Chapter 4

Results and Discussions I

Optimisation of polymersomes preparation and siRNA encapsulation

part of this work has been published in
Wang, L.G., et al., *Encapsulation of Biomacromolecules within Polymersomes by Electroporation.* Angewandte Chemie-International Edition, 2012. **51**(44): p. 11122-11125.

4.1. Introduction

As mentioned in *Chapter 2,* block copolymer, poly (2-methacryloyloxy)ethyl phosphorylcholine)-b-poly(2-(diisopropylamino)ethyl methacrylate (PMPC-PDPA) is able to form polymer vesicles or "polymersomes" to encapsulate large molecules including proteins (Canton, Massignani et al. 2013) and nucleic acids (Lomas, Canton et al. 2007). PMPC-PDPA comprises the always hydrophilic PMPC block and the pH sensitive PDPA block that is hydrophobic at pH higher than its pKa and hydrophilic at lower pHs.

For PMPC-PDPA, the most common method of formation of vesicle is the film rehydration. This involves gentle hydration of a thin polymer film under stirring for a given period of time. This method provides proper polymersome size but requires a long preparation time. Therefore, it is inappropriate for siRNA encapsulation as long preparation time will increase the risk of RNase contamination resulting in siRNA degradation. Here, PMPC-PDPA pH sensitivity can be used to encapsulate RNA. As explained previously in *Chapter 2: Literature Reviews*, this polymer is completely soluble at pH lower than 6.4 (the PDPA pKa in our working conditions, Room temperature and 100 mM PBS buffer). At this stage we can introduce the RNA molecules using two different approaches: (i) adding the RNA before raising the pH and triggering the formation of the polymersomes or (ii) loading the RNA after the polymersomes are formed using electroporation. Overview of the preparation methods is shown in *Figure 4.1*.

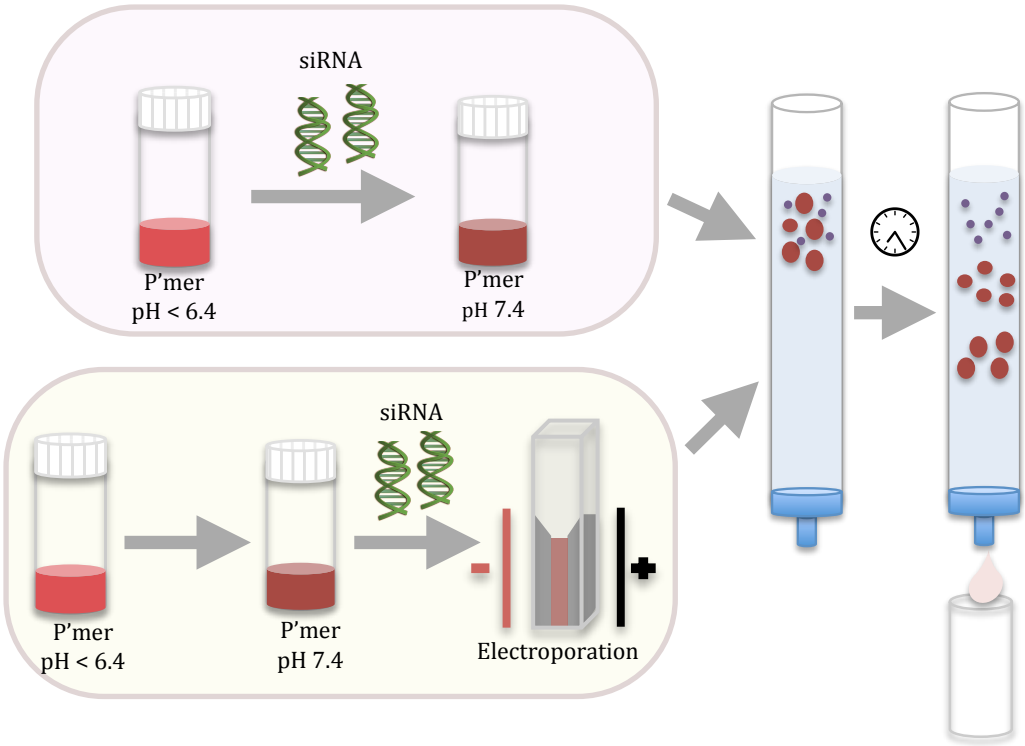

Figure 4.1: Polymersome preparation. Polymer was dissolved in PBS buffer pH 2.0 before addition of 1 M NaOH to adjust pH to 6.0. Two different methods were performed to encapsulate siRNA into polymersomes. First (on top panel) is to add siRNA into the polymer solution and increasing pH to 7.4 for vesicle formation. Another option (on bottom panel) is to make empty polymersomes by adjusting polymers solution to pH 7.4 and adding siRNA before performing electroporation. The polymersomes were separated by Gel Permeation Chromatography (GPC) using column packed with polymeric resins. The elution fractions were collected over time for further analysis.

4.2. Results and Discussions

4.2.1. Interaction of siRNA and polymer

PMPC-PDPA copolymers have been previously observed to interact with nucleic acid in a pH dependent manner (Lomas, Canton et al. 2007). The copolymer do not interact with plasmid DNA at pH higher than its pKa (pH>6.4) while at pH lower than the PDPA pKa, the negative charged DNA phosphate groups will bind to the the positive charged amino groups of PDPA. ζ-potential was performed to investigate the interaction between siRNA and PMPC-PDPA polymers. siRNA loaded polymersomes were characterised by ζ-potential at pH 7.4 (pH>pKa) and pH 2.0 (pH<pKa). The physiological pH here is the pH 7.4 which the pH that matched to the pH in cell cytoplasm. Polymersomes alone, siRNA loaded polymersomes and free siRNA were prepared at the siRNA and polymers concentration corresponded to a N/P ratio of 17.5 This is the ratio used in polymersome preparation and siRNA encapsulation in further experiments.

The ζ-potential in each sample was measured as shown in *Figure 4.2.* The difference can be observed after acidification at pH 2.0 (*Figure 4.2a*) where the ζ-potential of the polymer alone (+15.77 mV) is significantly higher than that of polymer with siRNA (+9.51 mV), suggesting possible interaction between polymers and siRNA. As at low pH, PDPA parts become hydrophilic with positive charge in their amine groups and can interact with the negative charge on siRNA phosphate backbone. This is correlated to the previous work (Lomas, Canton et al. 2007) however, PMPC-PDPA was reported to interact stronger with plasmid DNA, at physiological pH (pH 7.4, *Figure 4.2b*) both loaded and unloaded polymersomes show ζ-potential of 0. As expected, free siRNA gives a negative potential whilst the empty polymersomes give a neutral potential. Interestingly, the siRNA loaded polymersomes also display a neutral surface charge demonstrating effective encapsulation. If siRNA was free in solution or bound to the polymersomes surface, one would expect a negative potential. The ζ-potential of zero confirms that effective physical entrapment of the nucleic acid and hence its lack of electrophoretic mobility.

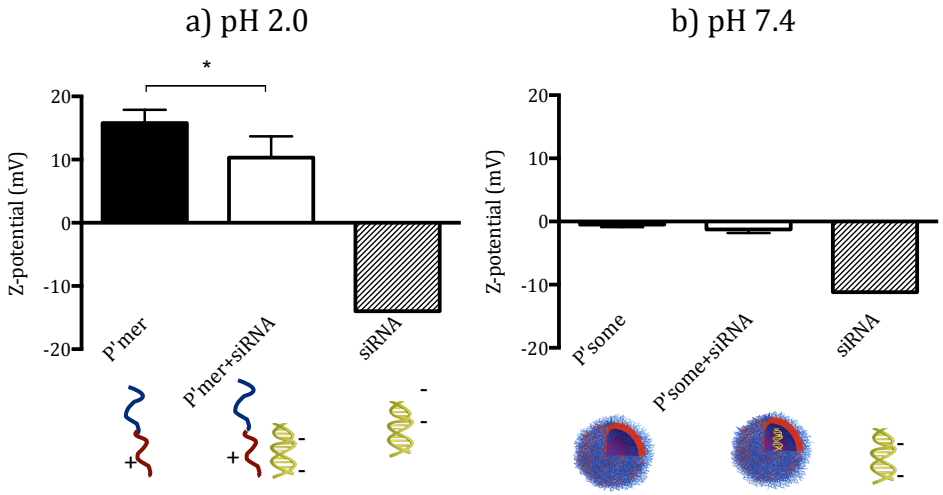

Figure 4.2: Zeta-potential of polymersomes and siRNA. Comparison between the surface charged of polymersomes with or without siRNA at different pH. a) pH 2.0, acidification condition, and b) pH 7.4, physiological pH. The ζ-potential of polymersomes, polymersomes with siRNA and free siRNA were shown with the N/P ratio of 17.5. The error bar represent the standard deviation for triplicate samples. Statistical analysis was performed by Student's *t*-test.

4.2.2. siRNA encapsulation by pH switch method

PDPA pKa depends on temperature and ionic strength (Giacomelli et al. 2006, Pearson, Warren et al. 2013). In the conditions herein used, the pKa is about 6.4 and this enables to control polymersome formation by changing the solution pH. Addition of substances into the block copolymer solution was done at pH 6.0 to ensure the packing of cargo during the polymersome formation. The mixture was

continuously stirred for ensuring homogeneity. The following step is to increase the solution pH by addition of NaOH up to pH 7.4 as described elsewhere (Lomas, Du et al. 2010, Massignani, Canton et al. 2010).

Effect of pH on siRNA

For pH switch method, siRNAs were added during polymersomes formation, in PBS solution in acidic conditions (pH 6.0). The integrity of siRNA during polymersomes preparation was confirmed by preparing siRNA in PBS buffer at different pHs, ranging from 2 to 7. The untreated control sample is siRNA prepared in RNase-free water and degraded control sample is siRNA prepared without RNase-free condition. The electrophoresis was performed to compare between six siRNA samples as shown in *Figure 4.3*. The results show that the band intensity of siRNA are quite similar at pH 2.0-7.4 with no detectable sign of degradation (comparing with degraded control siRNA).

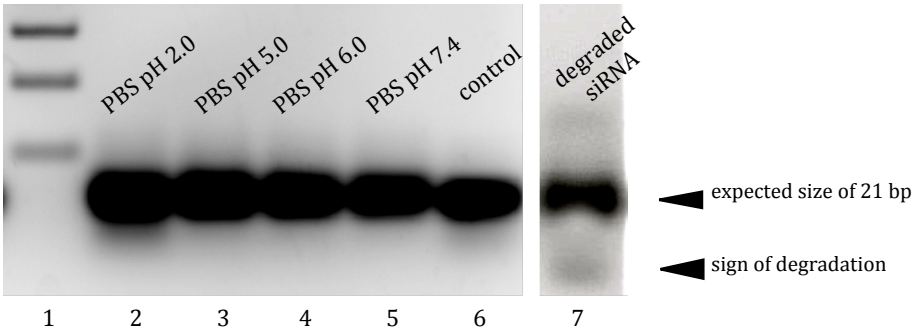

Figure 4.3: Effect of pH on siRNA. siRNA was prepared in PBS buffer at different pH before loaded onto the 5% agarose gel to perform the stability test. The gel electrophoresis was performed at 80 V for 1 h. Lane 1: Gene Ruler 50 bp DNA ladder (Thermo Scientific), Lane 2: 1 µg of siRNA at pH 2.0, Lane 3: 1 µg of siRNA at pH 5.0, Lane 4: 1 µg of siRNA at pH 6.0, Lane 5: 1 µg of siRNA at pH 7.4, Lane 6: 1 µg of siRNA untreated control, Lane 7: siRNA Degraded control.

RNA is less stable in alkaline condition but shows high stability under mild acidic condition. As RNA is prone to be hydrolysed in alkaline condition due to the presence of 2'OH (Voet and Voet 2011). According to the nature of RNA itself and the data presented in this work, it can be confirmed that RNA is not affected at pH between 2 and 7. The encapsulation of siRNA by pH switch method that requires an addition of siRNA into polymer solution at pH around 6 was therefore performed. It also worth noticing, that in siRNA stored solution which are not treated to remove RNAase, the degradation is rather quick. This emphasises once more the necessity to work in RNAase free conditions to ensure the nucleic acid integrity.

Polymersomes characterisation

As in pH switch method, siRNA was directly added into the polymer solution before increasing pH to 7, this experiment was performed to investigate the effect of siRNA on the polymersome formation. After polymersome preparation, size distribution of polymer sample in different experiments obtained from DLS were performed as shown in *Figure 4.4*. Slight differences in size distribution were observed in samples from each experiment, with the average size of 193±21 nm in diameter. Similar polymersomes morphology observed under TEM shows only spherical shape but with different sizes, suggesting the appearance of both polymersomes and micelles in the samples as presented in work from other people in our group (Lomas, Du et al. 2010, Pearson, Warren et al. 2013).

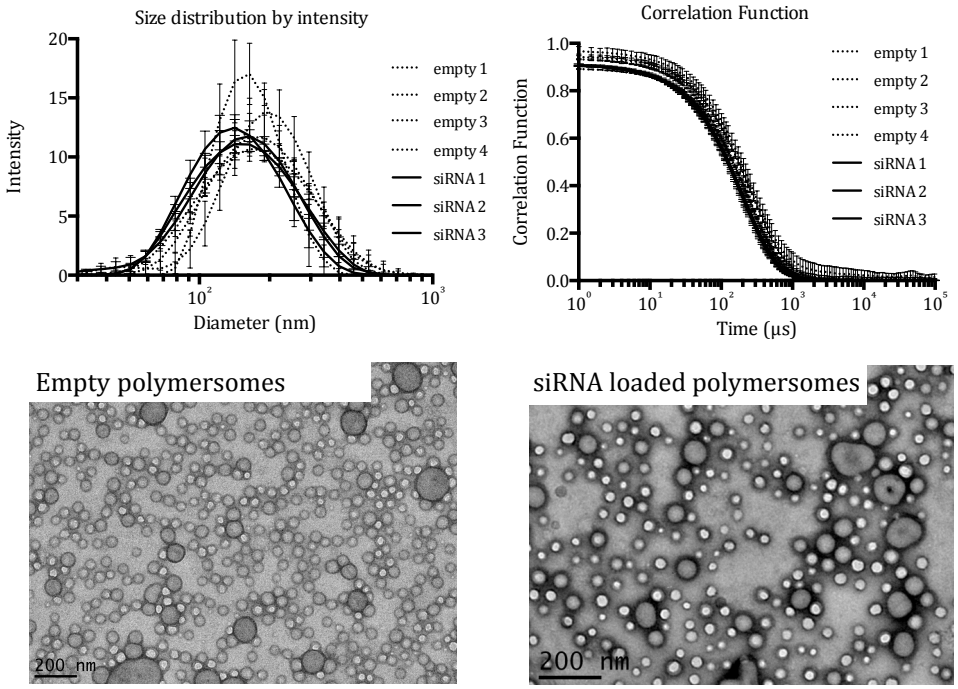

Figure 4.4: Characterisation of polymersome: encapsulation of siRNA into polymersomes using pH switch. Size distribution of polymer vesicles from 7 different samples (empty and siRNA loaded polymersomes) was measured by DLS (shown as size distribution by intensity and correlation function). Morphology of polymer sample was performed by TEM. The error bars in the plots represent the standard deviation obtained from three replicates of each sample.

Slight difference in polymersomes size was found among unloaded samples or siRNA loaded samples, this is according to the polymersome formation depends on rate of NaOH adding as well as the temperature. Such an effect in polymersomes formation was recently published by our group (Pearson, Warren et al. 2013), suggesting low temperature provide better condition for polymersomes formation according to the change of PDPA polymer pKa. In addition, not only polymersome but also micelle morphology was observed in this preparation method even with the

low temperature condition. This can confirm the occurrence of micelles in preparation by pH switch method in *Figure 4.4.*

PMPC-PDPA polymer self-assembly in solution could be in various forms as investigated previously (Blanazs et al. 2011). Polymers in unimer form starts to aggregate together and induce the micelles formation, following by continuous growth into cylindrical micelles. Next, cylindrical micelles are transformed into the bilayer membranes and finally become polymersomes. However, the micelle structure is quite stable in the solution, changing from micelles to other morphologies cannot occur without the presence of unimers in the solution. As unimer cannot be solubilised in solution according to its hydrophobic part, unimer avoid solubilisation by sticking onto the micelles surface. This causes asymmetry of micelles and leads to their morphological changes. The idea is presented in *Figure 4.5*, the first stage for polymer self-assembly is micelles, according to the less number of unimers aggregation required for micelles formation when compared with polymersome formation. In experimental condition, low concentration of unimers in solution was used, the number of unimers in limited area is not reach to the number required to form polymersomes, therefore the micelles can form in the first place. Additionally, pH switch method could affect the number of available unimers in solution. As most unimers reach to their pKa rapidly, therefore they tend to quickly aggregate together, this could lower possibility of free unimers in solution. Moreover, this preparation technique requires short period of time, this might restrict the chance of unimers to interact with micelles. Low number of available unimers in the solution as well as short preparation time of this technique could limit the morphological change of micelles to other shapes and lead to high population of micelles present in polymersomes preparation.

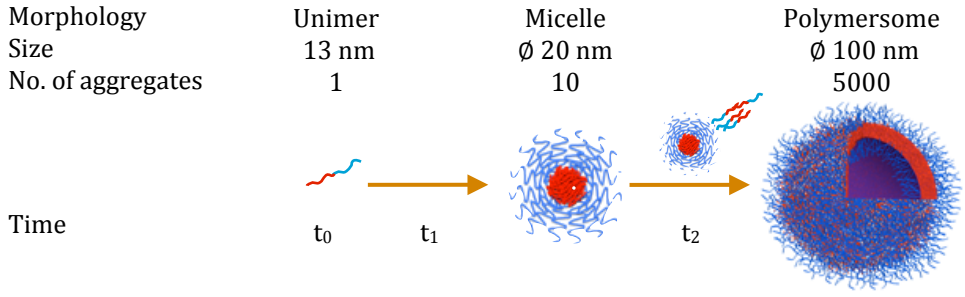

Figure 4.5: The polymersomes formation pathway. Polymersomes formation starts from presence of micelles and unimers in the solution, which can interact to each other by the aggregation of hydrophilicity. As the unimers grow on the micelles surface, this could induce the morphological change of micelles and transformed into polymersomes. This figure was adapted from pathway of polymersomes formation which was studied previously (Blanazs, Madsen et al. 2011).

The dissimilarity of polymersomes size as well as the occurrence of micelles suggests that further step is required to purify polymersomes from micelles and unencapsulated siRNA. In order to separate the micelles which also occurred in polymersome formation and study the siRNA encapsulation, purification of polymersomes from free siRNA was performed by the Gel Permeation Chromatography (GPC). This technique was introduced in 1964 (Moore 1964) and become widely used for protein and polymer purification. The separation is based on the pore size cross-linked polymeric resins typically made of polysulphobenze and or dextran, which is packed in a column. Small particles require longer elution time according to longer route of transport inside the resin. The purification was performed and characterised as shown in *Figure 4.6.* After polymersomes sample was prepared, it was loaded into the resin packed column, the elution fraction was collected by volume and used for further characterisation.

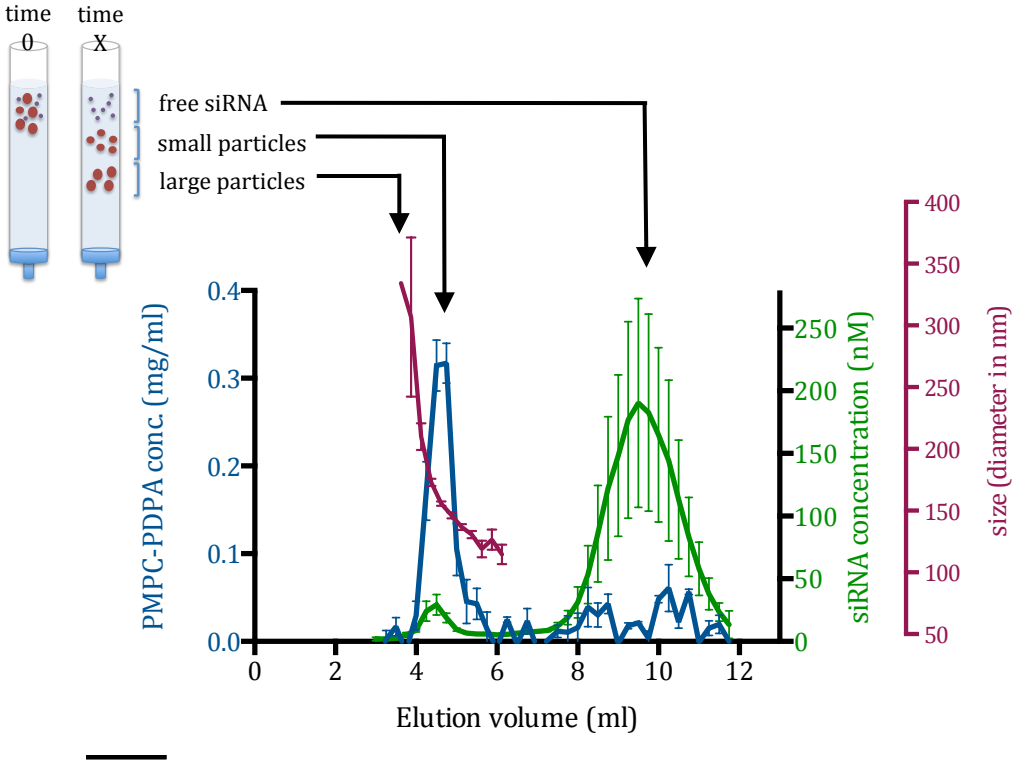

Figure 4.6: Gel Permeation Chromatography of siRNA/polymersomes samples. siRNA/ PMPC-PDPA polymersomes was prepared by pH switch method. After separation with GPC column, the elution samples were analysed to find the suitable fraction for further experiments. This plot shows the characterisation of each elution samples in terms of particle size (pink), polymer concentration (blue) and siRNA concentration (green). The error bars in the plots represent the standard deviation obtained from three replicates of each sample.

The plot in *Figure 4.6* shows the characterisation of the samples after separation in terms of their average size (pink), polymer concentration (blue), and siRNA

concentration (green). The average size of the elute was determined by DLS. The polymer concentration was measured using UV-Vis spectrophotometry, while siRNA concentration was analysed by the Picogreen assay (see *Chapter 3: Materials and Methods, section 3.2.8*). Both polymer and siRNA concentration were calculated from the calibration curve provided in the *Appendix B*

The chromatogram in *Figure 4.6* displays only one peak of polymer concentration (blue line) during the separation step at the elution volume of 4-6 ml. Two peaks of siRNA (green line) appear at elution volume of 4-6 ml and 8-12 ml which are clearly isolated from each other. The first peak of siRNA concentration is overlapped with polymer concentration. The average particle size of the first peak is about 120-200 nm which correlated to the polymersomes size. The second peak of siRNA concentration occurs at the late stage, which can be assumed to be free siRNA. After 6 ml elution volume, no polymersomes was detected by DLS and hence the size estimation could not be achieved. This experiment shows successful separation of polymersomes from the free siRNA using GPC column. The polymersomes samples collected at 4-6 ml of elution volume were the final purified polymersomes (after column samples) used for further analysis.

After GPC purification performed in *Figure 4.6*, TEM was used to observe the morphology of assembled polymers in samples before and after purification. Polymers can self-assemble as various sizes and shapes according to the preparation technique. Rapid change in hydrophobicity from pH switch method accelerates polymers aggregation, leading to diverse morphology such as micelles and polymersomes. Only polymersomes can be used to encapsulate siRNA in this work, while micelles have to be removed. As shown in *Figure 4.7*, the mean diameter of polymersomes before and after GPC column were 40±7 and 74±10 nm, respectively. Quantification of polymersomes size was performed by TEM pictures and shown in *Figure 4.7* (bottom panel), the polymersomes before separation process (bottom left) have various particle size ranging from 23-130 nm in diameter with the mean diameter of 45±17 nm. The polymersomes collected after separation (bottom right) with GPC column have narrow range of particle size from 81.25-123.44 nm in diameter, the average size is 98±20 nm. From the data, it can be confirmed that polymersomes were effectively purified from micelles with the separation by GPC column.

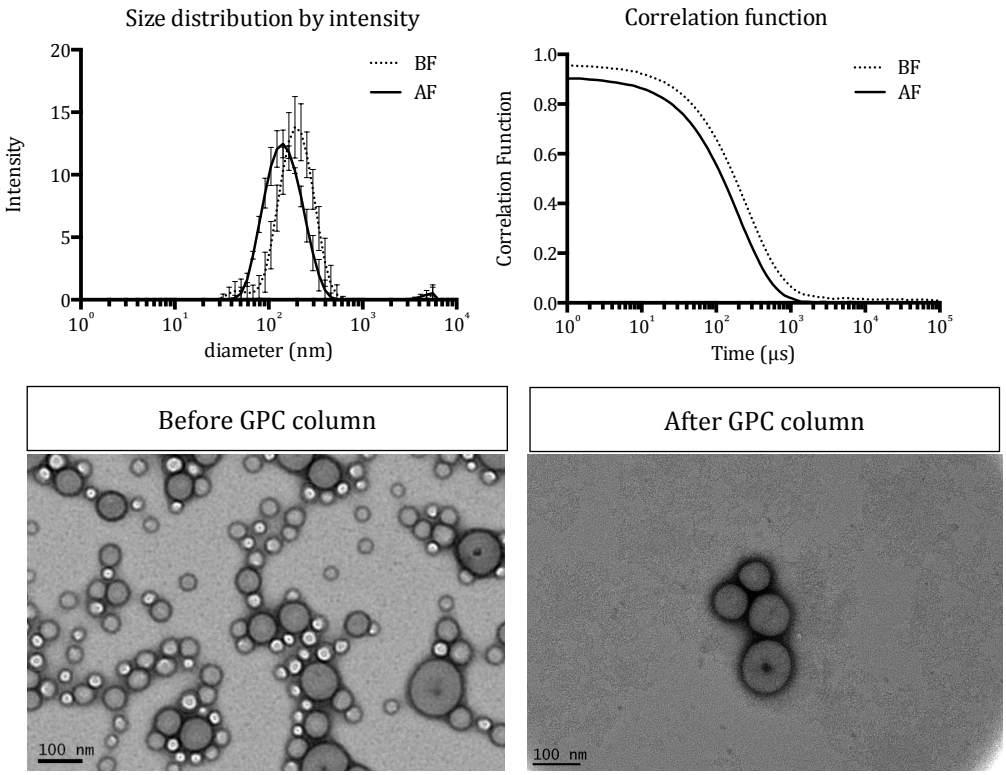

Figure 4.7: Morphology of polymeric vesicle with TEM. PMPC-PDPA polymersomes prepared by pH switch were measuring the size by DLS. The size distribution by intensity was presented (top left) along with the correlation (top right). The shape of polymersomes were observed under the TEM. The samples before (bottom left) and after (bottom right) separation with Gel Permeation Chromatography (GPC) were stained with phosphotungstic acid and visualised by a FEI Tecnai G2 Spirit TEM. The error bars in the size distribution by intensity plot represents the standard deviation obtained from three replicates of each sample.

Micelles and siRNA encapsulation

According to *Figure 4.4,* not only polymersomes was observed in pH switch method, but also micelles were presented in the samples. This might raise the question whether micelles can be used to encapsulate siRNA or not. In this experiment, the polymer micelles were prepared as described in micelles formation by increasing the temperature in polymersomes formation steps (see *Chapter 3: section 3.2.5*). As shown in *Figure 4.8*, this preparation technique produces only micelles with the diameter of 38±6 nm and no polymersome formation was observed.

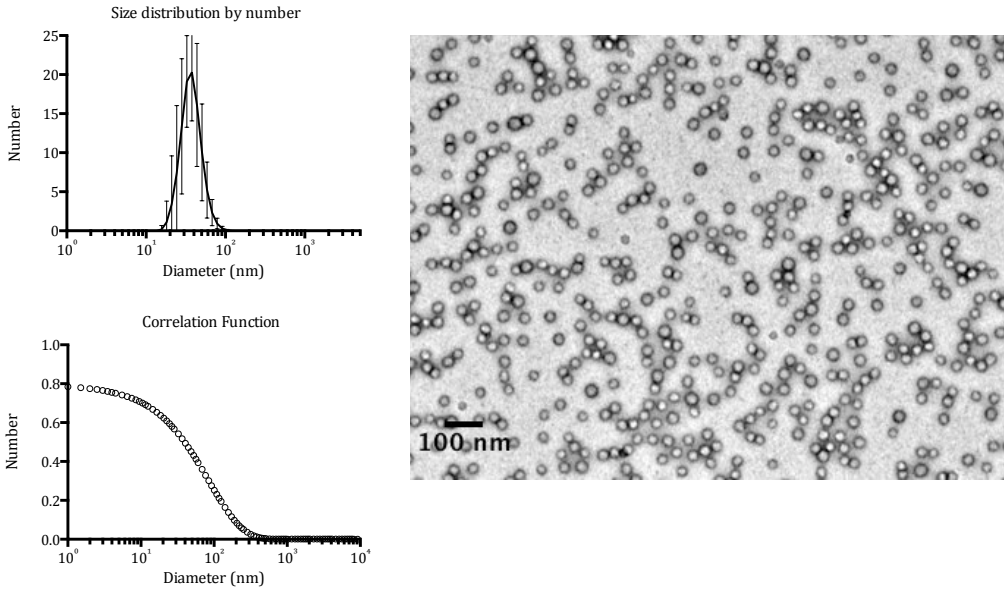

Figure 4.8: pH switch method resulting in micelles formation. PMPC-PDPA solution was rapidly adjusted its pH by addition of base solution to form only micelles. DLS and TEM were performed to investigate the size and morphology of self-assembly polymers. The error bars in the size distribution by intensity plot represents the standard deviation obtained from three replicates of each sample.

Similar purification step was performed with the sample obtained from the pH switch method for micelles preparation (as shown in *Figure 4.9*). Only siRNA concentration was used to investigate the ability of micelles to encapsulate siRNA. Presence of siRNA was observed in various elution volumes, ranging from 5-8 ml, suggesting that micelles might be able to pack siRNA. This is possibly due the some residual positive charges in polymer chain at the pH higher than pKa (Pearson, Warren et al. 2013), which is able to interact with negatively charged siRNA.

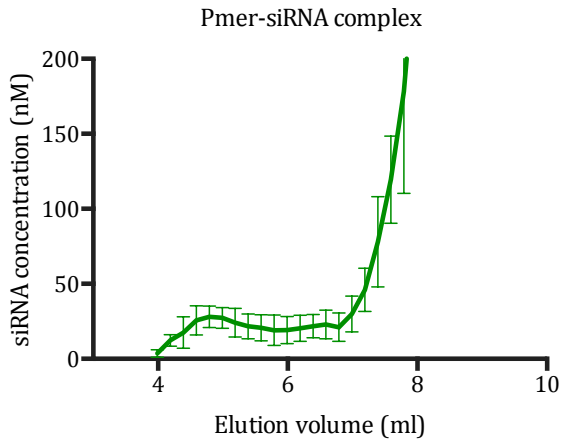

Figure 4.9: Gel Permeation Chromatography of siRNA/micelles samples. siRNA/PMPC-PDPA micelles were prepared by pH switch method. After separation with GPC column, the elution samples were analysed to find the suitable fraction for further experiments. This plot shows the siRNA concentration in each elution volume. The error bars represents the standard deviation obtained from three replicates.

However, as discussed in *Chapter 2: Literature Review, section 2.4.3*, the vesicles conformation is the only one that ensure effective endosome escape. Indeed micelles will disassemble into unimer within the endosomes, but the change in osmotic pressure will be orders of magnitude lower than the vesicles one. In this thesis, the presence of micelles in the sample can reduce the siRNA encapsulation efficiency of polymersomes and also affect the cellular uptake of polymersomes. Therefore, further studies were conducted to find the better encapsulation techniques to reduce the number of micelles contaminated in the polymersomes samples.

4.2.3. siRNA encapsulation by electroporation

As cellular uptake is size-dependent mechanism, but different siRNA sequences are required for normalisation and control experiments. Another encapsulation technique has been introduced to avoid the variability of size from encapsulation by pH switch. For many years, the most common way to temporary destabilising lipid membranes in living cells to introduce macromolecules has been the rapid application of an alternate electric field which induces the formation of pores in the membrane (Neumann et al. 1982, Wong et al. 1982). This method, accordingly named electroporation, relies on oscillatory applied electrical field in a electrode chamber to generates ion migration and charge poles across the lipid membrane. Lost in osmotic balance between external and internal membrane fluid, leads to osmotic pressure and induction of pore formation. This results in transport of external molecules across the membrane. Polymersomes membranes have the same supramolecular nature as lipid membranes and hence can be subjected to osmolysis and poration (Discher, Won et al. 1999, Aranda-Espinoza et al. 2001). On this basis, we have adapted the electroporation method to temporary destabilising polymersomes to load them with macromolecules post formation. The parameters to control electroporation are electrical field strength, number of pulses and pulse duration. Electrical field strength depends on applied electrical potential (V) and distance between the electrodes (d). The electrical field has to reaches to the critical value to generate membrane pores. While the number of pulses and pulse duration affect the number and size of the resultant pores. In addition, other factors such as membrane composition, ionic strength, temperature, are also involved. Polymersomes have entangled membranes with higher mechanical resilience than lipid membranes (Discher, Won et al. 1999). Electromechanical property of polymersome membrane (Aranda-Espinoza, Bermudez et al. 2001) have been studied, an increase in the hydrophobic length (thickness of polymersomes membrane) enhances breakdown potential when compared to liposome. As mentioned above, the nature of bilayer membrane of polymersome with their mechanical property allows the possibility of using of electroporation as an

approach to encapsulate macromolecules into polymersomes. In this work, electroporation is applied to transfer siRNA into the pre-formed polymersomes.

Effect of applied electrical potential and number of pulses on siRNA

In this experiment, the effect of two factors including number of pulses and applied voltage on siRNA have been studied. siRNA samples were transferred to the electroporation cuvettes and placed on ice before applied with voltage of 500 or 2500 V for one or five times. After electroporation, siRNA samples were performed the electrophoresis as shown in *Figure 4.10.* The band intensities of tested samples (lane 3-6) are quite similar to the untreated sample (lane 1) suggesting no degradation of siRNA in these conditions. The data was correlated to the encapsulation of plasmid DNA into polymersomes (Wang, Chierico et al. 2012b). Even at 2500 V, plasmid DNA shows no sign of degradation and can be transcribed in HEK293T cell.

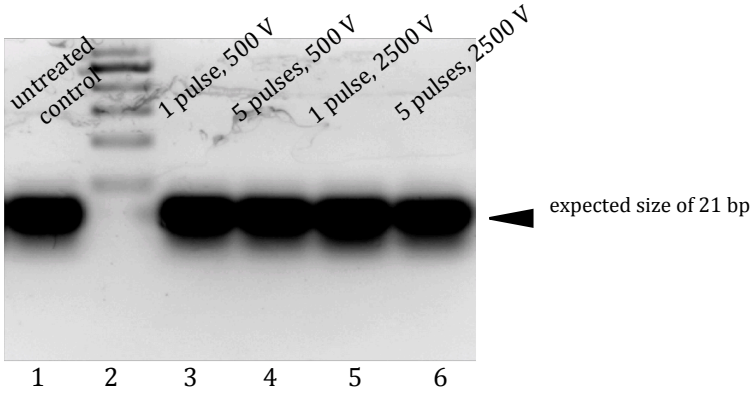

Figure 4.10: Effect of the number of pulses and the applied voltage on siRNA. siRNA was electroporated with different number of pulses and voltage before loaded onto the 5% agarose gel to perform the integrity test. The gel electrophoresis was performed at 80 V for 1 h. Lane 1: 1 µg of control siRNA without any treatment, Lane 2: Gene Ruler 50 bp DNA ladder (Thermo Scientific), Lane 3: 1 µg of siRNA with 1 pulse at 500 V, Lane 4: 1 µg of siRNA with 5 times pulse at 500 V, Lane 5: 1 µg of siRNA with 1 pulse at 2500 V, Lane 6: 1 µg of siRNA with 5 times pulse at 2500 V. The degraded siRNA control shown in Lane 7 in *Figure 4.3.*

Size distribution and morphology

The morphology and size of polymersomes samples before and after electroporation was investigated by TEM and DLS as shown in *Figure 4.11*. According to the data, size distribution of both samples were not different (*Figure 4.11a*). Polymersomes samples before and after electroporation have average diameter of 202±10 and 210±10 nm, respectively. The statistical analysis shows no significant difference between the mean value of these two samples (p-value > 0.05, Student's t-test). This is correlated to another experiment performed by Dr. Linge Wang for encapsulation of bovine serum albumin (BSA) (Wang, Chierico et al. 2012b). Similar polymersomes

size distributions were observed before and after electroporation (see *Figure 4.11b*), suggested no effect from electroporation on polymersome size. TEM pictures of polymersomes before and after electroporation without purification with GPC, in *Figure 4.11c and 4.11d*, show similar morphology of both samples as mixture of various size of polymersomes. This can be confirmed that electroporation can be performed for encapsulation of siRNA with no effect on polymersomes morphology.

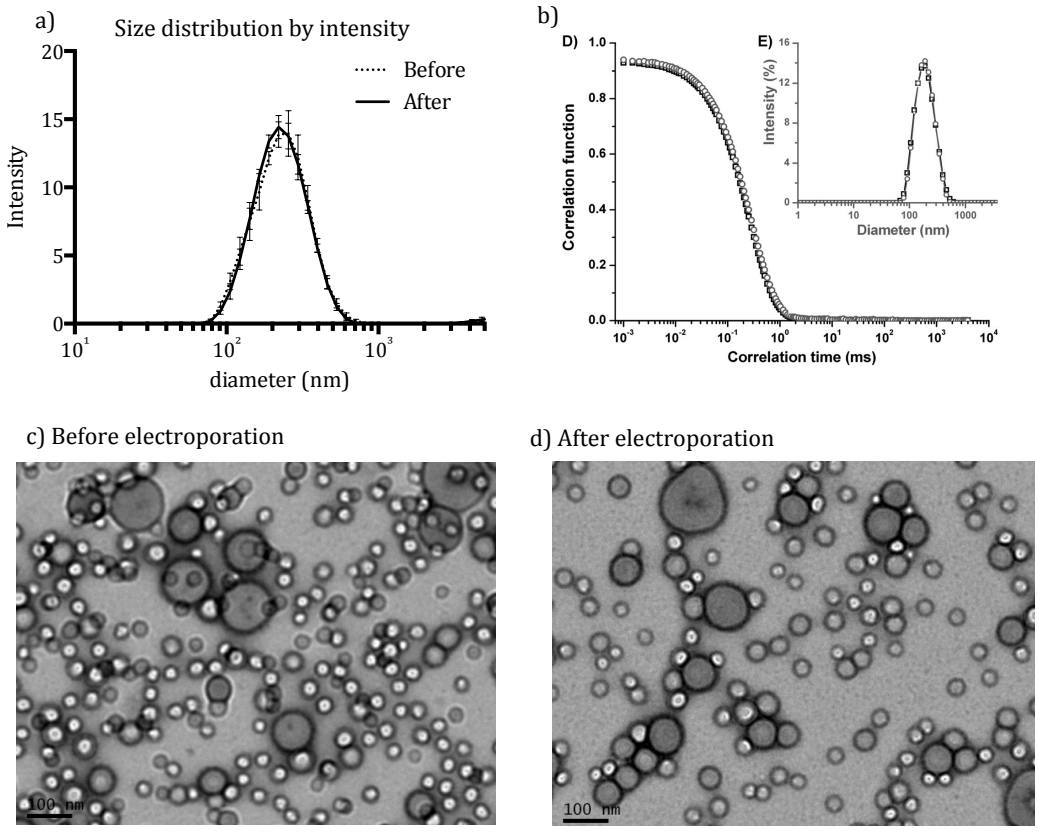

Figure 4.11: Characterisation of polymersome: encapsulation of siRNA using electroporation. Size distribution of polymersomes from samples before and after electroporation was measured by DLS of (a) siRNA encapsulation as well as (b) BSA, □ as before and ○ as after electroporation (figure obtained from (Wang, Chierico et al. 2012b). The morphology was performed by TEM (c) before electroporation and after electroporation. The error bars in the size distribution by intensity plot represents the standard deviation obtained from three replicates of each sample. The Student's *t*-test was performed for statistical analysis

Effect of siRNA sequence

Nucleotide sequence is an important variable when working with siRNA. As not only the target specific siRNA will be used to perform knockdown but also the negative control siRNA be used as a control for normalisation. The effect of different sequences of siRNA on polymersome size was investigated in this work. Empty polymersomes were prepared by pH switch followed by siRNA encapsulation using electroporation. Various siRNA including negative control siRNA (neg), anti-lamin

A/C siRNA (LMN), and cy3-labeled siRNA (Cy3) were used in this study. Their nucleotide sequences are different (see *Chapter 3: Materials and Methods*), as they specifically match to their target mRNA. Negative control siRNA was designed and validated to have no effect on any genes in human cells, in order to use as transfection control in the knockdown experiment. All siRNAs were encapsulated into the same batch of polymersomes. All polymersomes samples were purified and determined the size distribution compared with the non loaded polymersomes (PBS).

From *Figure 4.12*, small difference on average size distribution was observed, with no statistical significance between each sample suggesting there is no effect of siRNA sequence on encapsulation with electroporation.

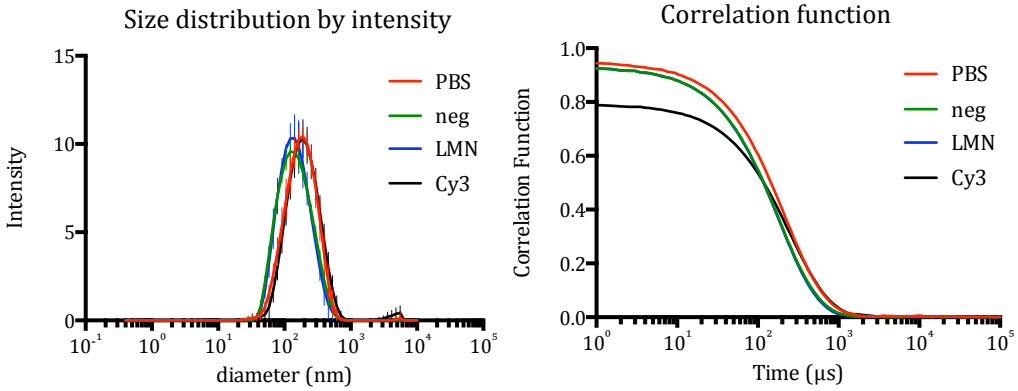

Figure 4.12: Different siRNA sequences have no effect on polymersomes size distribution. Polymersomes were encapsulated with 3 different sequence of siRNA using electroporation technique. The error bars in the size distribution by intensity plot represent the standard deviation obtained from three replicates of each sample. The Student's *t*-test was performed for statistical analysis.

Electroporation can be performed and used as the alternative method for encapsulation of siRNA as well as encapsulation of other macromolecules with controllable preformed polymersomes size.

4.2.4. Encapsulation of siRNA

The data were presented as the plot between percentage of retention efficiency or loading efficiency according to the method of encapsulation (see *Chapter 3: Materials and Methods* for the details on the calculation). The retention efficiency considers the ratio of initial versus final mass of siRNA as encapsulation efficiency, whereas, the loading efficiency considers the internal volume of polymersomes as the volume for encapsulation. As siRNA is expected to be encapsulated inside the empty space of polymersomes, therefore, the percentage of loading efficiency is more suitable to reflect the exact volume for siRNA encapsulation.

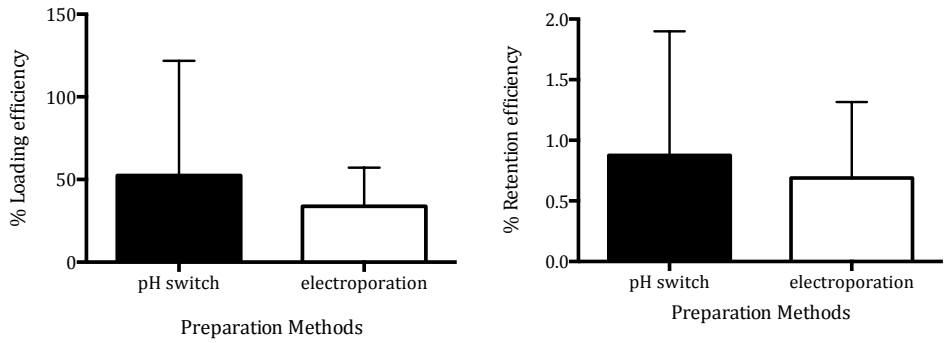

Figure 4.13: Efficiency of PMPC-PDPA in encapsulation of siRNA using pH switch and electroporation. Negative control siRNA were encapsulated in PMPC-PDPA polymersomes. The polymer concentration, siRNA concentration, and particle size distribution were measured and used as parameter to calculate the encapsulation efficiency. The encapsulation efficiency was presented in terms of percentage of retention efficiency and loading efficiency. Unpaired Student's *t*-test was performed but no significant difference was observed.

As shown in *Figure 4.13*, the percentages of retention efficiency obtained from pH switch method and electroporation were 0.88 and 0.74, respectively. In addition, the percentages of loading efficiency from pH switch method and electroporation were 52.44 and 31.69, respectively. Encapsulation efficiency acquired from pH switch was higher than that of electroporation, although no significant difference is observed. Large variation among experiments prepared by pH switch method suggests the high variability of this method. Moreover, high variation of polymersomes size prepared by pH switch method has to be considered as the particle size affects the cellular delivery.

Effect of number of pulses on siRNA encapsulation was performed. Pre-formed polymersomes was electroporated with the applied voltage of 2500 V, and one or five pulses, as previous experiment shows no siRNA degradation at these conditions (*Figure 4.10*). The encapsulation was calculated and presented in *Figure 4.14*. The percentages of retention efficiency obtained from one pulse and five pulses were 1.18 and 1.00, respectively (data not shown). In addition, the percentage of loading efficiency of one pulse and five pulses were 24.98 and 20.52, respectively. No significant difference was observed, suggesting that only one pulse is adequate for encapsulation. Other studies such as optimum siRNA concentration and maximum number of pulses can be performed to enhance siRNA encapsulation with this technique.

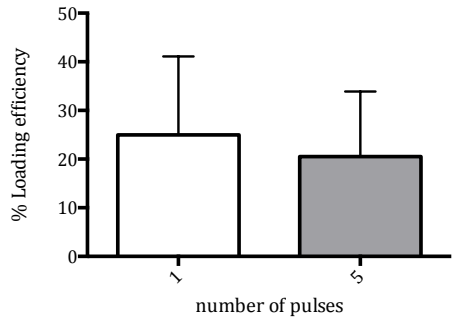

Figure 4.14: Number of pulses have no effect on siRNA encapsulation efficiency. Negative control siRNA were encapsulated in PMPC-PDPA polymersomes by electroporation, with 1 and 5 pulses at 2500 V applied voltage. The polymer concentration, siRNA concentration, and particle size distribution were measured and used as parameter to calculate the encapsulation efficiency. The encapsulation efficiency was presented in terms of percentage of loading efficiency. Unpaired Student's *t*-test was performed but no significant difference was observed.

Encapsulation of other macromolecules was studied; those that had the highest efficiency were compared with siRNA. BSA, IgG and plasmid DNA have loading efficiencies of 16.8%, 66.4% and 55.4%, respectively (data in supporting information from (Wang et al. 2012a)). Those encapsulation efficiencies were varied according to the mass of those macromolecules, which are much larger compared with siRNA molecular weight.

According to previous work from other groups, an siRNA encapsulation method frequently used was film rehydration, by directly adding siRNA into a polymer film during polymersome formation. This technique provides an encapsulation efficiency (loading efficiency) of 21.9% after 12 hours of stirring (Kim et al. 2013) and 50.2% after 48 hours of stirring (Pangburn, Georgiou et al. 2012). Another encapsulation technique is dialysis, which provides 30% encapsulation efficiency (Kim, Tewari et al. 2009). Both methods seem to provide higher encapsulation efficiency when compared with the encapsulation by electroporation technique from this work, however, the longer preparation time in those technique might not be applicable to siRNA work as it might increase the risk of siRNA degradation. In addition, the loading efficiencies of siRNA obtained (Kim, Tewari et al. 2009, Kim, Kim et al. 2013) were from the measurement of fluorescent signal from FITC-labelled siRNA, which might be an artifact from free FITC that can detach from the siRNA sample under specific test conditions.

To be mentioned here, the identification of encapsulated polymersomes and empty polymersomes, or unloaded polymersomes, cannot be performed in this work as no suitable method can separate the siRNA loaded polymersomes from unloaded

polymersomes. Therefore, polymersomes in the sample will be the mixture of both empty and siRNA encapsulated polymersomes. The concentration of siRNA in the polymersomes sample was calculated from overall mixture.

4.3. Summary

PMPC-PDPA shows no interaction with siRNA, moreover the surface charge of polymersomes can confirm the encapsulation of siRNA. siRNA encapsulation was performed by two methods, pH switch and electroporation. pH switch method provides potentially higher encapsulation efficiency when compared with electroporation. However, the size of polymersomes prepared from pH switch method is variable, and shown as micelles and polymersomes. Particle sizes have to be considered in transfection experiment, as the cells have to be transfected with both target siRNA and non-target siRNA as a control experiment. Variation of particle size might affect the cellular uptake of particles which results in the different uptake of siRNA and concentration of siRNA in the cells. A purification step was performed by Gel Permeation Chromatography, in order to separate polymersomes from micelles and free siRNA. This purification was verified and purified polymersomes were collected at 4-6 ml elution volume. Another method is the electroporation of siRNA into pre-formed polymersomes. This method confirmed the delivery of cargos into polymersomes with no effect on polymersomes size (Wang, Chierico et al. 2012b). Moreover, no effect from different siRNA sequences on polymersomes size was observed in this study. Electroporation method shows similar encapsulation efficiency when compared with pH switch method. It also has advantage over pH switch method on the polymersomes manipulation, especially in size controlling as size separation can be performed by GPC before encapsulation. This allows the further enhancement of the cellular uptake according to the size limitation as well as other manipulation such as attachment of peptide molecules on polymersomes surface, or encapsulation of more macromolecules into the polymersomes.