well suited to distinguish between these phenomena. With optical microscopy the particles were sometimes observed in pairs as they diffused through the solution, although a fusion event was not observed. The large particles that are present after ~ 20 minutes do not appear to be clusters of smaller ones. However, the resolution of the particles is not great, therefore fusion and/or aggregation cannot be ruled out. For the predominant growth mechanism to be ripening or fusion, the PBLG blocks must be mobile under these solvent conditions (2:3 v/v THF:PBS), and the particles may slowly approach the thermodynamic particle size. The experiment was repeated with a lower ratio of THF to PBS (0.5:3 v/v THF:PBS). The particles were smaller (average D_h ~ 650 nm, PDI ~ 0.2) and there was no particle growth (Figure A1). These two differences could be because the PBLG chains are effectively immobile in the more polar mixture, and the particles are therefore unable to ripen or fuse, or alternatively this may be the equilibrium size in this solvent mixture. The equilibrium size of polymersomes depends in part on the effective size of the corona K, which is expected to be larger in more polar solvent mixtures as then the K-solvent interaction is greater (Yu, Y. S.; Zhang, L. F.; Eisenberg, A. *Macromolecules* **1998,** 31, (4), 1144-1154. Adams, D. J.; Kitchen, C.; Adams, S.; Furzeland, S.; Atkins, D.; Schuetz, P.; Fernyhough, C. M.; Tzokova, N.; Ryan, A. J.; Butler, M. F. *Soft Matter* **2009,** 5, (16), 3086-3096).

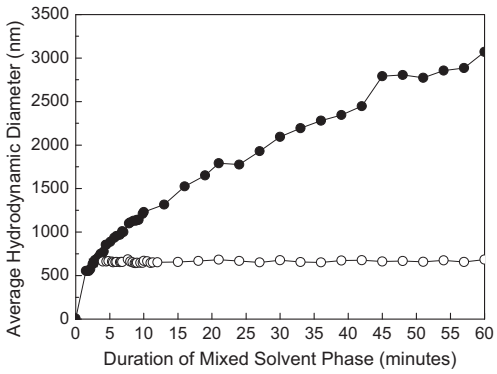

Figure A1. The change in average D_h of $PBLG_{50}K$ assemblies with time after addition of PBS (2:3 v/v THF:PBS (●), 0.5:3 v/v THF:PBS (○)).

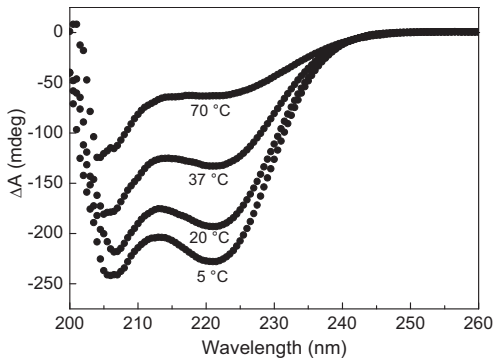

Figure A2. CD spectra of K at increasing temperatures. Conditions: [K] = 2 mg/mL, PB