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Evaluation of the effects of nanoprecipitation process parameters on the size and morphology of poly(ethylene oxide)-*block*-polycaprolactone nanostructures

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Keywords:

Block copolymers; Nanoparticles; Self-assembly; Nanoprecipitation; Statistical models; Process optimization

Abstract:

Nanoprecipitation is a straightforward method for the production of block copolymer nanoparticles for drug delivery applications. However, the effects of process parameters need to be understood to optimize and control the particle size distribution (PSD). To this end, we investigated the effects of material and process factors on PSD and morphology of nanoparticles prepared from an amphiphilic diblock copolymer, poly(ethylene oxide)-*block*-polycaprolactone. Using a Design of Experiments approach, we explored the joint effects of molecular weight, block length ratios, water volume fraction, stirring rate, polymer concentration and organic phase addition rate on hydrodynamic size and polydispersity index of the nanostructures and created statistical models explaining up to 94 % of the variance in hydrodynamic diameter. In addition, we performed morphological characterization by cryogenic transmission electron microscopy and showed that increasing the process temperature may favor the formation of vesicles from these polymers. We showed that the effects of process parameters are dependent on the polymer configuration and we found that the most useful parameters to fine-tune the PSD are the initial polymer concentration and the stirring rate. Overall, this study provides evidence on the joint effects of material and process parameters on PSD and morphology, which will be useful for rational design of formulation-specific optimization studies, scale-up and process controls.

Introduction

Nanoparticles (NPs) prepared from amphiphilic block copolymers (BCPs) have been the subject of intense study in the last three decades. Widespread interest in BCP NPs results from their attractive properties, such as high colloidal stability, synthetic tailorability, and ease of preparation via simple bottom-up type processes based on molecular self-assembly. One of the major application areas for BCP NPs is their use as colloidal drug delivery vehicles and such systems have already reached the clinics (Anselmo and Mitragotri 2019; Bobo, et al. 2016; Werner, et al. 2013).

Amphiphilic BCPs have the ability to assemble, in aqueous solutions, into aggregates of various morphologies, such as spherical micelles, worm-like micelles and vesicles (polymersomes). This microphase separation is due to the mutual repulsion between the constituent blocks and for a given polymer structure, the thermodynamic equilibrium morphology is essentially determined by the relative volumes occupied by the two blocks (Discher and Ahmed 2006; Mai and Eisenberg 2012), commonly expressed by the hydrophilic volume fraction, f. However, the morphology is also heavily influenced by the NP manufacturing method and parameters, such as solvent and temperature, as the polymer chains can be trapped into kinetically (meta)stable morphologies (Hayward and Pochan 2010).

In drug delivery applications, poly(ethylene oxide) is by far the most widely used polymer for the hydrophilic block (Adams, M. L., et al. 2003). Common choices for the hydrophobic block include degradable polyesters, such as poly(lactic acid), poly(glycolic acid), poly(lactic-co-glycolic acid) and poly(ε-caprolactone) (PCL), of which PCL shows the slowest degradation (Adams, M. L., et al. 2003; Woodruff and Hutmacher 2010). The diblock poly(ethylene oxide)-*block*-polycaprolactone (PEO-*b*-PCL) and slight variations thereof have been used as a carrier material for various active ingredients and imaging agents in the form of solid NPs (Ali, et al. 2017; Allen, et al. 1998; Deng, et al. 2014; Dong, et al. 2010; Elmowafy, et al. 2019; Gref, et al. 1994; Lim Soo, et al. 2005; Liu, J. and Gan 2014; Montazeri Aliabadi, et al. 2005; Park, Y. J., et al. 2002; Patel, et al. 2009; Ryu, et al. 2000; Shalaby, et al. 2016; Shi, et al. 2005; Villamil, et al. 2019) or vesicles (Ahmed and Discher 2004; Ghoroghchian, et al. 2006; Hannecart, et al. 2019; Lin, et al. 2018; Park, M., et al. 2015; Yang, et al. 2014; Zou, et al. 2015). The self-assembly behavior and critical values of f specific to PEO-*b*-PCL have extensively been studied elsewhere (Adams, D. J., et al. 2009; Du, et al. 2007; Giacomelli and Borsali 2006; Qi, et al. 2013; Zhou, et al. 2017).

For drug delivery systems, control over the particle size and shape is crucial, because they largely influence NP accumulation in various tissues upon intravenous administration, transport through physiological barriers, uptake by immune cells and clearance (Hoshyar, et al. 2016). Due to the unique self-assembly behavior of amphiphilic BCPs, the particle size and shape can be controlled by both material and process factors and it is necessary to evaluate the effects of these factors in detail in the laboratory scale to allow further formulation-specific process optimization, scale-up and process controls.

The nanoprecipitation method, also known as the solvent displacement or anti-solvent precipitation method, is one of the most popular methods for the manufacture of polymer NPs for pharmaceutical applications (Fessi, et al. 1989; Liu, Y., et al. 2020; Martínez Rivas, et al. 2017; Miladi, et al. 2016). It is a facile and rapid method and does not require high energy input or large amounts of toxic solvents (Miladi, et al. 2016). In this method, polymer and other hydrophobic agents dissolved in a water-miscible solvent are added to an aqueous non-solvent phase under stirring, resulting in the formation of NPs by precipitation. Several studies have been performed to explain the mechanisms of particle formation by nanoprecipitation (Lavino, et al. 2017; Lebouille, J. G. J. L., et al. 2014; Lince, et al. 2008; Saad and Prud'homme 2016). Besides the polymer structure and configuration, the most critical process parameters influencing the PSD are the choice of solvent, precipitation temperature, order of addition, solvent/non-solvent ratio and ionic strength of the aqueous phase (Liu, Y., et al. 2020).

In this work, we studied how various material and process factors influence the size and morphology of PEO-*b*-PCL aggregates prepared by the nanoprecipitation method in an acetone-water system. Using a statistical approach, we evaluated the effects of f, molecular weight, mixing speed, organic phase addition rate, polymer concentration in the organic phase, water volume fraction, and precipitation temperature on the hydrodynamic diameter and polydispersity index (PDI) and examined the corresponding morphologies by cryogenic transmission electron microscopy (cryo-TEM). To the best of our knowledge, detailed mapping of PEO-b-PCL morphologies generated by an acetone-water nanoprecipitation method has not been previously presented in the literature. Based on the gathered data, we created statistical models describing the joint effects of multiple process variables on the particle size distribution. Finally, we studied the thermal stability of the nanostructures and explored the role of kinetic control over particle size and morphology. By combining a Design of Experiments process optimization approach with a detailed morphological characterization of the nanostructures by cryo-TEM, this work provides useful insight into the underlying relationships between the process parameters and product quality for this particular method.

Materials and methods

Materials

Polycaprolactone-*block*-poly(ethylene oxide) methyl ether polymers (PEO-*b*-PCL) were purchased from PolymerSource, Inc. (Canada). According to the supplier, molecular weight distributions of the block copolymers were analyzed using size exclusion chromatography to determine the molecular weight of the PEO block and ^{1}H NMR to determine the final composition of the copolymer by comparing the integrated areas of PEO and PCL proton peaks. In addition, melting and glass transition temperatures of the polymers were analyzed using differential scanning calorimetry. Characteristics of the polymer samples are listed in Table 1. Volume fraction of PEO block (f_{PEO}) was calculated based on the molecular weights using literature values for solid densities of PEO and amorphous PCL at room temperature (1.15 and 1.081, respectively)(Crescenzi, et al. 1972; Rowe, et al. 2009). Acetone (≥ 99.5 %) was purchased from Sigma.

Table 1. Molecular weight data of the tested block copolymers. f_{PEO} is the PEO volume fraction calculated based on molecular weight and density of each polymer and N is the total degree of polymerization.

Product	PEO M_n (g/mol) [1]	PCL M_n (g/mol) [1]	M_w/M_n [1]	N	f_{PEO}	Short name
P5041-EOCL	2 000	1 000	1.10	54	0.65	PEO_{45}-*b*-PCL_9
P7170-EOCL	2 000	1 400	1.2	57	0.57	PEO_{45}-*b*-PCL_{12}
P9681-EOCL	2 000	10 000	1.19	133	0.16	PEO_{45}-*b*-PCL_{88}
P18129A-EOCL	5 000	5 000	1.18	157	0.48	PEO_{113}-*b*-PCL_{44}
P19764-EOCL	5 000	11 500	1.19	214	0.29	PEO_{113}-*b*-PCL_{101}
P19769-EOCL	5 000	25 000	1.33	332	0.16	PEO_{113}-*b*-PCL_{219}

[1] Reported by the supplier.

Experimental design

The effects of the volume fraction of water (ϕ_w), concentration of polymer in solvent (c_P), addition rate of solvent phase to the non-solvent (*A*) and rate of stirring (*S*) on the mean hydrodynamic diameter (d_H) and PDI were evaluated in this study using a Design of Experiments (DoE) approach. To allow the modelling of linear, quadratic and two-way interaction effects of each factor with a minimal amount of experimental runs, a D-optimal split-plot experimental design with three levels for each factor was created using the DoE functionality of JMP Pro 14 software (SAS). Similar approaches have been taken by several authors to optimize NP formulations (Elmowafy, et al. 2019; Günday Türeli, et al. 2016; Mohamadpour, et al. 2020; Tahir, et al. 2017).

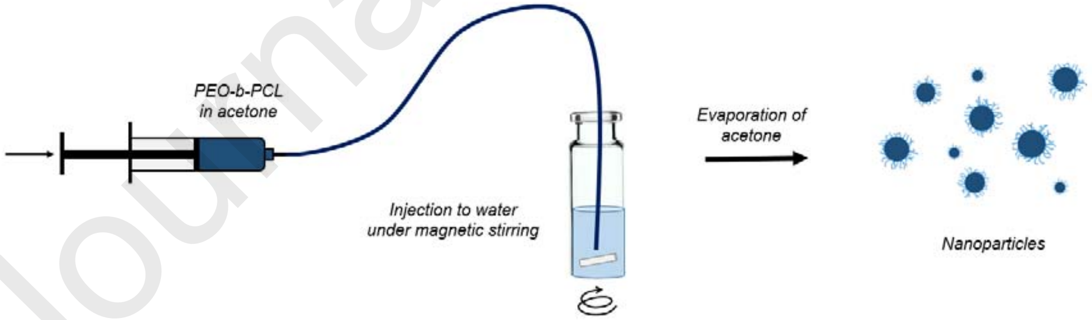
The chosen process parameters and their nominal values are shown in Table 2. The defined set of 24 experimental runs (Table S1) was completed for all six polymer samples. Firstly, the full 144 runs were performed by one operator. Secondly, the runs for 5000 g/mol PEO block polymers were repeated by a different operator to gain information on reproducibility of the nanoprecipitation method and data for model validation.

Table 2. Process parameters included in the study.

Factor	**Symbol**	**Unit of measurement**	**Nominal values**		
Volume fraction of water	ϕ_w	-	0.43	0.62	0.71
Polymer concentration in solvent phase	c_P	mg/mL	6	13	20
Addition rate of solvent phase	A	mL/h	10	130	250
Stirring rate	S	rpm	120	460	800

Nanoprecipitation

To prepare NPs by the nanoprecipitation method, the copolymer was firstly dissolved in acetone and loaded into a plastic syringe and a steel tip was attached. The syringe was then installed onto a syringe pump (PHD2000, Harvard Apparatus), a piece of low-density polyethylene tubing was attached and the solution was injected into 1.2 mL of Milli-Q water in a 4 mL glass vial under magnetic stirring at a controlled rate. During injection, the end of the tubing was immersed below the surface of water, close to the spinning magnet (see Scheme 1).



Scheme 1. Preparation of PEO-b-PCL nanoparticles by the nanoprecipitation method.

The resulting dispersions were stirred at room temperature for 20-24 h in open vials to allow evaporation of acetone. All samples were prepared at room temperature, unless otherwise specified, stored at room temperature in closed vials, and dynamic light scattering (DLS) analysis was performed within three days from preparation. Acetone was chosen as the organic solvent due to its miscibility with water, ability dissolve both polymer blocks, low toxicity (ICH Q3C Class 3) (EMA 2019), and facile removal by evaporation.

Dynamic Light Scattering

Particle size distributions were analyzed by DLS using Zetasizer Nano ZS (Malvern) at a scattering angle of 173 °. Attenuator setting, measurement position and the number of runs per measurement were chosen automatically by the instrument, compensating for variations in scattering signal strength arising from differences in particle size and concentration. The Zetasizer software (v. 7.11) calculates mean hydrodynamic diameter and PDI based on particle diffusivity. No pre-treatments (*e.g.*, filtering and extrusion) were performed prior to DLS measurement. All measurements were performed in triplicates.

Cryo-TEM analysis

Selected samples were imaged by cryo-TEM. No pre-treatments (*e.g.*, filtering and extrusion) were performed for the samples. A 5 µL drop of nanoparticle dispersion (2 mg/mL) was applied onto plasma-treated lacey carbon coated copper grids (Electron Microscopy Sciences). The drop was blotted with filter paper and plunged into liquid ethane (–170 °C) using an automatic plunge freezer (EM GP2, Leica) and stored in liquid nitrogen. The samples were inspected using JEM-3200FSC (JEOL) microscope at 300 kV acceleration voltage and images were acquired using Digital Micrograph software (Gatan).

Results and discussion

Effects of polymer configuration on size and morphology

To study the effects of polymer molecular weight and block lengths, nanoparticles were prepared by the nanoprecipitation method, as described above, and a general characterization by DLS and cryo-TEM was performed. The process parameters used in the preparation of these NP samples are listed in Table S2. Representative micrographs of the formed nanostructures are shown in Figure 2, including DLS size distribution histograms based on scattering intensity. A summary of the observed nanoscale morphologies is shown in Table 3.

Nanostructures of various shapes were observed in the studied samples. For PEO$_{45}$-*b*-PCL$_9$ ($f_{PEO} = 0.65$) and PEO$_{45}$-*b*-PCL$_{12}$ ($f_{PEO} = 0.57$), the dominant morphology was long thread-like micelles with a diameter of 13 ± 2 nm and 13 ± 3 nm, respectively, and length up to several microns (Figure 2 a-b). In addition, a small population of spheres with an average diameter of 12 nm was observed in both samples. For all other samples, the dominant nanoscale morphology was spherical. As measured from the micrographs, the diameter of the spheres was 26 ± 9 nm for PEO_{45}-*b*-PCL_{88}, 25 ± 9 for PEO_{113}-*b*-PCL_{44}, and 17 ± 7 nm for PEO_{113}-*b*-PCL_{101}. Besides spheres, PEO_{113}-*b*-PCL_{44} samples contained also notable amounts of worm-like micelles. PEO_{45}-*b*-PCL_{88} samples also contained small amounts of sheet-like and lamellar structures.

The morphology assumed by PEO-b-PCL has been shown to be highly dependent on both material and process factors and the overlapping of multiple different morphologies has been commonly reported even for narrow molecular weight distribution polymers. For example, Adams *et al.* studied vesicle formation induced by slow sequential addition of water into a solution of PEO-b-PCL in tetrahydrofuran (THF) and mapped the resulting aqueous equilibrium morphologies as a function of f_{PEO}. They showed that vesicles were the dominant morphology at $f_{PEO} \approx$ 0.15 – 0.23, whereas mainly worm-like micelles were present at $f_{PEO} \approx$ 0.25 – 0.32 and spherical micelles at $f_{PEO} \geq$ 0.3, with a partial overlap of different morphologies (Adams, D. J., et al. 2009). In another study, polymersomes were successfully generated from samples with f_{PEO} ranging from 0.14 to 0.27 and PEO molecular weight between 750 and 3800 g/mol using an aqueous hydration and extrusion method (Qi, et al. 2013).

In the present work, no vesicles were observed for any of the samples, even though polymers similar to PEO_{45}-*b*-PCL_{88} ($f_{PEO} = 0.16$) have been used to form vesicles, as described above, using different methods. Similar findings on the inability of the room-temperature solvent displacement methods to produce PEO-b-PCL polymersomes can be found in the literature (Sui, et al. 2015; Zhou, et al. 2017). Sui *et al*. demonstrated that PEO-b-PCL with f_{PEO} ranging from 0.17 to 0.32 formed spherical micelles by solvent displacement method using a THF-water system, whereas a mixture of spherical micelles and polymersomes was obtained by thin film hydration and direct hydration methods (Sui, et al. 2015). In another study, Zhou *et al*., successfully prepared PEO-b-PCL vesicles by solvent displacement using a THF-water system at elevated temperatures (> 50 °C), whereas an acetone-water system was unable to produce vesicles regardless of precipitation temperature (Zhou, et al. 2017).

The formation of high aspect ratio thread-like morphology of PEO-*b*-PCL with $f_{PEO} \geq 0.5$ precipitated from acetone (Figure 2a-b) in the present work is in agreement with the work of Giacomelli and Borsali (Giacomelli and Borsali 2006). In their work, PEO-b-PCL with $f_{PEO} =$ 0.50 – 0.58 formed spherical micelles when dialyzed from THF or dimethylformamide (DMF), but resulted in long thread-like micelles when acetone was used as the starting solvent.

The formation of vesicles is known to occur through a sequence of morphologies: individual polymer chains first aggregate to spherical micelles, which then transform to rods and finally vesicles as the water concentration is increased. This sequence can be explained as an attempt to minimize the free energy associated with the interface between the PCL core and the surrounding solvent by aggregate fusion and reorganization into structures of reduced curvature to minimize the interfacial area (Choucair and Eisenberg 2003). Besides interfacial energy, other important driving forces for these transitions include the entropic process of stretching of the core-forming block and possible electrostatic repulsion between corona-forming blocks (Choucair and Eisenberg 2003). In this work, a methoxy-terminated PEO was used as the corona-forming block, which is charge-neutral, hence electrostatic repulsion does not play a role here.

Compared to THF, acetone is a poorer solvent for PCL (Bordes, et al. 2010). Using a poorer solvent will reduce the degree of swelling of the core and the degree of PCL chain stretching. Diminished core chain stretching reduces the driving force for rearrangement into cylinders and vesicles and results in a kinetically trapped metastable morphology, a phenomenon that has been described in detail for polystyrene-block-poly(acrylic acid) polymers (Choucair and Eisenberg 2003). This can explain the appearance of thread-like micelles at $f_{PEO} \geq 0.5$ as well as the lack of vesicles at $f_{PEO} = 0.16$ when using this water-acetone system.

In addition to the nanostructures shown in Figure 4 and Table 3, all of the studied samples contained small amounts of larger objects of various shapes in the micron-scale. This was expected as the samples were not filtered or extruded. Such pre-treatments were omitted to obtain samples that are representative of the true results of the nanoprecipitation process. The observed large structures may result from contamination, impurities, agglomeration of particles or undissolved polymer.

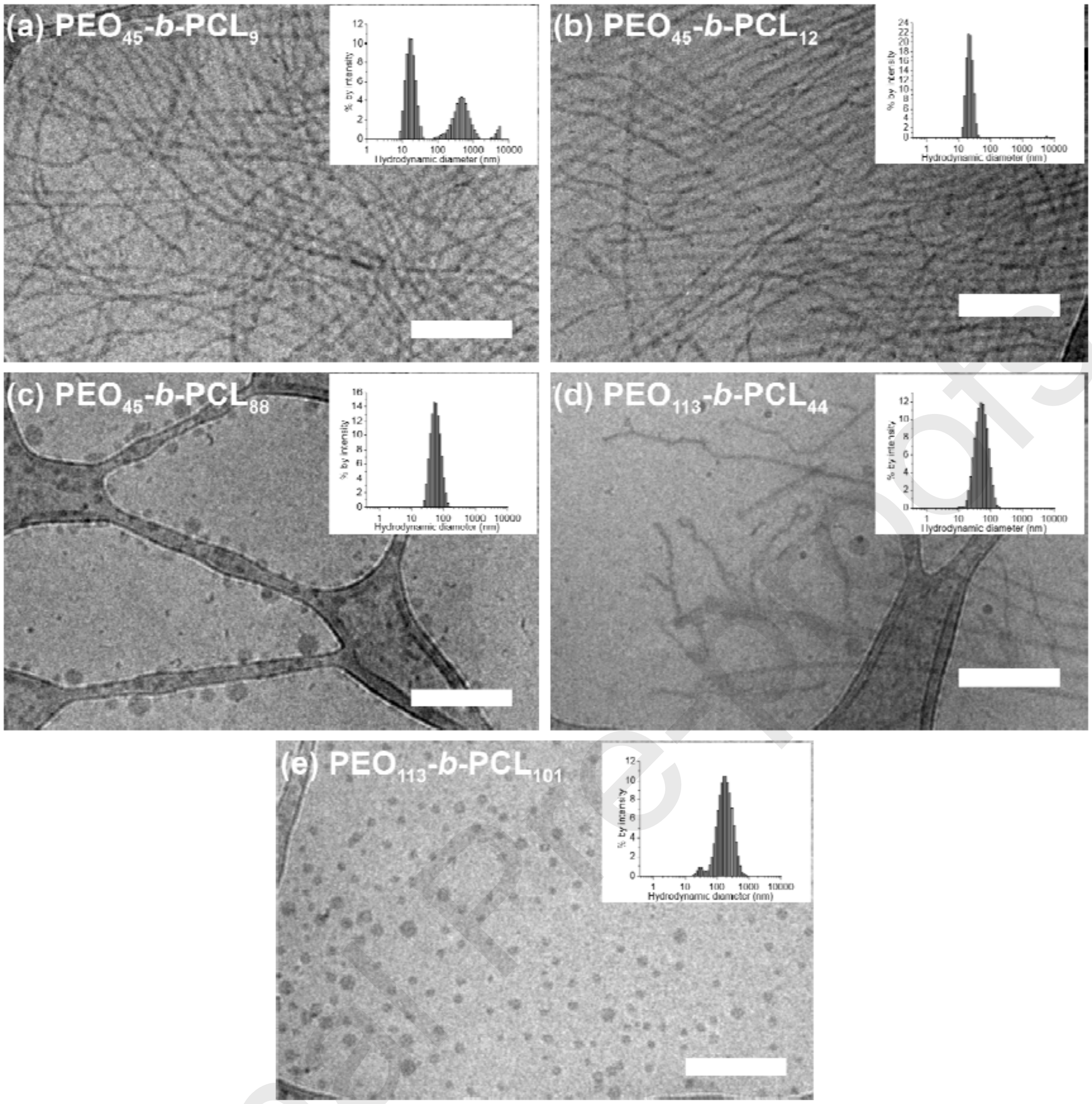


Figure 2. Representative micrographs of nanostructures formed from PEO-b-PCL with different block lengths. Size distributions obtained by dynamic light scattering are presented in inserts. Nanoprecipitation was performed at room temperature. Scale bars are 200 nm. Dark thick objects are the carbon film of the cryo-TEM grid.

Table 3. Summary of the observed nanoscale morphologies, where S = solid spheres, W = worm-like or fiber-like structures, L = lamellar or sheet-like structures, V = vesicles. Multiple morphologies are listed in order of abundance.

Polymer	**N**	f_{PEO}	**Nanoscale morphology**		
			Precipitation at R.T.	**Precipitation at 65 °C**	**Precipitation at R.T. + heat-treatment**
PEO_{45}-*b*-PCL_9	54	0.65	W, (S)	W, S	--
PEO_{45}-*b*-PCL_{12}	57	0.57	W, S	W, S	--
PEO_{45}-*b*-PCL_{88}	133	0.16	S, L	S, L, V	--
PEO_{113}-*b*-PCL_{44}	157	0.48	S, W	S, (W)	S, (W)
PEO_{113}-*b*-PCL_{101}	214	0.29	S	S	S, (V)

Statistical overview on the effect or process factors on PSD

Firstly, a simple linear correlation analysis was performed for the obtained DLS data. PEO_{45}-*b*-PCL_9 ($f_{PEO} = 0.65$) and PEO_{45}-*b*-PCL_{12} ($f_{PEO} = 0.57$) samples were excluded from all statistical analyses due to a non-spherical morphology. Linear correlations between the variables were evaluated by calculating Pearson correlation coefficients (Table S3), also presented in the form of heat maps in Figure 3. The numerical data (Table S3) shows that d_H and PDI correlated significantly ($p \leq 0.05$) only with *N*, f_{PEO}, ϕ_w and with each other. The effects of *N*, f_{PEO} and ϕ_w are shown in Figure 4 in the form of dot plots. The data shown in Figures 3-4 and Table S3 contain all the acquired d_H values, including those associated with very high PDI and multimodal PSD. As the PDI values approach unity and multiple peaks appear, it becomes meaningless to use d_H to describe the PSD. Despite these limitations, some conclusions can be made based on the obtained data.

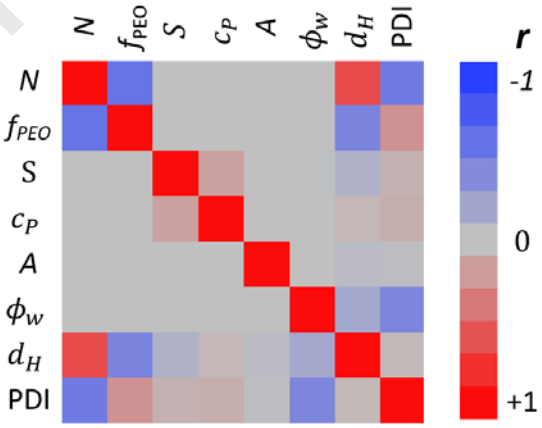


Figure 3. Linear correlations of variables visualized by heat maps, where N = polymerization degree, f_{PEO} = PEO volume fraction, S = stirring rate, c_P = concentration of polymer in organic

phase, A = addition rate of organic to water, ϕ_W = water volume fraction, d_H = mean hydrodynamic diameter and PDI obtained by DLS. Data from polymers with a non-spherical dominant morphology have been excluded from the analyses. For the colored figure, the reader is referred to the web version of this article.

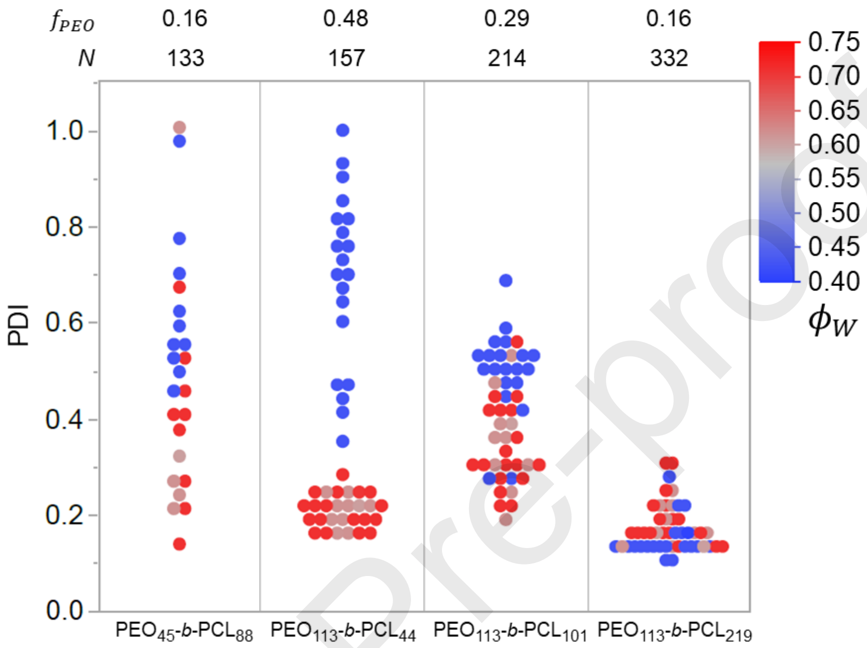


Figure 4. Dot plot of polydispersity index (PDI) values as a function of PEO block volume fraction (f_{PEO}), total degree of polymerization (N) and water volume fraction (ϕ_w). For the colored figure, the reader is referred to the web version of this article.

Firstly, the degree of polymerization (or molecular weight, MW) had a negative correlation with PDI, indicating that higher MW polymers resulted in a narrower PSD. This can be partly explained by the higher degree of supersaturation achieved for high MW polymers due to their lower solubility. The nanoprecipitation method is based on rapid reduction of solvent quality, leading to the precipitation of NPs. High supersaturation favors nucleation over particle growth, leading to small particles with a narrow PSD (Johnson and Prud'homme 2003).

Another explanation for high PDI values at low MW is that a wider range of morphologies is obtained with lower molecular weight polymers, as can be seen from Figure 2 and Table 3. This explanation is supported by the findings of Du *et al*., who showed by TEM analysis that PEO-*b*-PCL with higher PEO block lengths (5000 g/mol) assumed a spherical morphology through a wide range of PCL block lengths, whereas polymers with shorter PEO blocks (2000 g/mol) showed more diverse morphologies (spheres, worm-like micelles, and lamellae) over the PCL block lengths studied (Du, et al. 2007).

Secondly, for PEO_{113}-*b*-PCL_{44} and PEO_{113}-*b*-PCL_{101}, significantly higher PDI values were obtained at low ϕ_W and this effect was more pronounced for PEO_{113}-*b*-PCL_{44}. This can be explained by partial solubility of the hydrophobic block in the dispersing medium when the PCL block length and ϕ_W are both small, resulting in low supersaturation and possible agglomeration of NPs. The effect of ϕ_W on the PSD and morphology is explored in more detail later in this work.

Selection of data for statistical modelling

A set of criteria was put in place to choose what data to include for a more detailed statistical analysis of process parameter effects on d_H and PDI. Using d_H to describe the PSD is meaningful only when the particles are spherical, their PSD is unimodal and PDI is relatively low. Therefore, only polymers for which unimodal PSDs were typically obtained, were included in the study. Only PEO_{113}-*b*-PCL_{219} and PEO_{45}-*b*-PCL_{88}, polymers with $f_{PEO} = 0.16$, fulfilled this criterion. Additionally, only data points corresponding to PDI < 0.5 were included in statistical models.

Model of process parameter effects for PEO_{113}-b-PCL_{219}

The lowest PDI values were obtained with the highest molecular weight polymer, PEO_{113}-*b*-PCL_{219}. Therefore, the effects of process parameters on PDI and d_H were first evaluated by creating a model exclusively for this polymer. Both linear regression models and quadratic mixed models were created by standard least squares method using the Fit Model function of JMP Pro 14 software. Model coefficients are available in Tables S4-S6. Qualities of fit of each model are summarized in Table 4.

A linear regression model for PEO_{113}-*b*-PCL_{219} using the four process variables could predict d_H reasonably well ($R^2_{adj} =$ 0.83, $p \ll$ 0.05), while a linear model for PDI was not able to explain the variance ($R^2_{adj} = 0.41$, $p = 0.74$). Fixed effect tests of the d_H model showed that the effects of S, c_P and ϕ_w were statistically significant ($p <$ 0.05), whereas the effect of addition rate (A) was not significant ($p =$ 0.31). The directions and magnitudes of each effect are shown as leverage plots in Figure S4.

According to the linear model, an increase in the stirring rate and water fraction decreased the average particle size, which can be explained by increased local supersaturation, leading to nucleation-favoring conditions. The model also implies that particle size was increased by an increase in polymer concentration in solvent phase. These qualitative findings are well supported by other literature reports (Johnson and Prud'homme 2003; Lebouille, J. G. J. L., et al. 2014). Particle size was not strongly affected by changes in addition rate within the range studied here (10 to 250 mL/h).

Quadratic models containing all squared effects and two-way interactions (a total of 14 effects), resulted in $R^2_{adj} = 0.93$ and 0.37 for d_H and PDI, respectively. To simplify the quadratic models, effects were manually excluded one-by-one to maximize the coefficient of determination, while minimizing the amount of remaining effects and the root-mean-square error of prediction (RMSE). The effects c_P^2, S^2, ϕ_W^2, $c_P A$ and $c_P \phi_w$ were removed from the model of d_H, resulting in a model with nine effects and $R^2_{adj} = 0.92$. Similarly, all squared interactions were removed from the PDI model, which greatly improved model quality in terms of adjusted R^2 and RMSE (See Table 4).

The simplified quadratic models for PEO_{113}-*b*-PCL_{219} are visualized in Figure 5 using response surface plots. The effects of ϕ_W, S and c_P on d_H were very similar to the linear model. The effect of addition rate was small in magnitude. Looking at the standardized model coefficients (Table S6) it can be seen that an increase in the terms of ϕ_W, S , A and $c_P S$ was associated with a decrease in particle size, whereas increasing the c_P, A^2, $\phi_W S$, $\phi_W A$ and SA terms was associated with an increase in particle size in this particular data set.

Table 4. Qualities of fit of the statistical models of process parameter effects created for PEO_{113}-b-PCL_{219}. RMSE = root-mean-square error of prediction and R^2_{adj} is the adjusted coefficient of determination.

Response	Model type	Number of effects	R^2	R^2_{adj}	RMSE
d_H	Linear	4	0.86	0.83	9.99
	Quadratic, full	14	0.97	0.93	7.26
	Quadratic, simplified	9	0.96	0.94	6.69
PDI	Linear	4	0.52	0.41	0.036
	Quadratic, full	14	0.75	0.37	0.031
	Quadratic, simplified	10	0.76	0.57	0.028

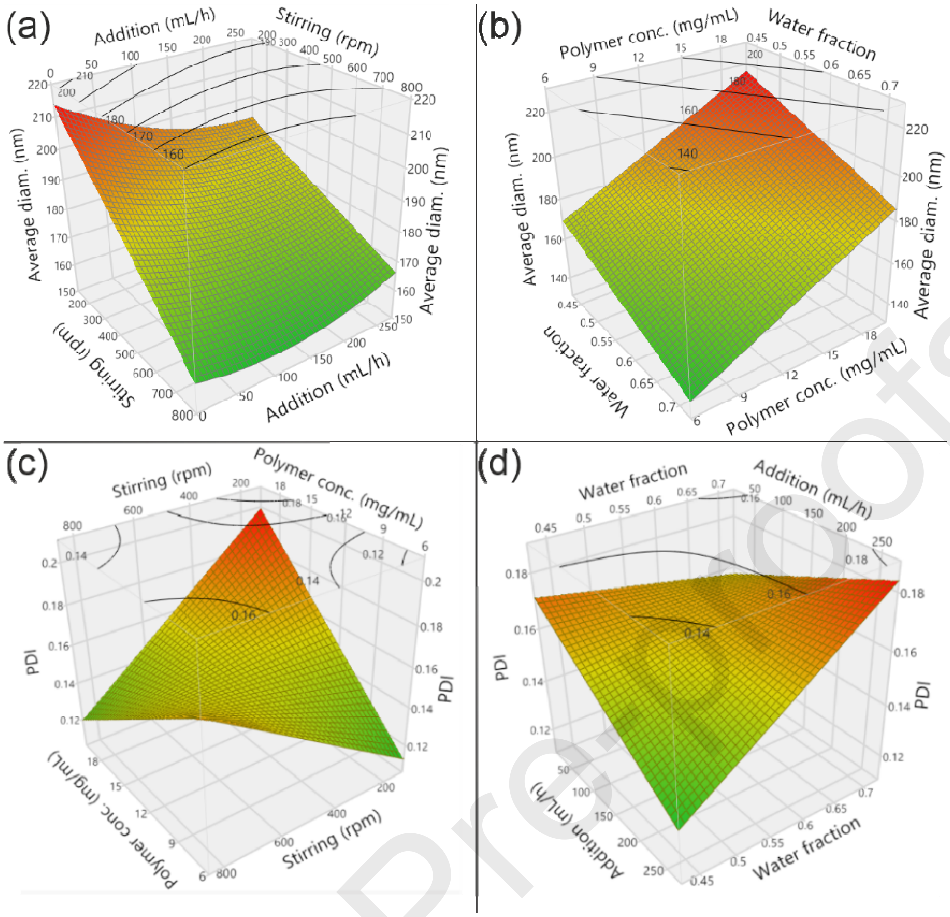


Figure 5. Response surface plots of the simplified quadratic model for PEO$_{113}$-b-PCL$_{219}$ on the effect of nanoprecipitation parameters on mean hydrodynamic diameter (a-b) and PDI (c-d).

Reproducibility and the operator effect

To evaluate the reproducibility of the nanoprecipitation method and to provide data for model validation, the experimental runs for 5000 g/mol PEO block polymers were repeated by a second operator, yielding a second data set. Both operators performed the experimental runs with identical protocols, equipment, parameters and instructions. Figure 7 shows a scatterplot of d_H values of the two data sets plotted against the prediction of the simplified quadratic model, which is shown as a straight line. The model was created based on the first data set. It can be seen that both data sets clearly correlated with the model. However, the fit was poorer for the second data set. There was visible systematic error as the second set contained, on average, higher values than the first.

Reproducibility of the basic nanoprecipitation method is limited due to insufficient control over mixing conditions. In this work, the method was modified to specifically reduce the variation in mixing conditions by injecting the polymer solution via a tube at a controlled rate. In spite of this modification, systematically higher d_H values were observed with the second operator. Besides person-to-person differences in sample preparation, another possible explanation for this variance is the partial hydrolysis of PCL during storage, in either the dissolved or the solid state. Solid polymers were stored in dry, closed vials in a refrigerator for several months between the two data sets. Polymer solutions were stored in tightly sealed flasks at room temperature and storage time varied from days to weeks. However, hydrolysis of PCL is expected to reduce the molecular weight, and thus, particle size, which is opposite to the general trend seen in Figure 6. Therefore, it is unlikely that the differences between data sets are caused simply by polymer hydrolysis. Yet another possible source of variance is the lack of filtering of solutions prior to nanoprecipitation and samples prior to analysis, which may result in contamination that can affect the size distribution.

In light of the possible confounding effects listed above and considering that only two operators were included in these tests, no strong conclusions can be made on the operator effect. Nevertheless, the results underline the challenges in reproducibility of this method and the need for strict control of both material and process factors for obtaining a homogeneous end product.

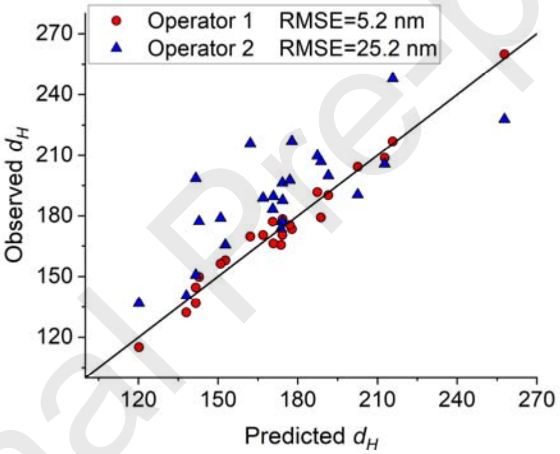


Figure 6. Observed *vs*. predicted values plot for the simplified quadratic model for average hydrodynamic diameter. Observed d_H values of the first data set (operator 1, red spheres) and second data set (operator 2, blue triangles) are plotted against the prediction of the model, which has been created based on the first data set. Straight line represents a perfect fit.

Combined effects of molecular weight and process factors

To include the effect of molecular weight in the models, new models were created based on data from both f_{PEO} = 0.16 polymers (PEO_{45}-*b*-PCL_{88} and PEO_{113}-b-PCL_{219}) using the same method as above. Data sets from the both operators were included, but operator effect was not included as an independent variable. Data points with PDI > 0.6 were excluded (n = 6). The full quadratic models, containing 19 effects each, were able to explain 92% of the variance in d_H (R^2_{adj} = 0.92, RMSE = 14.6 nm) and 79% of the variance in PDI (R^2_{adj} = 0.79, RMSE = 0.059). The models were simplified by manually removing the effects with minor contributions and recalculated.

The simplified models, containing eight effects for d_H and five effects for PDI, were able to explain 90% (R^2_{adj} = 0.90, RMSE = 14.5 nm) and 80% (R^2_{adj} = 0.80, RMSE = 0.060) of the variances of d_H and PDI, respectively. Fixed effect tests showed that all the included effects were statistically significant ($p < 0.05$). The coefficients of both models are listed in Table S7. Response surface plots based on the simplified model are presented in Figure 7. According to the standardized model coefficients, the strongest predictor for particle size is the molecular weight and other important variables are the polymer concentration, stirring rate and water fraction. Once again, addition rate did not strongly affect the responses.

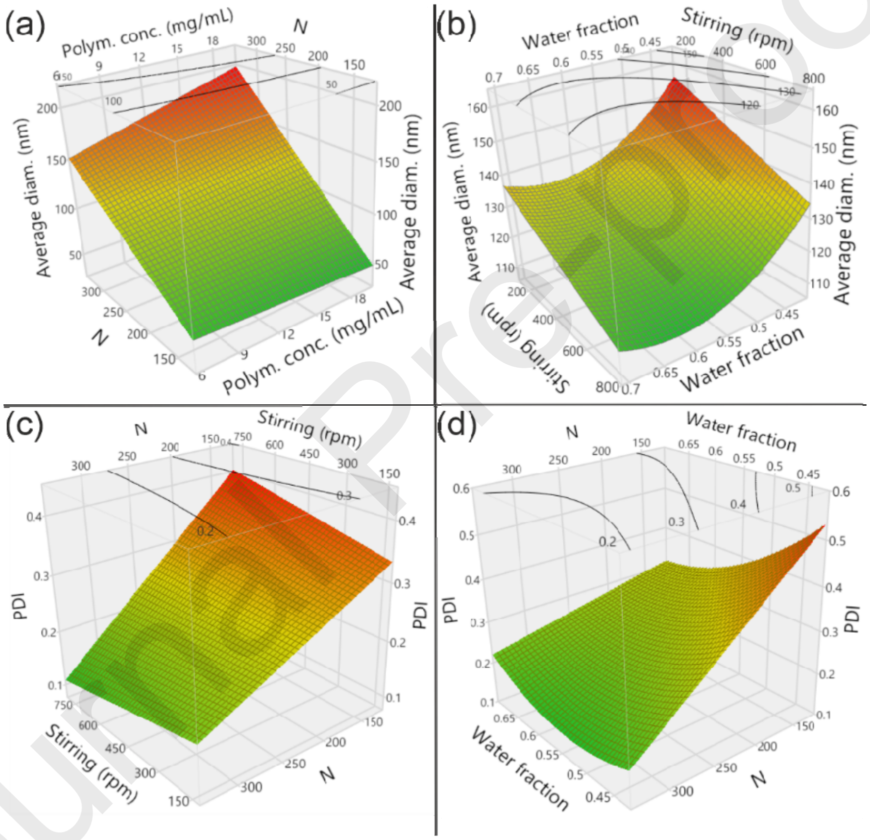


Figure 7. Response surface plots of simplified quadratic models for hydrodynamic diameter (a-b) and PDI (c-d) for polymers with PEO volume fraction of 0.16. This model includes the data from both operators.

Effect of water volume fraction

As previously discussed, PDI was found to correlate with water volume fraction, ϕ_w, especially strongly for PEO$_{113}$-*b*-PCL$_{44}$ and PEO$_{113}$-*b*-PCL$_{101}$. To study this dependency in more detail, additional experiments were conducted with ϕ_w ranging from 0.40 to 0.875 (see Table S2 for details). The obtained values for d_H and PDI are plotted as a function of ϕ_w in Figure 8.

Representative low-magnification micrographs and DLS data from selected samples are shown in Figure S1.

According to Lince *et al.*, the solubility of PCL (14 000 g/mol) in acetone-water mixtures drops significantly at water mole fractions of 0.4 to 0.5 (Lince, et al. 2008), which correspond to ϕ_w of 0.14 to 0.20. This means that to obtain a high level of supersaturation, ϕ_w far in excess of 0.20 should be used. Our data of PEO$_{113}$-*b*-PCL$_{101}$ in Figure 8 shows that for this micelle-forming polymer, nanoprecipitation with ϕ_w as low as 0.4 did not cause widening of the PSD. However, for PEO$_{113}$-*b*-PCL$_{44}$ with a shorter PCL block, very wide PSDs were obtained below $\phi_w = 0.5$. This is explained by the higher solubility of a shorter PCL block in the dispersing medium: a shorter PCL block requires a stronger reduction in solvent quality to achieve a high enough supersaturation to obtain a narrow PSD. Additionally, the data shows that further increase of ϕ_w above 0.55 did not reduce PDI or average hydrodynamic size for these polymers, showing that this parameter is a poor choice for fine-tuning of particle size for these polymers.

According to the cryo-TEM analyses, a population of large structures appeared for PEO$_{113}$-b-PCL$_{44}$ at low ϕ_W (Figure S1a), which explains the associated high PDI values. These flat objects have an oval cross-section resembling a rice-grain and range from 1 to 8 µm in length and they were abundant in PEO$_{113}$-b-PCL$_{44}$, but scarce in the PEO$_{113}$-b-PCL$_{101}$ samples. Closer investigation of these oval-shaped structures suggests that they consist of agglomerated worm-like micelles (Figure S2) and they seem to form in large numbers when ϕ_w is reduced below ≈ 0.5. For the micelle-forming PEO$_{113}$-*b*-PCL$_{101}$ ($f_{PEO} = 0.29$), size and PDI are largely unaffected by changes in water volume fraction within the studied range, and some larger agglomerates were found regardless of ϕ_w (Figure S1 d-f). Dominant nanoscale morphologies did not change as a function of ϕ_W for either of the samples tested.

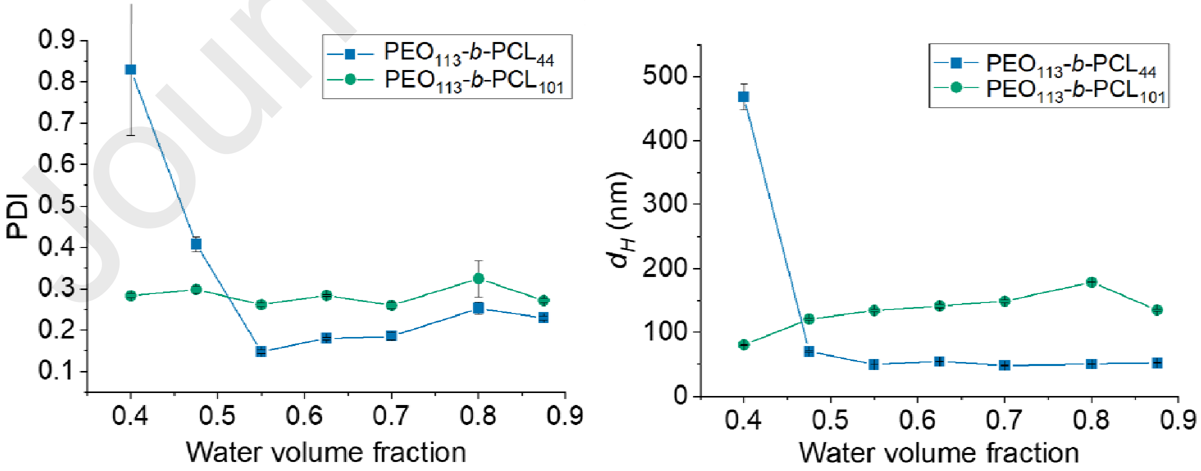


Figure 8. Polydispersity index (PDI) and mean hydrodynamic diameter (d_H) as a function of water volume fraction. Error bars show ± standard deviation (SD) of three repeat measurements of a single sample. Lines connecting the data points have been added only for visual clarity.

Effect of precipitation temperature and evaluation of stability above melting point

As discussed above, kinetic trapping of polymer chains into non-equilibrium morphologies plays a role in BCP nanoprecipitation. This was further evidenced in the present work by the fact that no vesicles were formed from PEO_{45}-*b*-PCL_{88} ($f_{PEO} = 0.16$) by simple nanoprecipitation. The PCL NP core can crystallize (Du, et al. 2007; Rajagopal, et al. 2010), which further increases the kinetic barrier for chain reorganization into vesicles. Therefore, we were interested to see whether maintaining temperature above the melting point of PCL ($T_m \approx$ 55 °C) during nanoprecipitation, effectively preventing crystallization, could affect the morphology (Zhou, et al. 2017).

In this experiment, nanoprecipitation was performed as previously, but in a heated water bath. The temperature of the water phase was adjusted to 65 °C ± 1 °C prior to injection of the organic phase (see Table S2 for parameters). After injection, acetone quickly evaporated as the temperature was above its boiling point. The temperature of the mixture was not monitored after injection. After solvent evaporation, the samples were analyzed by DLS and imaged by cryo-TEM, as previously described. The obtained d_H and PDI values are shown in Figure 9 and representative micrographs are shown in Figure 10a-e. Light scattering data is presented only for the polymers that formed spherical particles.

It can be seen that performing nanoprecipitation at elevated temperatures caused an increase in PDI and d_H, with the exception of the micelle-forming polymer PEO_{113}-*b*-PCL_{101}, for which no significant change in particle size was observed as a function of temperature. Size increase was the strongest for PEO_{45}-*b*-PCL_{88}. Cryo-TEM analyses showed that the increase in precipitation temperature caused only minor changes in morphology. Dominant morphologies for each polymer remained identical to the corresponding samples at room temperature. In the high-temperature sample PEO_{45}-*b*-PCL_{88}, a small population of vesicles appeared in addition to solid spherical particles (Figure 10c), even though no vesicles were observed in the room temperature sample of this polymer (Figure 2c). In addition, lamellar or ladder-like structures were observed, examples of which can be seen in Figure S3.

Zhou *et al*. developed a temperature-assisted nanoprecipitation method for the production of PEO-*b*-PCL vesicles. They showed that when THF was used as the organic solvent, increasing the precipitation temperature above 50 °C allowed the formation of vesicles from polymer with $f_{PEO} =$ 0.12, whereas precipitation at room temperature was unable to produce vesicles. They reported that precipitation from acetone was unable to produce vesicles from this polymer even at elevated temperatures (Zhou et al. 2017). Our data also shows that a small population of vesicles did form from polymer with $f_{PEO} = 0.16$ at 65 °C, when using acetone as the organic solvent.

In another experiment, we wanted to study whether heating the already prepared NPs could change their morphology, when heated above the melting point of PCL. Aliquots of analyzed room-temperature precipitated samples of PEO$_{113}$-*b*-PCL$_{101}$ and PEO$_{113}$-*b*-PCL$_{44}$ were placed in a 65 °C ± 1 °C water bath with moderate magnetic stirring for 30 min. After equilibrating to room temperature, the heat-treated samples were re-analyzed by DLS and cryo-TEM. The obtained d_H and PDI values are shown in Figure 9, and the representative micrographs are shown in Figure 10f-g. The data shows that heat treatment did not have a strong effect on hydrodynamic size. A small increase of PDI was seen with PEO$_{113}$-*b*-PCL$_{44}$. Cryo-TEM investigation showed a very small population of vesicles in the sample of PEO$_{113}$-*b*-PCL$_{101}$ after heat-treatment, whereas no vesicles were observed in the same sample prior to the heat treatment (Figure 2e).

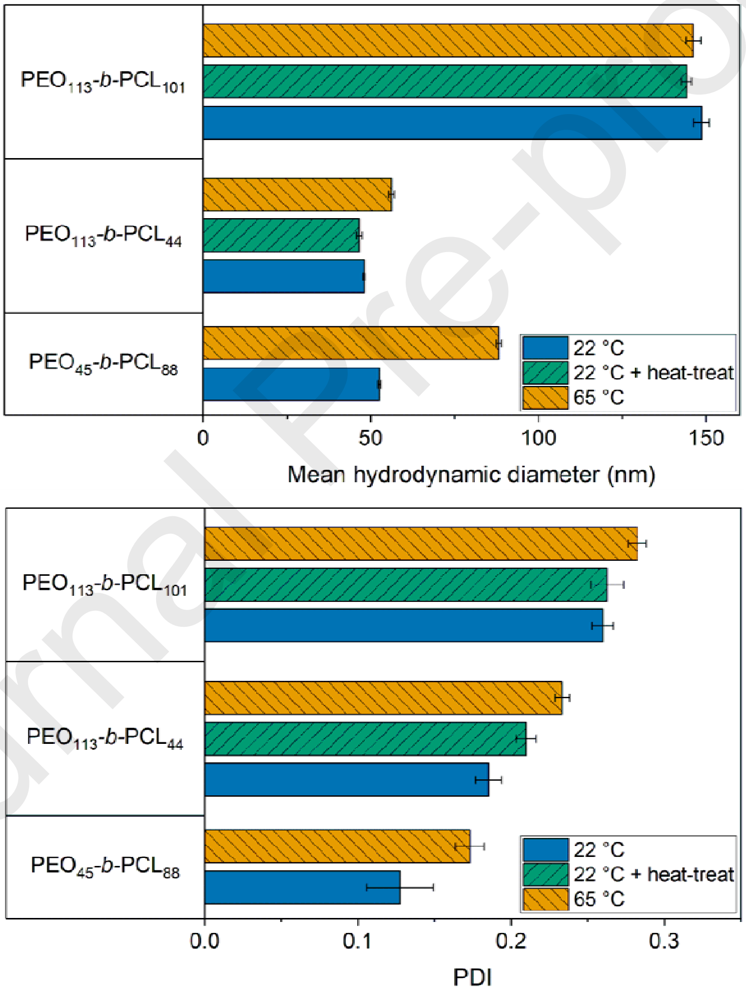


Figure 9. PDI and mean hydrodynamic diameter of samples precipitated at room temperature (≈ 22 °C) and at 65 °C, as well as room-temperature samples heat-treated at 65 °C for 30 min. All samples were analyzed at room temperature. Error bars show ±SD of three repeat measurements of a single sample.

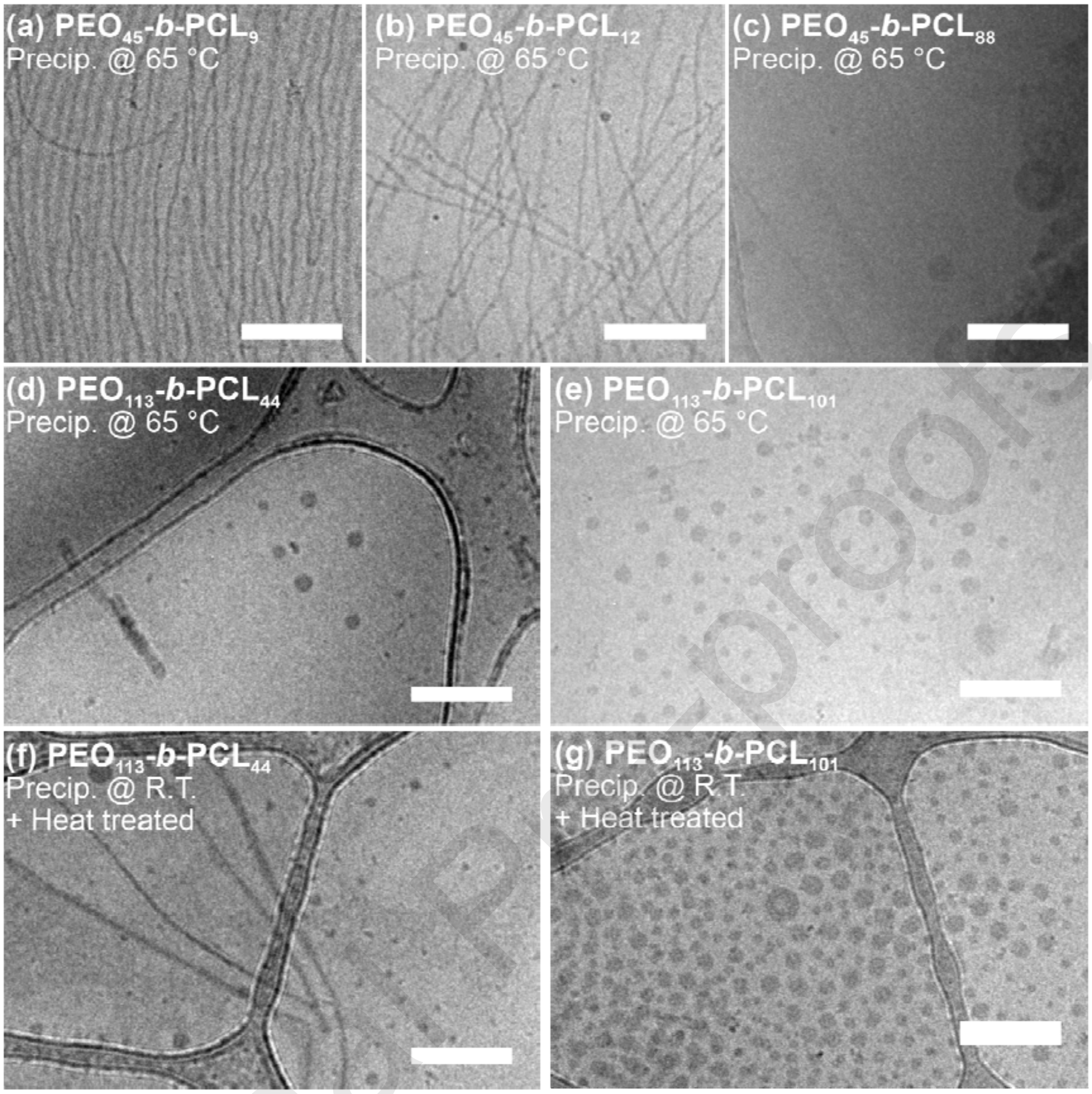


Figure 10. Representative micrographs of the formed nanostructures when nanoprecipitation was performed at 65 °C (a-e) or when the nanoprecipitation was performed at room temperature and the samples were heat-treated (30 min, 65 °C) after solvent evaporation (f-g). Scale bars are 200 nm.

In contrast to lipids or low-molecular weight surfactants, the ability of polymer chains to re-arrange after the initial precipitation step is limited. Due to this lack of molecular mobility, the choice of manufacturing method and parameters is crucial in obtaining the desired morphology. Our observations underline that the morphology of BCP NPs is dictated not only by polymer configuration, but largely also by the manufacturing process. Block copolymers that have a vesicular aqueous equilibrium morphology can be used to prepare solid NPs by the kinetic control enabled through rapid quenching by nanoprecipitation. Formulating solid NPs from BCPs with long hydrophobic blocks, such as the f_{PEO} = 0.16 polymers used here, can be beneficial in terms of achieving a higher loading capacity of hydrophobic drugs per NP (Lim Soo, et al. 2005; Shi, et al. 2005).

Conclusions

We have evaluated the effects of nanoprecipitation process parameters on the PSD and morphology of PEO-*b*-PCL aggregates using a statistical modelling approach. Depending on the molecular weights of the polymer blocks, long fiber-like micelles (f_{PEO} = 0.57 to 0.65) or spherical particles (f_{PEO} = 0.16 to 0.48) were obtained. For the polymers forming spherical particles, the most important variables affecting the PSD were identified and visualized and statistical models were created capable of explaining up to 94% in the variance of d_H. In addition, the effects of water volume fraction and precipitation temperature were examined in detail and we found that the critical water content for obtaining low polydispersity was strongly dependent on the length of the PCL block and that increasing the precipitation temperature could facilitate the formation of vesicles from the polymer when PEO fraction was low (f_{PEO} = 0.16). The results highlight that the morphology is dictated by both material and process factors and that these factors need to be well-controlled to achieve reproducible production of nanoparticles. Overall, the findings of this work can enable the rational choice of parameters for any formulation-specific optimization studies and we hope that this work inspires more mechanistic studies on preparation methods of colloidal drug delivery systems from block copolymers.

Conflict of Interests

V. Balasubramanian is an employee at Bayer Oy (Finland). The other authors declare no conflict of interest.

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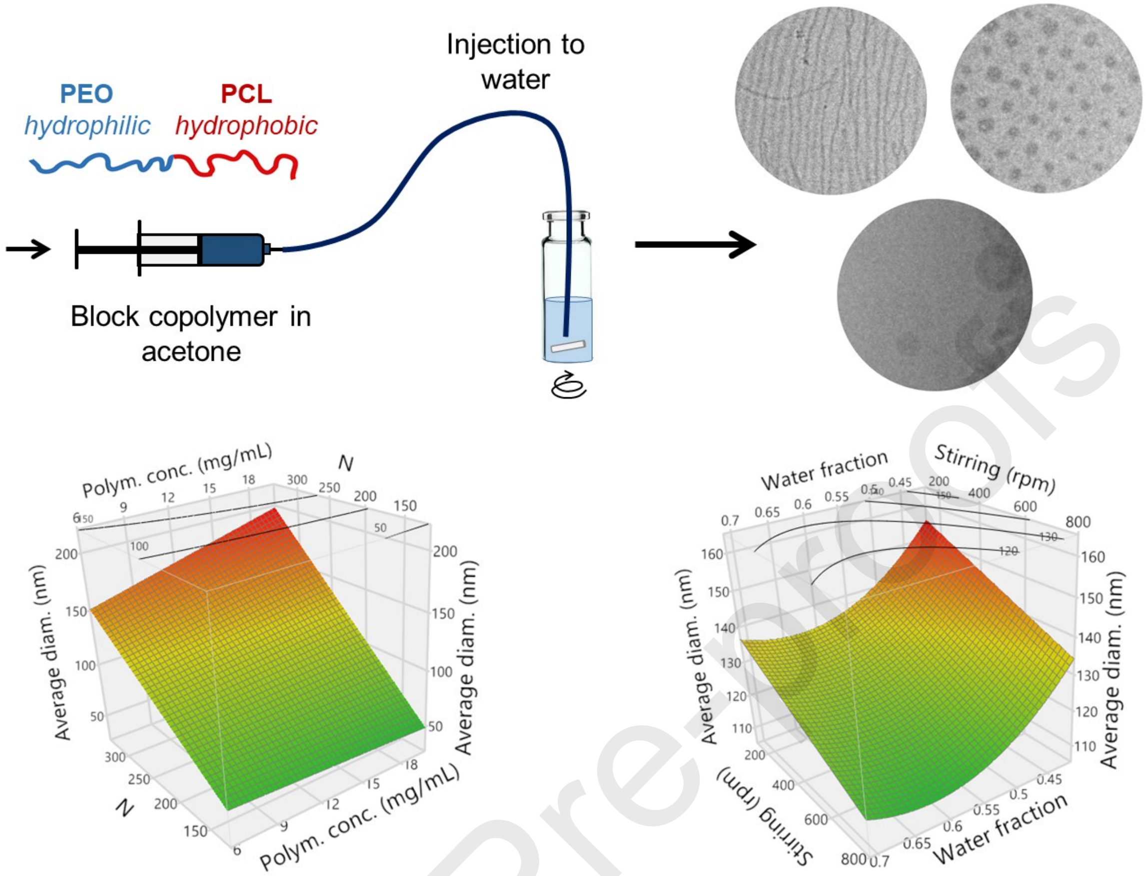
Credit author statement

Voitto Känkänen, Vimalkumar Balasubramanian and Hélder A. Santos conceived and designed the experiments. Voitto Känkänen and Henri Tuovinen performed the experiments, except for cryo-TEM imaging. Jani Seitsonen performed the cryo-TEM imaging. Janne Ruokolainen supervised the cryo-TEM imaging. Voitto Känkänen analyzed and interpreted the data. Voitto Känkänen, Vimalkumar Balasubramanian, Hélder A. Santos and Jouni Hirvonen wrote the paper. All authors have seen and accepted the contents of the manuscript.


PEO
hydrophilic
PCL
hydrophobic
Injection to water
Block copolymer in acetone
Polym. conc. (mg/mL)
N
Average diam. (nm)
Water fraction
Stirring (rpm)
Average diam. (nm)