Chapter 5

Results and Discussions II

Cellular delivery of siRNA

5.1. Introduction

The discovery of RNAi in 1998 (Fire, Xu et al. 1998) provided scientists and clinicians with a powerful tool to manipulate genes in order to study complex biological events or to "cure" malfunctioning processes. However, one of the major hurdles to RNAi translation is its effective delivery within cells.

The cell membrane functions as barrier to protect the intracellular organelles from the outside environment, as well as controlling the transport of substances into the cell. The cell membrane is composed of amphiphilic phospholipids that self-assemble into a bilayer, resulting from the balance of hydrophilic and hydrophobic interaction. The resulting hydrophobic layer allows the diffusion of non-polar molecules but restricts the permeation of polar molecules through the membrane. More complex transport can be achieved by hosting protein transporters such as ion channels or molecular transporters. In eukaryotic cell, more efficient transport can also be achieved by endocytosis processes which involve membrane deformation, invagination, budding and fusion. Endocytosis pathways include steps of sorting and recycling typically within early endosomes and late endosomes, and enzymatic degradation inside lysosomes.

siRNA, as well as any other nucleic acids, is an anionic polymer with a molecular weight of between 10000 to 20000 Da. Understandably, both the size and polarity of RNA hinder any passive transport across lipid membranes. In addition to this, RNAs are extremely sensitive molecules and require protection from biological fluids.

siRNA requires effective carriers to achieve cellular uptake. Polymersomes fulfil this requirement by providing protection against RNA degradation and enhancing the delivery of siRNA into the cell cytoplasm by promoting endocytosis, as well as causing endosomal escape to bypass the endocytic degradation.

The cell model chosen in this study is HeLa cell. This is an epithelial immortalised cell line derived from a cervical cancer cell with low variation over time and passage number (Gey et al. 1952) and is the most common cell model used for mechanistic studies including several RNAi development (Elbashir et al. 2001, Dalby et al. 2004, Kakizawa et al. 2004, Segura et al. 2007, Suzuki et al. 2007).

In this Chapter, we present and discuss the quantitative analysis of empty and siRNA loaded polymersomes in HeLa cells. Intracellular uptake was studied by Flow Cytometry and Microscopy. Flow cytometry relies on population-based fluorescent intensity, which allows kinetic study of cellular uptake in overall populations. Microscopy is a more singe cell-based approach where difference can be observed in single cells, and is therefore suitable for the sub-cellular localisation studies. Moreover, this method provides higher detection sensitivity. All polymersomes with and without siRNA were prepared, purified, and characterised according to *Chapter 4* before applying to the cell in this chapter.

5.2. Results and Discussions

5.2.1. Cellular uptake of Polymersomes

Flow cytometry

Polymersome uptake has been studied previously by showing the ability of intracellular delivery of encapsulated fluorescent probes (Massignani, Canton et al. 2010), or fluorescent labelled-polymers in several cells types. In this study, uptake of polymersomes was performed in HeLa cells, by using rhodamine-conjugated $PMPC_{25}$-$PDPA_{70}$ to observe the fluorescent intensity. Cells treated with PBS buffer were used as negative control. The mean fluorescent signal obtained from each sample was subtracted by the fluorescent signal from untreated control in each incubation period. Three set of experiments were examined with two replicates per experiment, the mean fluorescent intensity of each sample was normalised against the fluorescent intensity of the longest incubation time (as maximum intensity) to standardised the signal obtained from different experiments. The results were summarised in *Figure 5.1*, as the plot between normalised mean fluorescent intensity versus incubation periods.

Within 10 minutes of incubation, an increase in fluorescent intensity has been observed, but the uptake is statistically significant at 60 minutes of incubation with *p*-value equals to 0.0158. Cellular uptake continued over time as can be seen from the increase fluorescent intensity at 360 and 1200 minutes (*p*-value < 0.001).

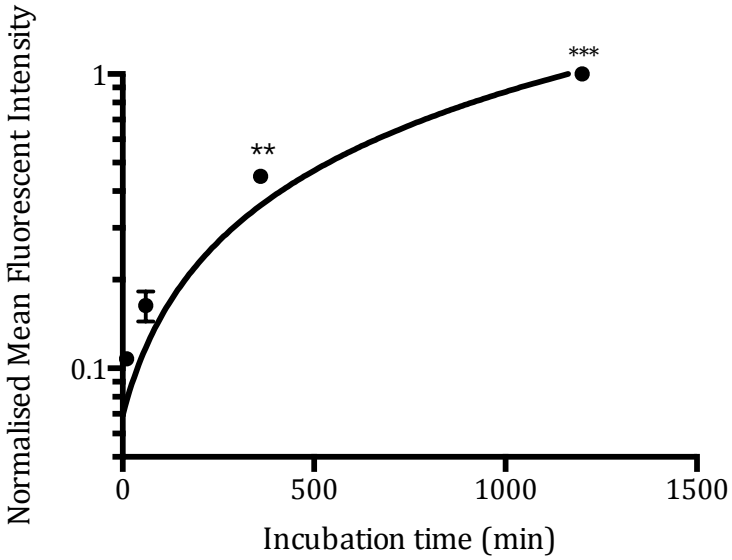

Figure 5.1: The kinetic uptake of Rhodamine conjugated PMPC25-PDPA70 in HeLa cells. HeLa cells were treated with Rho-$PMPC_{25}$-$PDPA_{70}$ polymersome (ratio of Rho-$PMPC_{25}$-$PDPA_{70}$: $PMPC_{25}$-$PDPA_{70}$ is 1:9, at concentration of 0.5 mg/ml). HeLa cells treated with PBS buffer were used as the negative control. Rho-PMPC25-PDPA70 polymersomes were incubated with HeLa cells at different incubation periods (10, 60, 360, and 1200 minutes). The fluorescent intensity of positive cells were detected by Flow cytometry. The normalised mean fluorescent intensity of rhodamine was obtained by subtracting the positive signal by the negative control, and divided by maximum fluorescent intensity (the mean intensity at 1200 minutes time point). The experiment was performed in replicates and three repeats. Statistical comparisons were performed using one-way ANOVA and Dunnett's multiple comparison. (*p*-value: $^{**}p<0.01$, $^{***}p<0.001$).

Further experiments were performed with longer incubation periods (as shown in *Figure 5.2*). Cellular uptake of polymersomes have been observed at an incubation period of 1440 minutes (20 hours) with no significant difference being detected at 48 and 72 hours (2880 and 4320 minutes, respectively). This suggests a 20 hour incubation is sufficient for cellular uptake of polymersomes with no additional incubation period required. An increase in fluorescent intensity at longer incubation periods suggests that the cells continue their uptake of polymersomes, which might be the result of higher uptake by a single cell, or from uptake by new cells produced via the proliferation process. The uptake remains unchanged after 20 hours (about 1200 minutes) of incubation, suggesting the equilibrium between polymersomes uptake (endocytosis) and secretion (exocytosis).

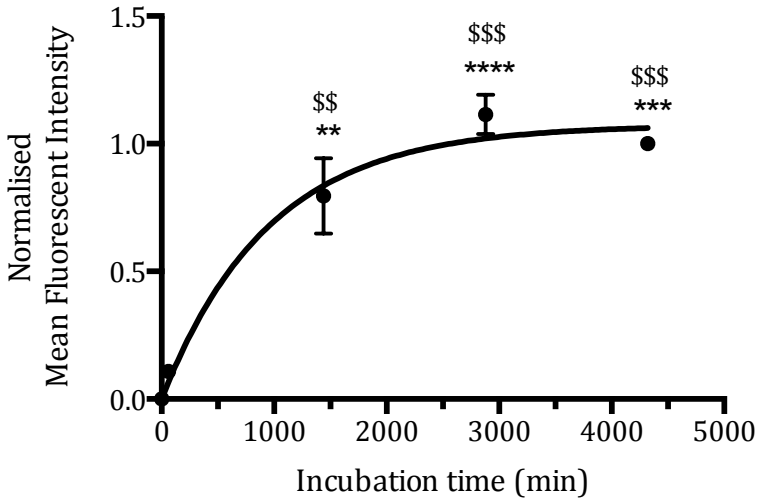

Figure 5.2: The cellular uptake of Rhodamine conjugated $PMPC_{25}$-$PDPA_{70}$ in HeLa cells in long incubation time. HeLa cells was treated with Rho-$PMPC_{25}$-$PDPA_{70}$ for various incubation time (0, 60, 1200, 2880 and 4320 minutes). HeLa cells treated with PBS buffer was used as the negative control. The fluorescent intensity of positive cells were detected by Flow cytometry The normalised mean fluorescent intensity of rhodamine was obtained by subtracted with the negative control, and divided by maximum fluorescent intensity. The experiment was performed in replicates and three repeats. Statistical analysis has been performed by ANOVA, with Tukey's multiple comparisons (*p*-value: **, $$ $p<0.01$, ***, $$$ $p<0.001$, ****$p<0.0001$, where * comparison with untreated control and $ compared with 60 minutes).

Rho-$PMPC_{25}$-$PDPA_{70}$ polymersomes in three different sizes of 65, 85 and 160 nm in diameter were prepared and characterised in the collaboration with Dr. Linge Wang (see *Chapter 3: Materials and Methods, section 3.2.2).* The cells were incubated with 1 mg/ml of Rho-$PMPC_{25}$-$PDPA_{70}$ polymersomes for 60 and 360 minutes before being washed and prepared for analysis of rhodamine fluorescent intensity by Flow cytometry. As shown in *Figure 5.3,* no significantly difference in fluorescent intensity was observed at 60 minutes of incubation among the three tested polymersome sizes. However, at 360 minutes of incubation, the cellular uptake of smaller particles (65 and 85 nm) differs from that of the larger size (160 nm) with no significant difference between 65 and 85 nm. Although the smaller particle shows higher uptake, larger polymersomes can encapsulate considerably more siRNA as previously observed (in *Chapter 4, Figure 4.7*) that the polymersomes size of about 150-200 nm shows the ability to encapsulate siRNA but the encapsulation can not be seen with the smaller size (smaller than 100 nm). The effect of polymersomes size is omitted in this work, as we need to find a trade-off between optimising internalisation vs. RNA delivery. However, insight studies about effect of polymersomes size on cellular uptake have been conducted by a colleague within our group.

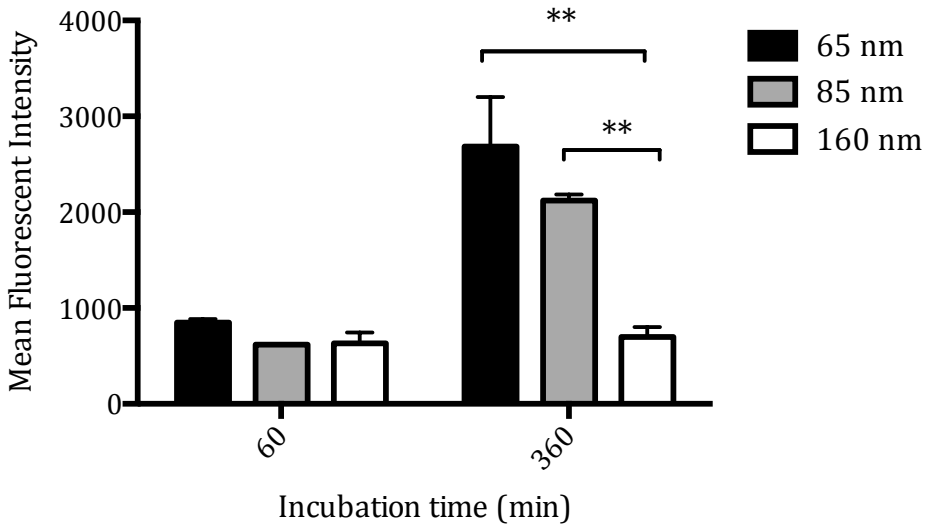

Figure 5.3: Effect of polymersomes size with cellular uptake. HeLa cells were treated with polymersomes at the size of 65, 85 and 160 nm in diameter and incubated for 60 and 360 minutes. Flow cytometry was used to measure cellular uptake. Three sets of experiments were performed in replicates. The statistical analysis used in this study was ANOVA followed by Tukey test for multiple comparison between each size (*p*-value: ** $p<0.01$).

Microscopy

Study of polymersomes uptake in HeLa cell was also observed in microscopy in live and fixed cells. HeLa cells were incubated with Rho-$PMPC_{25}$-$PDPA_{70}$ polymersomes, as in previous experiments. In fixed cells, HeLa cells were washed with PBS buffer after the desired incubation period, 10, 60, 360 and 1200 minutes. The cells were fixed with 3.7% *p*-formaldehyde and stained with nucleic acid staining, SYTO® 9 (Molecular probes®).

Confocal imaging was used to visualise the fluorescent signal of both polymersomes and nucleic acid as shown in *Figure 5.4*. Within 10 minutes of incubation, the rhodamine signal could be visualised, suggesting that polymersomes uptake occurs within 10 minutes. The signal was first located at the border of the cells, and gradually it spreads throughout the cytoplasm. Fluorescent intensity increased over the incubation period which might be the result of an increase in the cellular uptake of polymersomes.

Further investigation was performed with live cell imaging, following the same procedure apart from in the staining and fixation steps. HeLa cells were stained this time with Hoechst 33342 (Molecular probes®), and placed in imaging media. As shown in *Figure 5.5*, the polymersome signal has been observed as expected and

correlated to Flow cytometry and fixed cell imaging. The fluorescent signal obtained from Rho-PMPC-PDPA was observed throughout the cells after as little as 10 minutes of incubation.

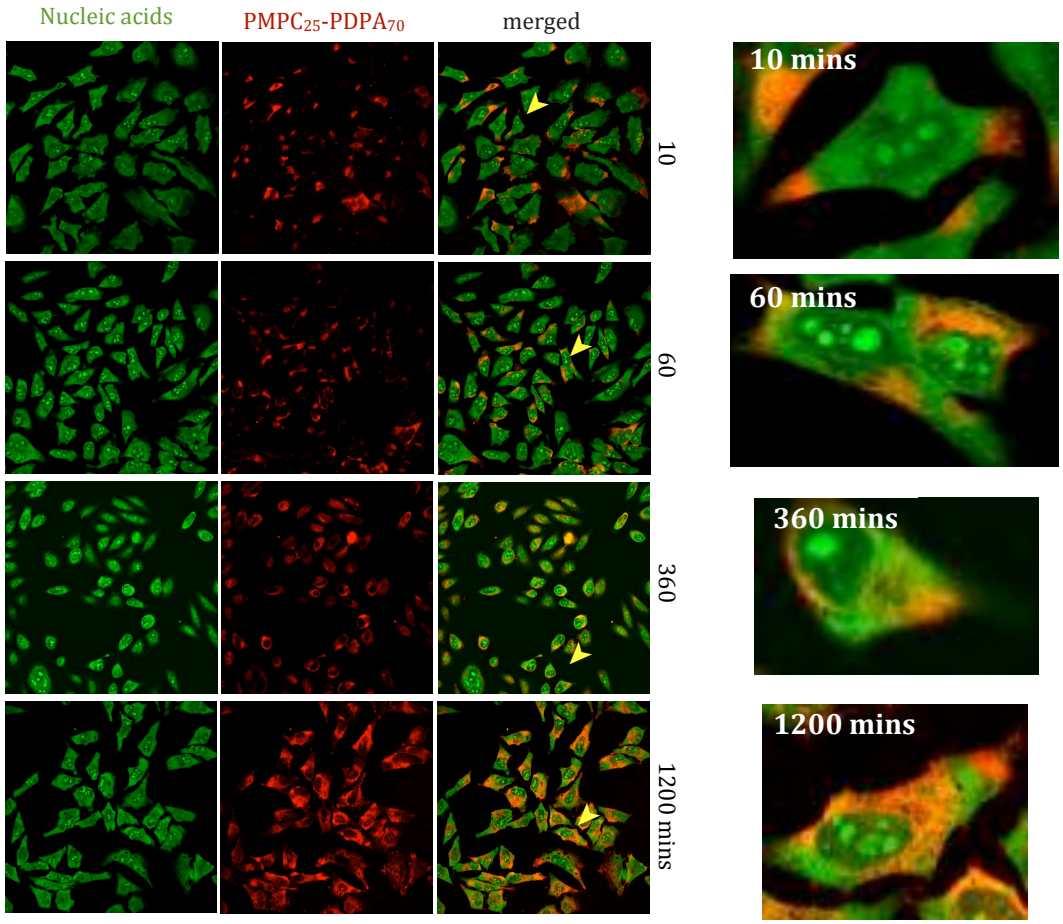

Figure 5.4: The imaging of cellular uptake of polymersome. The HeLa cells were treated with rhodamine-conjugated $PMPC_{25}$-$PDPA_{70}$ (Red) at ratio of 1:9 (Rho-$PMPC_{25}$-$PDPA_{70}$: $PMPC_{25}$-$PDPA_{70}$) polymersomes for 10, 60, 360, and 1200 minutes and stain with SYTO® 9 (green) before observed under the Olympus FV1000 confocal microscope with 40x objective lens. Selected cells (cells with arrows) were enlarged and shown as single cells on the right panel.

The polymersome signals observed in live cells (*Figure 5.5*) differ from those in fixed cells (*Figure 5.4*). The fixed cells show less fluorescence and different subcellular localisation. This is very likely due to the fixation process indicating that the polymer associated with the lipid membrane after endosome escape, see (Massignani, LoPresti et al. 2009).

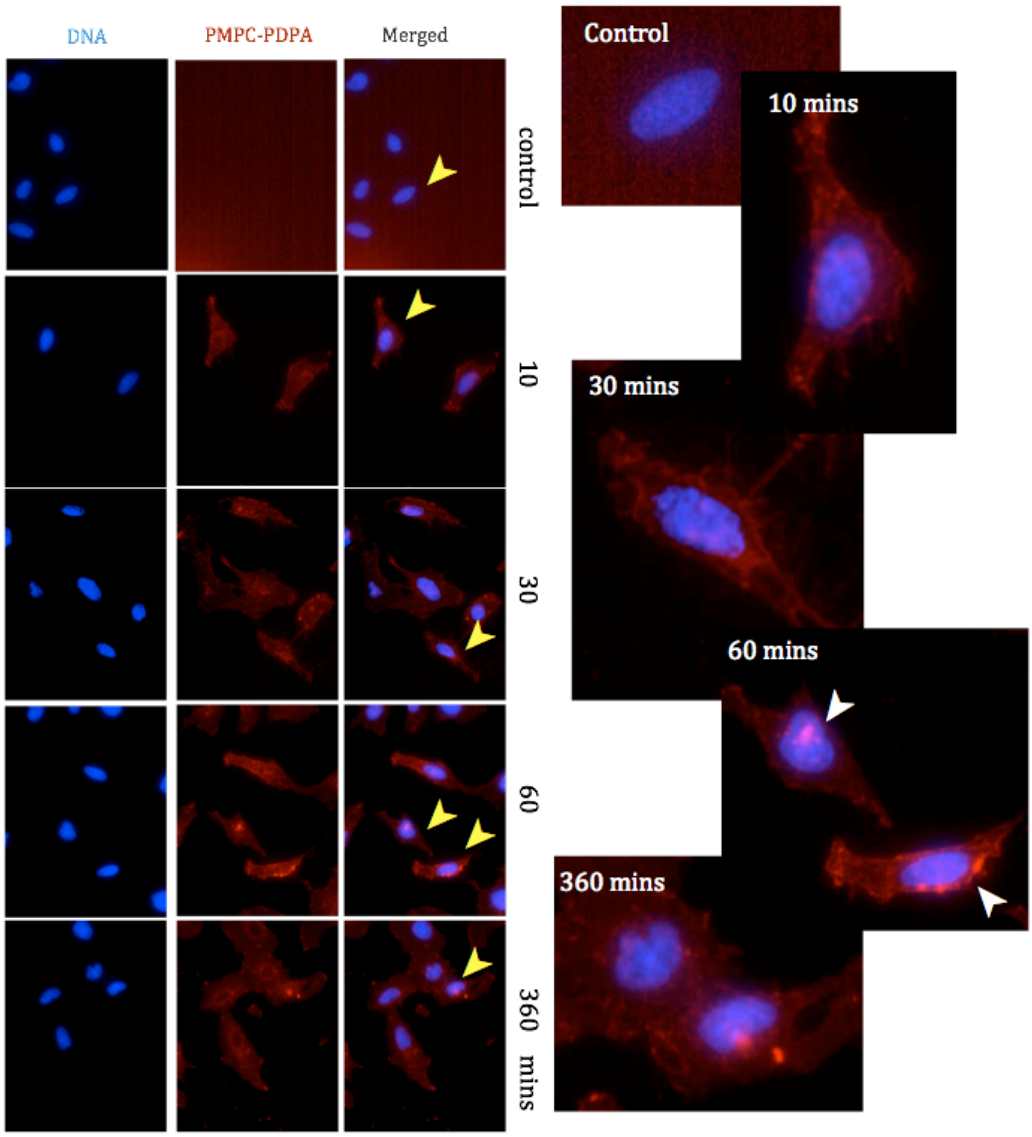

Figure 5.5: Fluorescent microscope image of HeLa with different time. HeLa cells were treated with rhodamine-labelled polymersomes for 10, 30, 60, and 360 minutes plus the untreated cells as a control experiment. The cells were washed and stained with Hoechst 33342 (nucleus) before being visualised under the BD Pathway™ 855 microscope with 40x objective lens. Selected cells (cells with yellow arrows) were enlarged and shown as single cells on the right panel.

The plot in *Figure 5.6* was obtained by the image analysis which is the comparing the average intensity of rhodamine between 2 areas in the cells, the cytoplasm and the membrane. The image analysis was achieved by the MATLAB software with support from Adrian Steve Joseph (see *Appendix C* for the script). In brief, the fluorescent signal obtained from Hoechst 33242 (blue) was used to determine the nuclear region of the cells, and the area of the cell was identified by rhodamine signal (red). The rhodamine intensity was divided into the membrane (the edge of the cell) region and cytoplasm (the cell area subtracted by the nuclear region and

membrane). All areas were normalised with the total rhodamine intensity and plotted as shown in *Figure 5.6*.

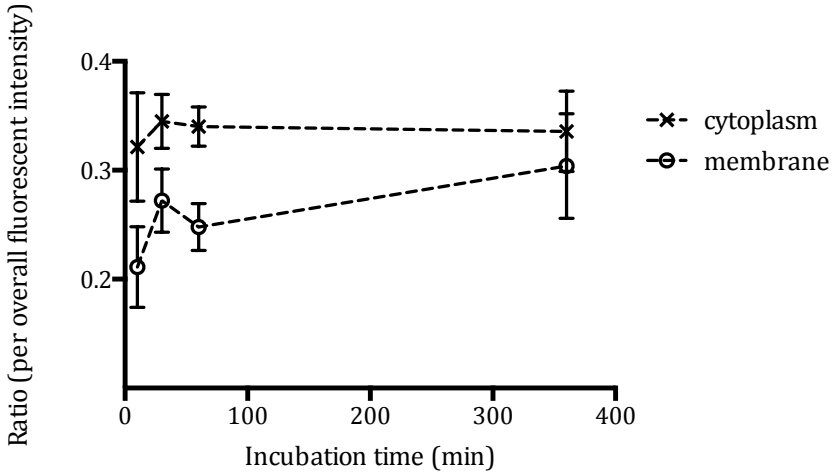

Figure 5.6: The ratio of polymer in different areas of the cells over time. Image analysis was performed using the MATLAB to determine the rhodamine intensity to represent the polymer signal inside the cells. Two different areas were studied, cell cytoplasm and cell membrane. All regions was normalised with the overall mean fluorescent intensity and presented as a plot of ratio of each region divided by total fluorescent intensity.

Fluorescent intensity in cell cytoplasm was quite stable along overall incubation time as well as signal from cell membrane. A similar ratio of fluorescent intensity in different areas suggests that polymersomes can spread throughout the cells and not be contained or accumulated in specific areas during experimental periods. However, the signal in cell cytoplasm was slightly decreased at late time point while in cell membrane was increased. This suggests the accumulation of polymers on the cell membrane as it has to be secreted out.

5.2.2. $PMPC_{25}$-$PDPA_{70}$ polymersome mediated siRNA delivery

Flow cytometry

Alexafluor® 647-labelled siRNA encapsulated in $PMPC_{25}$-$PDPA_{70}$ polymersomes (Alexafluor® 647-siRNA/polymersomes) were incubated with HeLa cells for 10, 60, 180, 360, and 1200 minutes at 1, 10, 50, and 100 nM siRNA concentration. HeLa cells were prepared and loaded into BD FACSArray™ Bioanalyzer to determine the fluorescent intensity of Alexafluor® 647 as siRNA was delivered inside the cells. The results are shown in *Figure 5.7* as the scatter plots and line plot. The scatter plots show the measurement of Alexafluor® 647 intensity (x-axis, Red-A) versus size scattering (y-axis, SSC-A). Gating was performed according to the FSC threshold in order to cut down the cell debris. The line plot shown as semi-logarithmic scale where x-axis as incubation periods and y-axis as normalised mean fluorescent intensity obtained from cells treated with siRNA encapsulated in polymersomes.

The mean fluorescent intensity of each sample was divided by the maximum fluorescent intensity (100 nM siRNA, 1200 minutes) to normalise the different in fluorescent signal from each set of experiment. The signal obtained from untreated control was used to set up the base line.

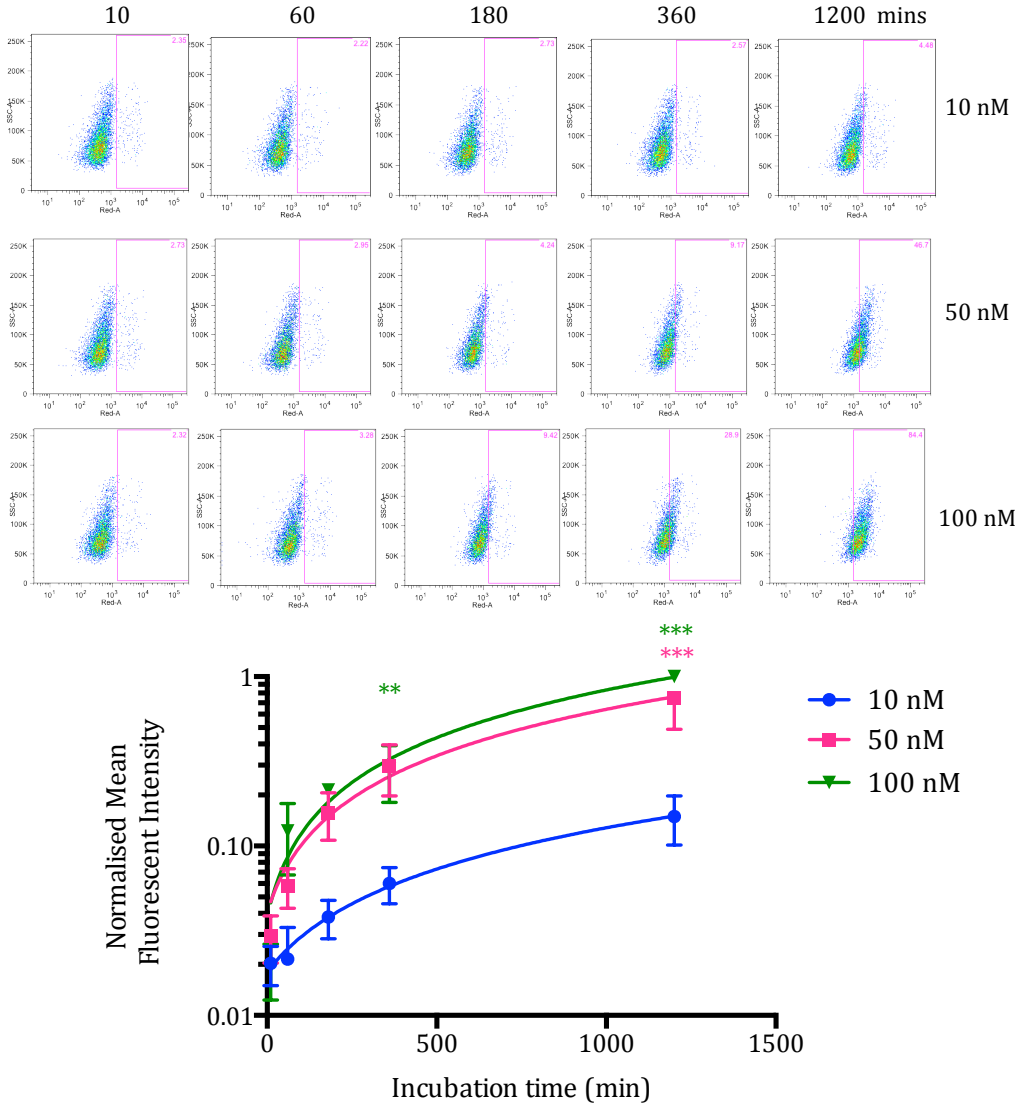

Figure 5.7: Cellular uptake of Alexfluor® 647-siRNA/polymersomes. HeLa cells were treated with various incubation times and various siRNA concentrations before trypsinisation and centrifugation. The siRNA concentration shown in the plot is the final siRNA concentration in the media. The cell pellets were resuspended with cold PBS buffer and the fluorescent signal measured using BD FACSArray™ Bioanalyzer. The cells with positive fluorescent signal of polymersomes were shown as the mean fluorescent intensity, compared with untreated control and normalised with maximum fluorescent intensity (100 nM siRNA at 1200 minutes). The experiment was performed in replicates and three repeats. The statistical analysis used in this study was ANOVA followed by Dunnett's multiple comparison test between each siRNA concentration and untreated control (p-value: $^{**}p<0.01$,$^{***}p<0.001$).

Uptake can be seen at the minimum siRNA concentration of 50 nM for 1200 minutes (p-value < 0.001) and increased at siRNA concentration of 100 nM for 360 (p-value = 0.0051) and 1200 minutes (p-value < 0.001). Similar trends of siRNA uptake can be

observed at lower siRNA concentration but show no statistical difference due to the detection limits of the instrument.

Comparison of polymersomes (Rhodamine-conjugated $PMPC_{25}$-$PDPA_{70}$) and siRNA (siRNA encapsulated in polymersomes) uptake was performed as shown in *Figure 5.8*, revealing a similar uptake trend. At 10 minutes of incubation, uptake of empty is statistically different from uptake of siRNA in polymersomes at 50 nM (*p*-value = 0.0006) and 100 nM (*p*-value = 0.0003). This discrepancy might be due to the sensitivity of flow cytometry, in which intensity obtained from empty and loaded polymersomes especially at early stage can be under detection limits. However, no significant difference among samples was observed at longer incubation times, also no significant difference compared with untreated control. These results suggest similar rate of uptake between empty and loaded polymersomes in HeLa cells.

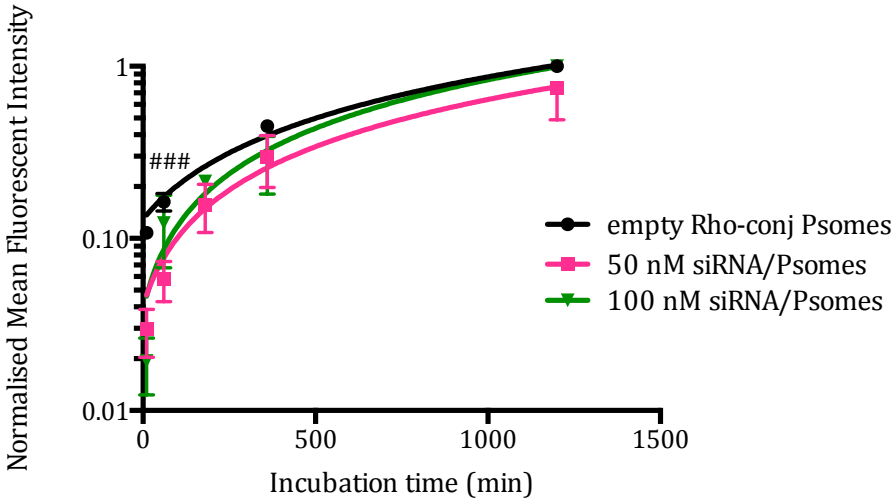

Figure 5.8: Cellular uptake of polymersomes alone and siRNA with polymersomes as a carrier. Comparison of the uptake of empty polymersomes (Rho-labelled $PMPC_{25}$-$PDPA_{70}$) and siRNA uptake (siRNA encapsulated in polymersomes) was performed according to the data from Flow cytometry. The statistical analysis used in this study was ANOVA followed by Tukey test for multiple comparison among cellular uptake of polymersomes and siRNA uptake (siRNA encapsulated in polymersomes) (*p*-value: ###$p<0.01$).

Microscopy

After treatment with Cy3-labelled siRNA encapsulated in $PMPC_{25}$-$PDPA_{70}$ polymersomes (Cy3-siRNA/polymersomes), HeLa cells were fixed and visualised under BD Pathway™ 855 microscope. The cells were stained with DNA staining, Hoechst 33342 to identify the area of the nucleus. From *Figure 5.9,* cellular uptake of siRNA can be visualised at 60 minutes of incubation, with the fluorescent signal obtained in cytoplasm. Further increased in the fluorescent signal was detected with longer incubation times. Moreover, the siRNA was spread throughout the cells at

1200 minutes of incubation. siRNAs were detected in cytoplasm which is the desired site for RNAi machinery, as well as in the nuclear region. It is likely that the siRNA is accumulated in nucleolus, where ribosomal RNA is located. This experiment confirms that polymersomes can effectively carry siRNA into the cytoplasm as expected.

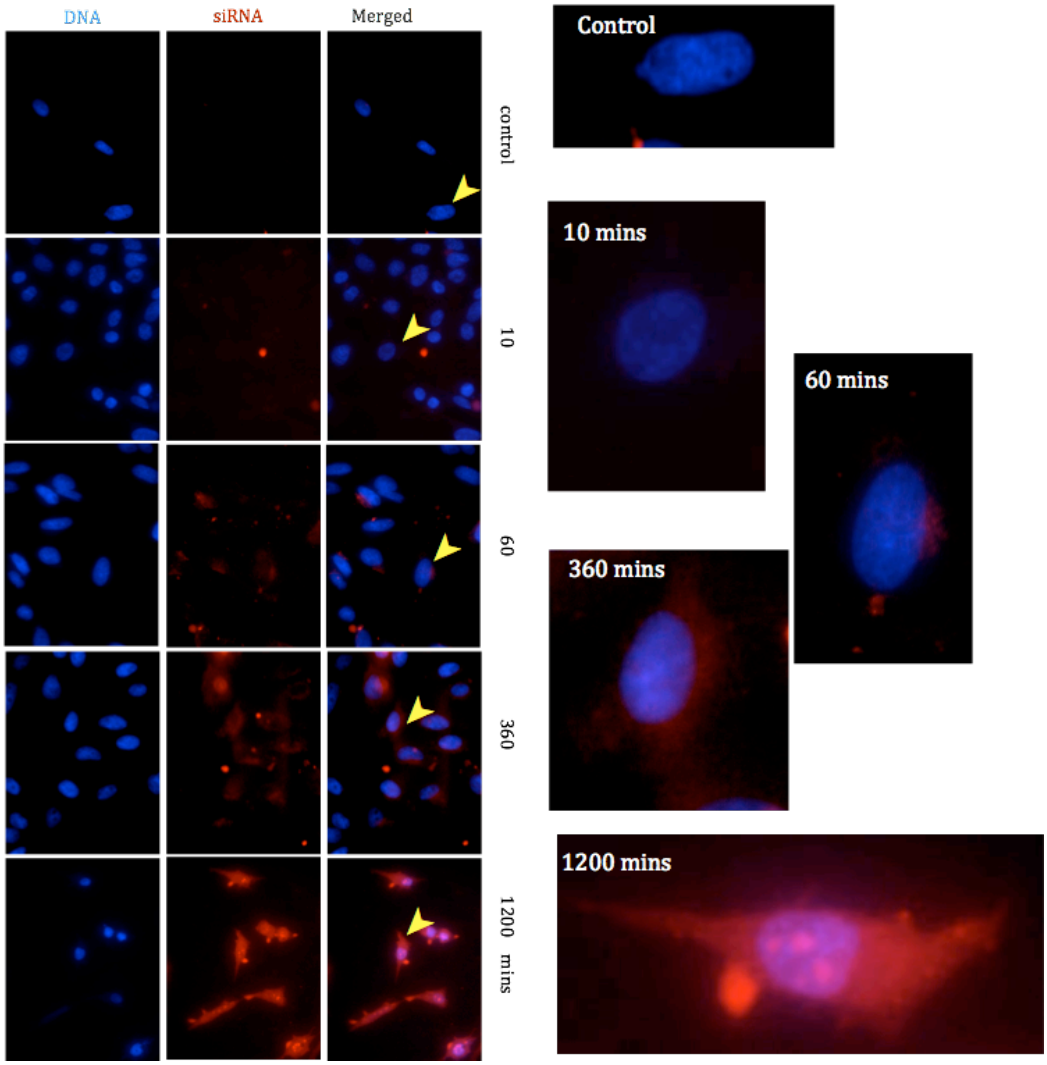

Figure 5.9: Fluorescent microscope image of siRNA/polymersomes uptake in HeLa with different incubation time. HeLa cells were treated with Cy3-siRNA/polymersomes at about 40 nM for 10, 60, 360, and 1200 minutes plus the untreated cells as a control experiment. The cells were washed and fixed with 3.7% formaldehyde and stained with Hoechst 33342 (nucleus) before being visualised under the BD Pathway™ 855 microscope with 40x objective lens. Selected cells were enlarged and shown as single cells on the right panel.

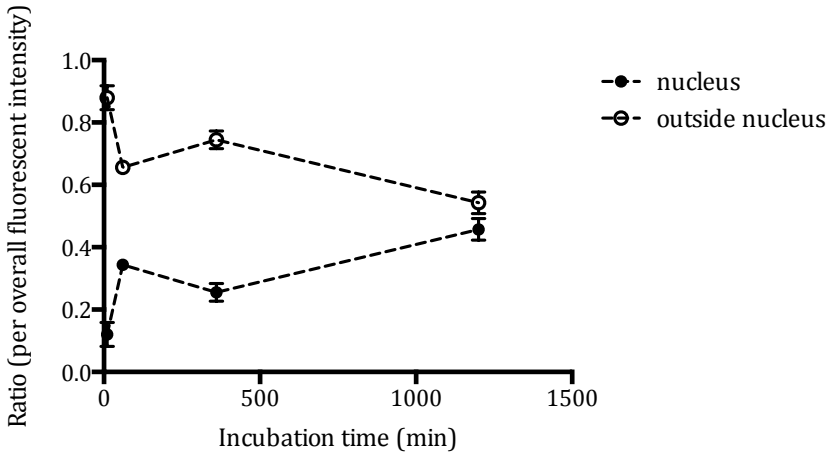

Figure 5.10: The ratio of siRNA within the cells over time: siRNA/polymersomes. Image analysis was performed using the MATLAB to determine the Cy3 intensity to represent siRNA signal inside the cells. Fluorescent intensity of Cy3 was divided into two areas, the nuclear region which was determined by nuclear staining Hoechst 33342, and the rest of the cells. All regions were normalised with the overall mean fluorescent intensity and presented as a plot of ratio of each region divided by total fluorescent intensity.

The image analysis was performed as shown in *Figure 5.10*. The nuclear region was determined by Hoechst 33242, corresponding to the nucleus ROI, and the rest of the cells. The results show that Cy3 siRNA starts to be delivered inside the cells with the higher signal at early incubation time. The fluorescent signal in the nucleus was very low initially but increased overtime, whereas, the signals from the rest of the cells was high in early incubation time and dropped to an amount almost equal to those in nucleus at 1200 minutes. Even though no previous report of effects from fixative reagent on nucleic acid has been found, cellular uptake of siRNA was confirmed in live cells as shown in *Figure 5.11.*

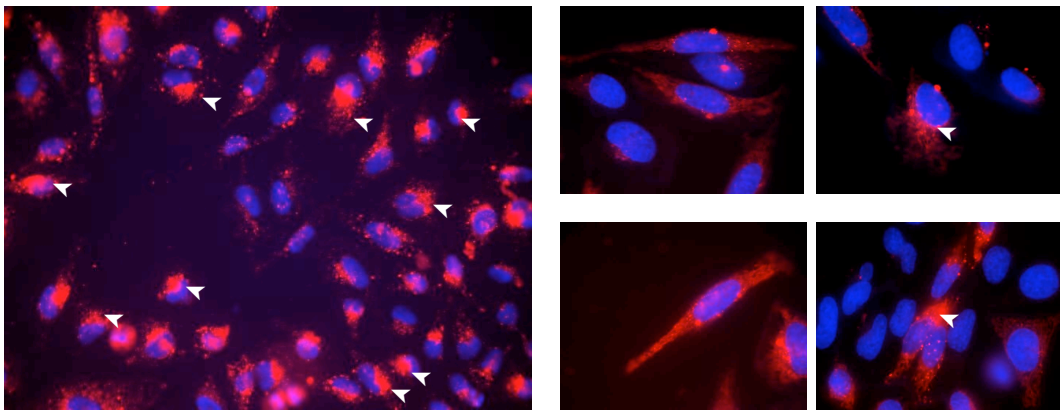

Figure 5.11: Live cell imaging of siRNA/polymersomes uptake in HeLa. HeLa cells were treated with Cy3-siRNA/polymersomes at about 40 nM for 1200 minutes. The cells were washed and stained with Hoechst 33342 (nucleus) before being visualised under the BD Pathway™ 855 microscope with 20x (left) and 40x objective lens (right).

Cy3 fluorescent signal was mostly found in cytoplasm and also in the nucleus of some cells. Moreover, the signal in cytoplasm is strongly observed in specific areas near nuclear membrane (white arrows), which very likely to be the Endoplasmic Reticulum (ER).

Delivery of siRNA with polymersomes was compared with the commercially available transfecting reagent, Lipofectamine™ 2000 (Invitrogen) (Chu et al. 2009) which is widely used for both DNA and RNA delivery. Lipofectamine™ 2000 is a liposome with cationic lipids able to form complex with anionic charge on phosphate group in nucleic acids.

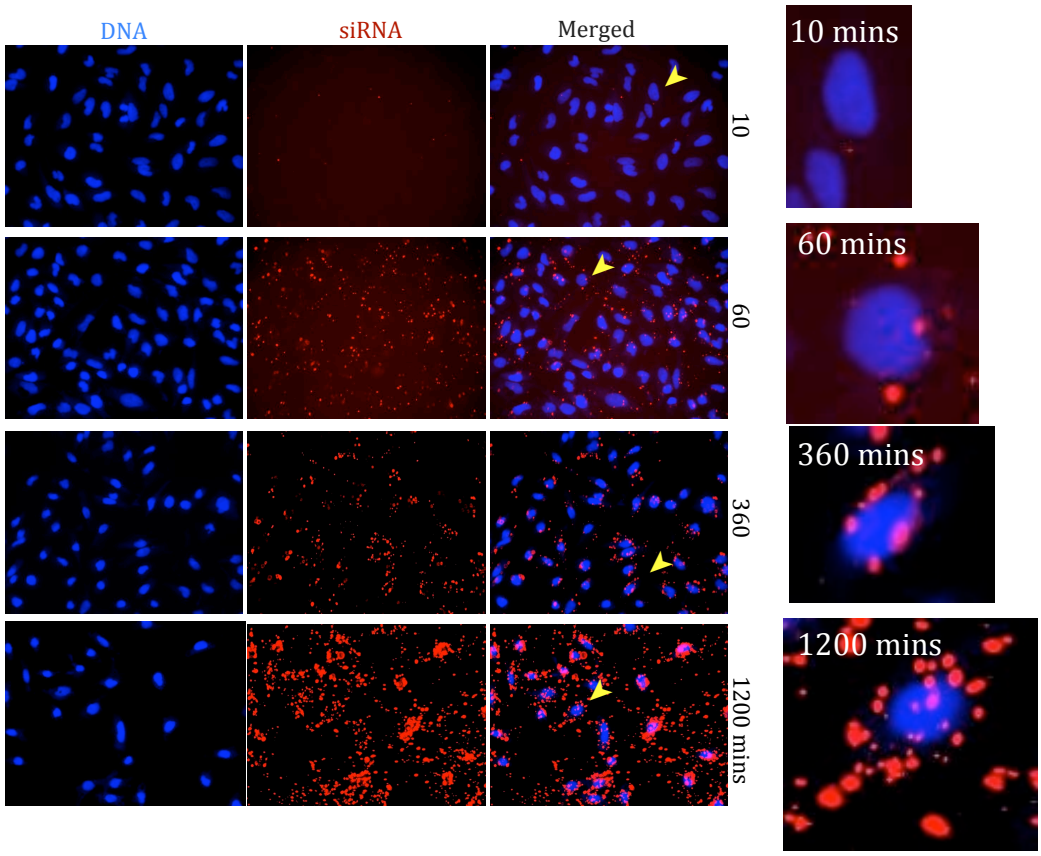

Figure 5.12: Fluorescent microscope image of siRNA/Lipofectamine uptake in HeLa with different incubation times. HeLa cells were treated with Cy3-siRNA/Lipofectamine for 10, 60, 360, and 1200 minutes The cells were stained with Hoechst 33342 (nucleus) before visualisation under the BD Pathway™ 855 microscope with 20x objective lens. Selected cells were enlarged and shown as single cells on the right panel.

As shown in *Figure 5.12*, siRNA delivery using Lipofectamine™ 2000 provides uptake within 60 minutes of incubation. However, the siRNA signal is punctuated and mostly confined to what appear to be large vacuoles. Increase in the fluorescent signal was observed at 1200 minutes, however, the localisation is still quite punctuated and no different from earlier time points. This suggests the majority of

siRNA was not released in the cytosol. Such an effect has been recently quantified by using lipid nanoparticles loaded with siRNA conjugated colliodal-gold particle, with analysis of the amount of siRNA in the cytoplasm. It was observed that only 2% of siRNA is actually released by the endosomal compartments (Gilleron et al. 2013a).

No evidence of the size of siRNA/Lipofectamine in this work and previous work. The size of Lipofectamine™ 2000 itself and pEGFP/Lipofectamine (plasmid DNA of enhanced green fluorescent protein) was observed as about 128.5 and 205.9 nm, respectively in the previous work (Kong et al. 2012). The endosomal escape and releasing mechanism of siRNA delivered by Lipofectamine™ 2000 and other liposome also remains unclear. However, the proposed mechanism is through the "fusion" of liposomes to the endosomal membrane or cell membrane with the requirement of helper pH-sensitive lipid, DOPE (Wrobel et al. 1995b, Almofti et al. 2003a). However, liposome might form complex with nucleic acid in different forms such as complex lamellar, resulting in ineffective release of nucleic acids (Tresset 2009a). The images of HeLa cells with siRNA delivered by polymersomes and Lipofectamine™ 2000 were compared in *Figure 5.13.* There is obvious difference in fluorescent signal where polymersomes can deliver siRNA throughout the cytoplasm including the nucleus. On the contrary, a small amount or almost no release of siRNA is obtained when using Lipofectamine™ 2000 as a carrier. Similar siRNA localisation was observed in the poly[(DMAEMA)-b-(BMA)-co-(DMAEMA)-co-(PAA)], the pH sensitive micelles (Convertine et al. 2010), siRNA-conjugated Quantum rod micelles (Law et al. 2012). These systems show diffuse siRNA across the cytoplasm, whereas discrete punctuated signal was observed in siRNA/ Lipofectamine system. In addition, punctuated signal from siRNA delivered by Liposome system was also detected in previous studies (Barreau, Dutertre et al. 2006, Tagalakis et al. 2011). This might be an evidence of the ineffective "fusion" mechanism of Lipofectamine™ 2000 which was proposed (mentioned previously in *Figure 2.*10) as endosomal escape and siRNA release.

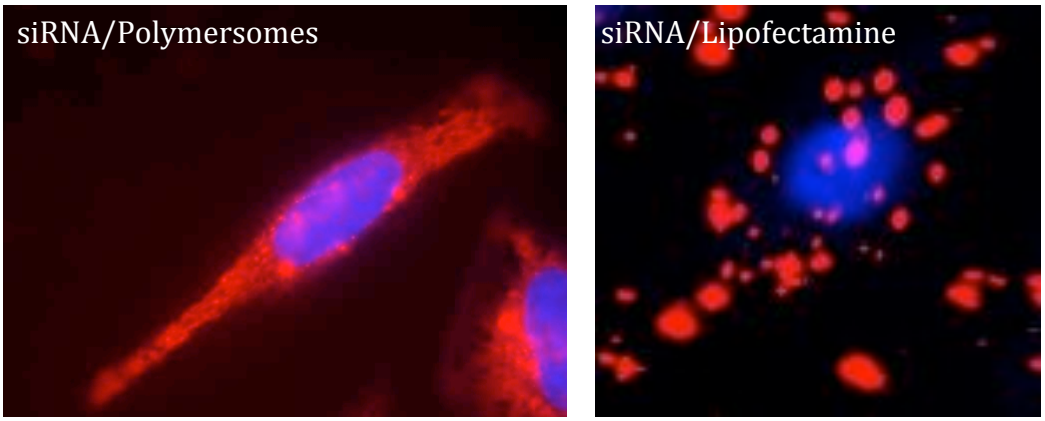

Figure 5.13: Comparison of siRNA delivery by two different carriers. HeLa cells were incubated with polymersomes containing siRNA (left) and Lipofectamine™ 2000 containing siRNA (right) for 1200 minutes before visualisation under the BD Pathway™ 855 microscope. Nuclear staining by Hoechst 33342 was performed.

Image analysis was performed to investigate the location of siRNA delivered by Lipofectamine™ 2000 as shown in *Figure 5.14,* using similar method as siRNA/ polymersomes in *Figure 5.10*. The results reveal the low accumulation of siRNA found in the nucleus after extended incubation periods. Moreover, most fluorescent signal was obtained in the rest of the cells with not much change occurring over all incubation periods.

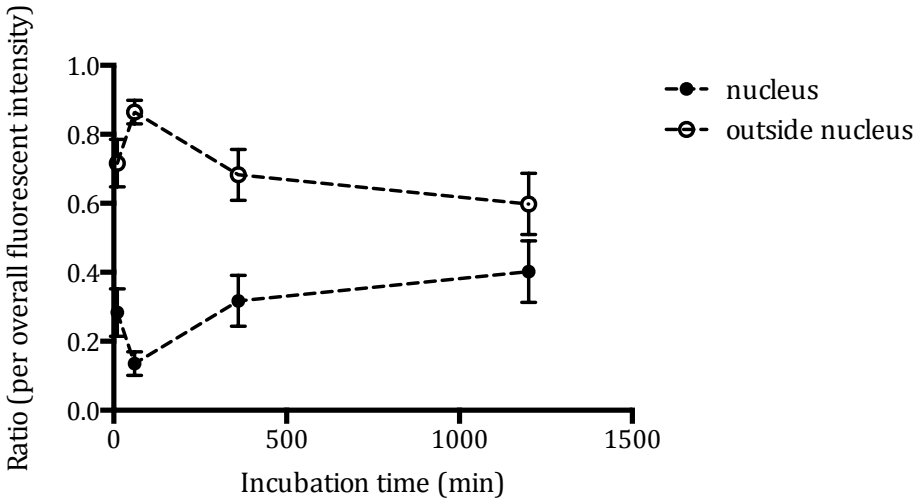

Figure 5.14: The ratio of siRNA within the cells over time: siRNA/Lipofectamine. Image analysis was performed using the MATLAB to determine the Cy3 intensity to represent siRNA signal inside the cells. Fluorescent intensity of Cy3 was divided into two areas, the nuclear region which was determined by nuclear staining Hoechst 33342, and the rest of the cells. All regions was normalised with the overall mean fluorescent intensity and presented as a plot of ratio of each region divided by total fluorescent intensity.

Further experiments were performed to observe the siRNA localisation in HeLa cells. To investigate further, the cells have been transfected with CellLight™ Early Endosomes marker, the modified virus containing Rab5 fused with GFP expression

system. Rab5 (Zahraoui et al. 1989) is the peripheral membrane protein generally found in early endosomal membranes, which functions as a regulator of early endosomal membrane fusion (Chavrier et al. 1990). It is used widely as an early endosome marker. Therefore the early endosome can be tracked and visualised under microscope together with Cy3-siRNA/polymersomes and nuclear staining. HeLa cells which stained with the early endosomal marker were treated with Cy3-siRNA/polymersomes for one night before imaging. As discussed previously, internalisation of siRNA/polymersomes still be active even at 20 hours of incubation time, therefore the uptake and endocytosis of siRNA could be observed at this time point.

In *Figure 5.15*, two cells were selected to analyse the colocalisation with early endosome, both cells show strong siRNA signals (red) throughout the cell and most of signals are not colocalised with Rab5 endosome marker (green). According to the data from Milagros Avila-Olias, the colleague in Battaglia's group who study the internallisation of $PMPC_{25}$-$PDPA_{70}$ in mammalian cells, the $PMPC_{25}$-$PDPA_{70}$ polymersomes can be internalised to the cell via receptor mediated endocytosis and the presence of PMPC-PDPA polymersomes in the endosome was confirmed by time-lapse imaging (unpublished data). Together with the data from this work, it confirms the siRNA delivery inside the cytoplasm. and the release of siRNA out of early endosome. Colocalisation of siRNA (red) and nucleus (blue) was investigated but very low colocalisation has been observed. Moreover, some Cy3 signals from nuclear region were detected in the area of low or no Hoechst 33342 staining (see *Figure 5.15, DNA and siRNA)*, which correlated with previous data (*Figure 5.9*) that siRNA might accumulated in the nucleolus. Most signals were detected at the nuclear membrane which is close to the location of ER. This suggests the different location of siRNA delivered by polymersomes.

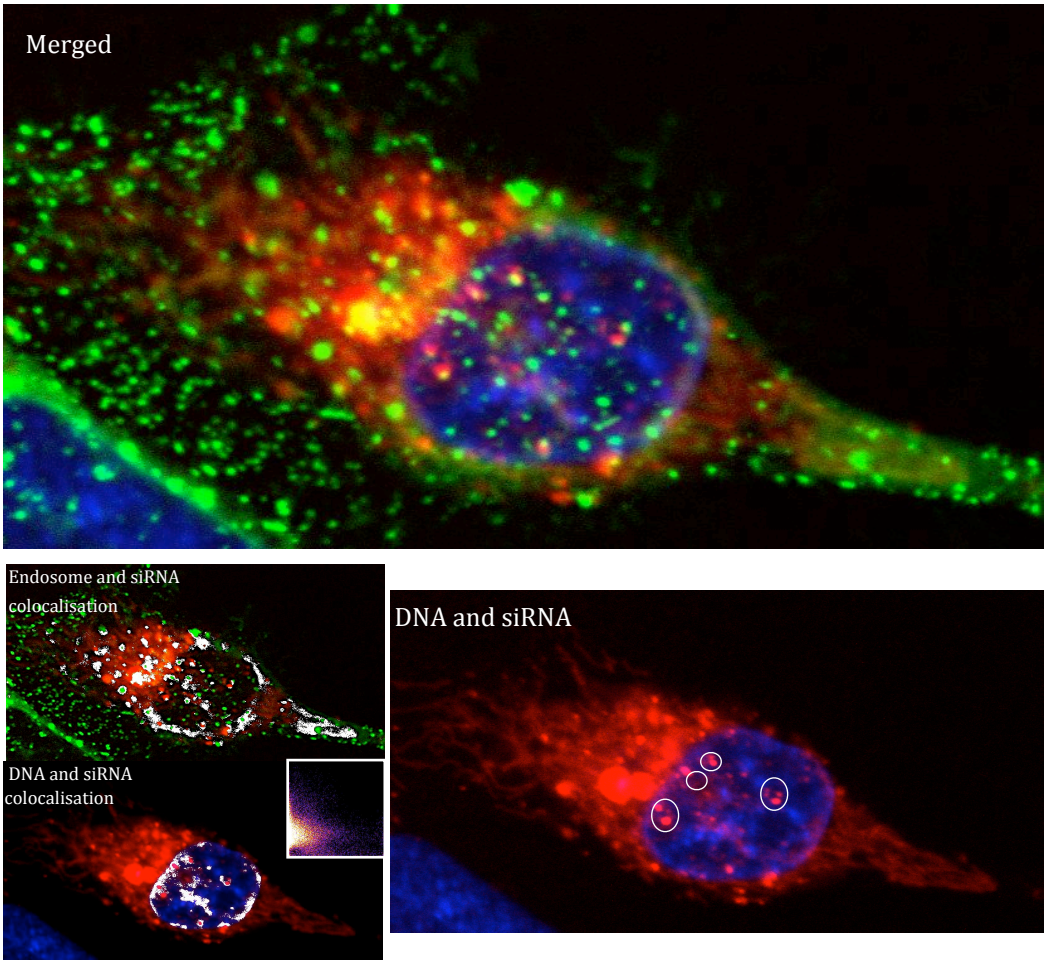

Figure 5.15: Live cell imaging of siRNA uptake in HeLa cells. HeLa cells were incubated with CellLight™ Early Endosomes-GFP before treatment with Cy3-siRNA/polymersomes overnight. The cells were washed with PBS buffer to remove of excess siRNA/polymersome, and added with phenol red-free DMEM. The cells were imaged under the Zeiss LSM510 confocal laser scanning microscope with 20x objective lens at 37°C with 5% CO_2 to maintain the cell viability. This figure shows three different fluorescent signals including Cy3-siRNA (red), GFP-CellLight™ Early Endosomes-GFP (green), nucleus staining-Hoechst (blue).

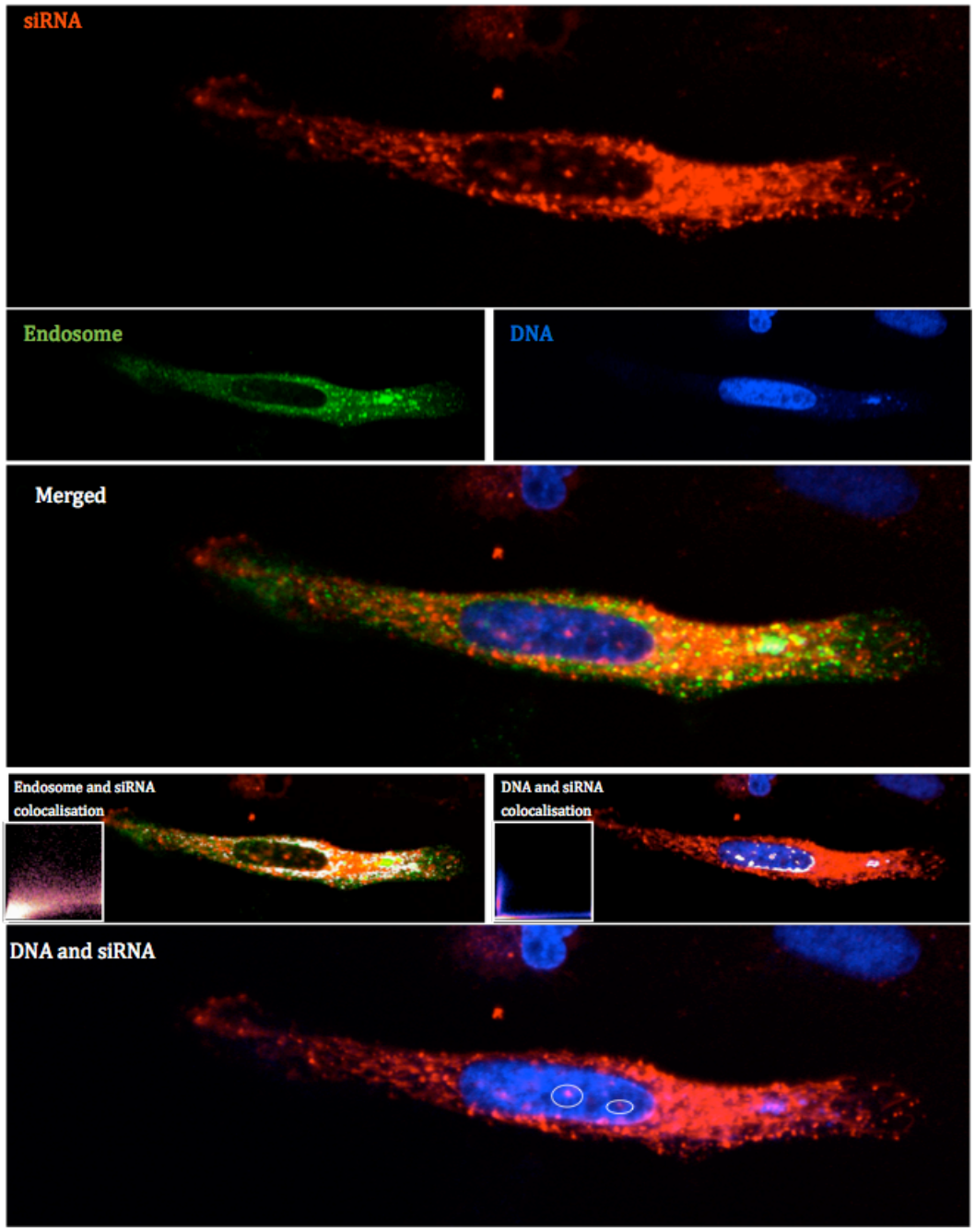

Figure 5.15: Live cell imaging of siRNA uptake in HeLa cells (cont.). HeLa cells were incubated with CellLight™ Early Endosomes-GFP before treatment with Cy3-siRNA/ polymersomes overnight. The cells were washed with PBS buffer to remove of excess siRNA/polymersomes, and added with phenol red-free DMEM. The cells were imaged under the Zeiss LSM510 confocal laser scanning microscope with 20x objective lens at 37°C with 5% CO_2 to maintain the cell viability. This figure shows three different fluorescent signals including Cy3-siRNA (red), GFP-CellLight™ Early Endosomes-GFP (green), nucleus staining-Hoechst (blue).

5.3. Summary

Cellular uptake of PMPC-PDPA polymersomes was observed within 10 minutes of incubation, whilst an increasing of uptake can be found with extended periods. The uptake reaches to its equilibrium at around 20 minutes in HeLa cells. It has to be noticed that the rhodamine signal in this work was obtained from rhodamine-labelled polymers, to represent all polymers but the actual amount of polymer is higher than the rhodamine signal as only 10% of the polymersome polymers were labelled.

PMPC-PDPA polymersomes can effectively deliver siRNA into the cytoplasm within 60 minutes but the longer incubation is required for greater fluorescent intensity, which is a consequence of greater polymersomes uptake. The support evidence of the internalisation of polymersomes occur through receptor-mediated endocytosis (unpublished data from the colleague in our group), together with the data from this work that the delivered siRNA shows low colocalisation with early endosome suggesting the endosomal escape of polymersomes and payloads.

When compared to another commercially available transfection system, Lipofectamine™ 2000, the siRNA can be delivered into the cells within 60 minutes of incubation, similar to delivery by polymersome. However, the siRNA obtained under fluorescent microscope appeared as intense spots with increased numbers, but remain punctuated over incubation times.

The diagrams in *Figure 5.16* show the cellular uptake of polymersomes and siRNA delivered by polymersomes and Lipofectamine™ 2000. Internalisation of both polymersomes and Lipofectamine™ 2000 occurs via endocytosis but endosomal escape mechanisms are different. For PMPC-PDPA polymersomes, pH-sensitive PDPA plays an important role as it changes from hydrophobic to hydrophilic in low pH conditions within the early endosome. This leads to the polymersomes disassembly and increase in osmotic pressure in endosome followed by membrane osmolysis. Both siRNA and polymers can be released from the endosome. PDPA becomes hydrophobic at physiological pH, and integrates with the endogenous membrane and consequently accumulated in membrane rich organelles such as the ER or Golgi. siRNA, on the contrary, is able to diffuse in the cytoplasm. Our data suggest that once in the cytosol, siRNA can diffuse freely reaching several subcellular compartment including the ER and the cell nucleus. Whether such trafficking is simply diffusional or associated with endogenous mRNA transport is still subject of study.

Whereas in Lipofectamine™ 2000, the fusion of lipid to endosomal membrane is proposed as endosomal escape. This is ascribed as a mechanism to deliver siRNA to the cytoplasms. However, the majority of the fluorescent signal was punctuated, suggesting low siRNA release in cytoplasm, unlike the signal obtained from siRNA delivery with polymersomes. In a recent work, only 2% of siRNA delivered by liposome shows releasing from the endosomal compartments (Gilleron, Querbes et al. 2013a). On the contrary, both flow cytometry and microscopy data suggest that PMPC-PDPA polymersome enable much larger quantity of RNA in the cytosol. While this bodes well for the RNA activity, such high concentration of RNA and more importantly wider distribution within the cells can induce more off target effect.

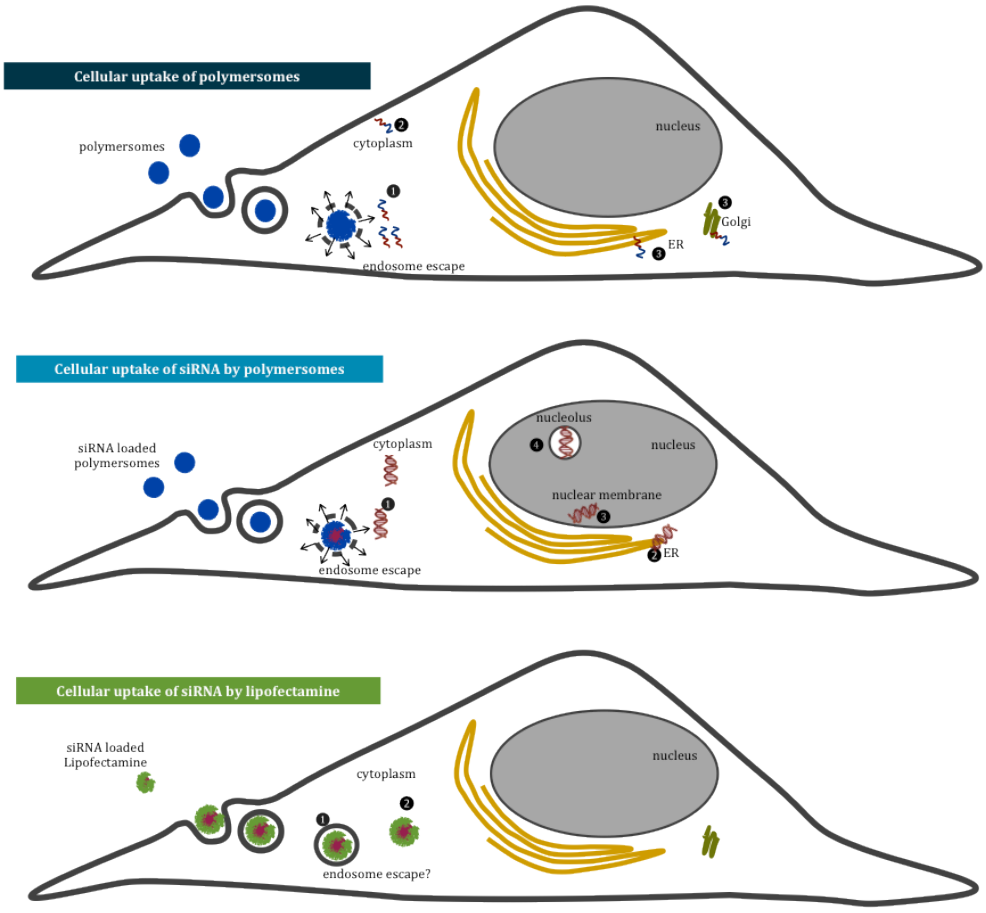

Figure 5.16: Overview of cellular uptake of polymersomes and siRNA. Proposed mechanism and of polymersomes delivery (upper panel), siRNA/polymersomes (middle panel) and siRNA/Lipofectamine (bottom panel) where different localisation of polymer and siRNA was proposed according to the results from this Chapter.