Chapter 7

General Conclusions and Future Directions

7.1. General conclusions

In this thesis, delivery of siRNA was performed by using PMPC-PDPA polymersomes as a carrier. Here, polymersomes have been tested and confirmed for their ability to encapsulate siRNA. siRNA was transferred into polymersomes by pH switch method, and newly introduced technique, electroporation. These two methods have advantages as well as limitations.

For pH switch, the advantage of this method is that it is fast and involves few steps. However, the limitation is the presence of high population of micelles in solution. Micelles are required at the beginning in polymersome formation steps, interaction of unimers and micelles lead to formation of metastable micelles that eventually evolve into polymersomes. With this method, micelles can be formed easily and quickly according to rapid change in the solution pH, resulting in low amount of leftover unimers. As micelles are quite stable under the experimental conditions, and low rate of unimers presented in the solution, small population of polymersomes are produced by this preparation method. Even though micelles are able to encapsulate small amount of siRNA via condensation, they are not useful in siRNA delivery as insufficient amount of unimers to create the osmotic pressure in endosomes. Therefore, polymersomes purification steps have to be performed.

For the electroporation technique, this method requires more preparation steps, as pre-formed polymersomes have to be produced in advance. Additionally, after electroporation, purification step is necessary to separate polymersomes from free siRNA. However, this method provides better option for siRNA encapsulation, as polymersomes and purification of polymersomes from micelles can be prepared in

advanced to ensure the suitable polymer morphology as well as size-limit for siRNA encapsulation.

Intracellular delivery of siRNA and polymersomes were proven by flow cytometry and microscopy. siRNA signal is presented throughout the cells, in the cytoplasm and the nucleus. This confirms the successful delivery of siRNA and also support the endosomal escape mechanism of polymersomes and their cargoes. The siRNA delivered by polymersomes was compared with another available delivery system, Lipofectamine™ 2000, which showed less distributed and punctuated siRNA signal throughout the cell. These two methods are different in the pathway of endosomal escape. Polymersomes provide efficient endosomal escape by the pH-responsive property, that could produce the osmotic pressure to the endosome, leading to the release of siRNA into the cytoplasm. On the other hand, Lipofectamine™ 2000 is a liposome with the cationic surface charge that complex with the anionic charges on nucleic acids phosphate backbone. The mechanism of endosomal escape of Lipofectamine™ 2000 are proposed as fusion between the lipid and the endosomal membranes (Wrobel and Collins 1995b). Difference in intracellular delivery of siRNA between this two systems creates dissimilar cellular responses which are described more in details in the following paragraph.

After cellular delivery of siRNA was confirmed, the next study was focussed on the investigation of the knockdown efficiency of polymersomes loaded with siRNA as well as Lipofectamine™ 2000. Polymersomes, with efficient intracellular delivery of siRNA, yield almost no silencing activity at both mRNA and protein level. This raised the question on why siRNA delivered by polymersomes, which provide efficient intracellular delivery and endosomal escape, fail to perform its silencing activity. According to this work, two factors which possibly influence the knockdown activity of siRNA/polymersomes are proposed as (i) siRNA pathway and (ii) effect of carriers.

First, from a cellular biology point of view, RNA interference process has been fully characterised in several organisms and considered to be a defense mechanism against viral infection. Such a response is conserved in most eukariotic cells. However, in mammalian animals where the immune system is more developed, RNAi has never been considered as possible anti-viral process. However, recent evidences in both mouse embryonic stem cells (Maillard, Ciaudo et al. 2013) and 7-day-old mice (Li, Lu et al. 2013) showed that RNAi has an antiviral activity, also in mammalian systems which is gradually subsided by more complex cellular responses as the immune system developed.

As described in the introduction, we can now induce RNAi using synthetic nucleic acid and in mammalian cells, using siRNA. This is very much dependent on its affinity and specificity as well as its intracellular concentration. Synthetic siRNA is exogenous materials and often can be interpreted as viral infection to the cells causing off-target responses. Previous studies showed that siRNA concentration higher than 20 nM (extracellular concentration) induced the stress and apoptotic genes (Semizarov et al. 2003). In addition, until now, localisation of the siRNA machinery has not been clarified. As the siRNA interact with RISC in the P-bodies (Liu, Valencia-Sanchez, et al. 2005, Sen and Blau 2005), which are thought to be located in the cytoplasm. However, RISC formation and turnover involves the endosomal pathway, suggesting association between RISC and the membrane trafficking (Gibbings et al. 2009, Lee et al. 2009). Additionally, a recent study measured that in a delivery system very similar to the Lipofectamine™ 2000 used herein, only 2% of siRNA is able to escape out of endosomes (Gilleron et al. 2013b), this might support the hypothesis of non cytoplasmic localisation of the siRNA machinery.

Second, the delivery system is another parameter which affect the knockdown activity of siRNA. Different carrier systems can lead to different cellular responses. In this work, polymersomes showed low effect on cellular response, hardly any effect in cell viability and no induction of pro-inflammatory responses unlike Lipofectamine™ 2000, which has been used as nucleic acids carriers for many years. Improvement in its composition, and other optimisation on transfection protocols such as culture condition, incubation time, have been tested to increase cell viability as well as transfection efficiency. However, the main problem of cytotoxicity still remains. Its toxicity is caused by its cationic nature which interact with the cell membrane very strongly (Dokka et al. 2000). Lipofectamine™ 2000 shows induction of cytotoxicity and other cellular stresses, such as pro-inflammatory response, pro-apoptotic signal. This might somehow relate to the knockdown by siRNA. Based on this work, addition of Lipofectamine™ 2000 cannot enhance the knockdown efficiency of polymersomes, which means that the stress caused by Lipofectamine™ 2000 cannot directly improve the silencing activity. On the contrary, polymersomes induced some rescue effect in target mRNA expression, as the mRNA expression in polymersomes plus Lipofectamine™ 2000 was higher than in Lipofectamine™ 2000 alone. Based on PCR array data, polymersomes can induce ISG20 which might leads to degradation of exogenous dsRNA and hence lower the siRNA concentration, resulting in less knockdown activity. Regarding to the studies mentioned earlier (Li, Lu et al. 2013, Maillard, Ciaudo et al. 2013) about activity of siRNA was interfered with presence of immune response in adult mammalian cells, it might explained that

polymersomes-promoted interferon involved genes can cause lower activity of RNAi mechanisms.

In addition, the effect of Lipofectamine™ 2000 on global gene expression has been proposed, with 2500 non-target genes alteration (Tagami et al. 2008). This might support the evidence of unstable expression of genes selected as reference genes. As relative gene expression relied on the selection of reference genes for normalisation, numerous changes in global gene expression can affect the chosen reference genes, leading to misinterpretation of the knockdown activity.

Lastly, almost all of siRNA studies are performed with lipid carrier systems, which completely differ from polymersomes system herein discussed, in terms of their compositions, siRNA releasing mechanism, cellular responses. This could lead to biased analysis on polymersomes study, as all normalisation and assay are set up based on dissimilar system.

7.2. Future directions

Enhancement of polymersomes as delivery system

Many factors which can be varied in order to improve the encapsulation of siRNA. For electroporation technique, the effect of other important parameters such as polymersomes size and ions in solution could be studied. Large polymersomes could provide additional space for siRNA encapsulation efficiency. Additionally, the ion concentration in polymer solution could affect the encapsulation of siRNA as it provides charges on the polymersomes surface during electroporation. Understanding of factors that influence the encapsulation efficiency of siRNA can also be used in other nucleic acids encapsulation. In addition, development of RNAse-free encapsulation technique along with standard protocol set up for replicable encapsulation have to be developed. Finally, finding of suitable separation techniques for polymersomes purification have to be conducted to separate empty polymersomes from siRNA-contained polymersomes to limit the contamination of unencapsulated polymersomes.

Intracellular delivery and silencing activity of siRNA delivered by polymersomes

Deep investigation of localisation study of siRNA inside the cells are required, such as co-localisation of siRNA and RISC or other organelles, to ensure that delivered siRNA are in the suitable locus and ready to perform its action. In addition, knockdown with other carriers or systems could be performed in order to investigate the difference on siRNA localisation and activity between polymersomes and other available systems. For example, polymersomes with less endosomal escape property could be used to investigate the ability of siRNA delivery and

knockdown activity in comparison to Lipofectamine™ 2000. Moreover, knockdown of different genes might be performed to ensure that the problem of no silencing activity is on the chosen target gene or the delivery system. Global gene expression of polymersomes treated cells have to be investigated and compared with available work on Lipofectamine™ 2000 systems as this might lead to finding of genes involved with the RNAi mechanism.