Chapter 6

Results and Discussions III

Knockdown efficiency and Cellular response of polymersomes mediated siRNA

6.1. Introduction

In the previous Chapter, polymersomes have been confirmed as efficient siRNA carriers into HeLa cells. Successful delivery of siRNA is only the first step, as its interference activity has to be determined, as well as other side effects on the cells. For the knockdown efficiency, the specificity of siRNA sequence has to be considered, as only 11 nucleotides matched to their target mRNA can cause gene silencing (Jackson et al. 2003b). Use of bioinformatics tools can fulfill this requirement, as RNA sequences can be designed to match with target gene (with less similarity to any other genes) in order to limit the possible non-target knockdown.

Lack of specificity can lead to unwanted off target effects such as the silencing of the non-targeted gene, which may affect the target gene indirectly. The off target effect can cause false positive mRNA expression of target gene. As mRNA expression is regulated by many steps such as transcriptional and translational regulations, the effect on one gene might be related to many genes which are directly or indirectly involved. Side effects on cells including carrier-mediated response and off-target effects from siRNA have been considered in this work. The well known gene delivery system, Lipofectamine™ 2000, which has been used to validate siRNA, has been shown to affect cell viability (Maurisse et al. 2010) and global gene expression (Fiszer-Kierzkowska et al. 2011). On the contrary, polymersomes shows low effect on the cell viability when treated for 24 hours on 23 different cell types (Massignani,

LoPresti et al. 2009). However, investigation of other cellular responses from polymersomes treatment is still required. Off target effects also depend on siRNA concentration, as overloading might leads to immune stimulation (Caffrey et al. 2011) as well as saturation of the endogenous RNAi machinery (Grimm et al. 2010).

In order to determine the knockdown ability of siRNA carried by polymersomes in this work, Lamin A/C was chosen as a model protein. Lamin A/C is a nuclear envelope protein which is transcribed from LMNA gene (gene ID: NM_005572, NCBI). Lamin A/C is an important nuclear structural protein but its knockdown does not cause cytotoxic effects (Sullivan et al. 1999). It was chosen as model for endogenous gene knockdown to translate RNAi into mammalian cells (Elbashir, Harborth et al. 2001, Elbashir et al. 2002). Anti-Lamin A/C siRNA sequence was obtained from previously published work by Elbashir, et al (Elbashir, Harborth et al. 2002). Knockdown of Lamin A/C was performed with several siRNA concentrations of the selected sequence. The knockdown efficiency was compared with commercially available siRNA carriers, Lipofectamine™ 2000. Furthermore, polymersomes as well as siRNA itself have been investigated for their effects on the cells regarding cytotoxicity and other intracellular responses, including pro-inflammatory response and interferon response. The aim of this study is to examine the siRNA interference activity as well as to investigate, in detail, the effect of the delivery system to achieve the most effective knockdown efficiency.

6.2. Results and Discussions

6.2.1. Cell viability

Foreign compounds internalised in cells could be toxic and lead to loss in biological function and cell death. Therefore, a cell viability test has to be performed to assure that the cells could survive and maintain their functions. Cell viability can be monitored by several assays, including cell counting, cell growth, membrane integrity (such as vital staining), and enzymatic activity (Lactate Dehydrogenase or LDH assay, MTT assay). MTT assay, which was used in this study, is based on an investigation of the metabolic activity of mitochondrial enzyme which can be used to estimate cell viability. MTT is the short name for 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide, substrate of NAD(P)H-dependent oxidoreductases and dehydrogenases (Mosmann 1983b, Berridge et al. 2005). MTT can be reduced and converted to insoluble formazan. The colour substrate, MTT, is in yellow while the insoluble product, formazan, is in purple. Therefore, production of formazan can be determined by measuring the absorbance at a wavelength of 570 nm. This assay (van Meerloo et al. 2011) has been used broadly in the *in vitro* study of cytotoxic effects of drugs on various cell types (Vistica et al. 1991, Ciapetti et al. 1993, Tully et

al. 2000). Moreover, this technique showed higher sensitivity than that of LDH, and protein assay (Fotakis et al. 2006).

In this work, an experiment was performed to test the cell viability on the polymersomes treated cells in polymer concentration of 5 and 10 μM (or 1 and 2 mg/ml in the culture media) after 24 hours incubation. Cell counting from hemocytometry and MTT assay were performed and compared to the PBS treated cells as the negative control (100% cell viability). The results are shown *Figure 6.1* as cell counting (grey bar) and MTT assay (white bar). No significant difference in cell counting was observed among various polymer concentrations. However, for MTT test, the cell viability of 10 μM PMPC-PDPA polymer treated cells decreased to 80% (p-value=0.0424). In this experiment, MTT assay is a more suitable technique as the reaction occurs in active mitochondria that can represent living cells, whereas cell counting might not be sensitive enough. In addition, high cell viability of polymersomes treated cells suggests very low cytotoxicity of this polymer, with up to 80% of cells remaining in very high polymer concentration (about 10 times of normal polymer concentration used for siRNA delivery).

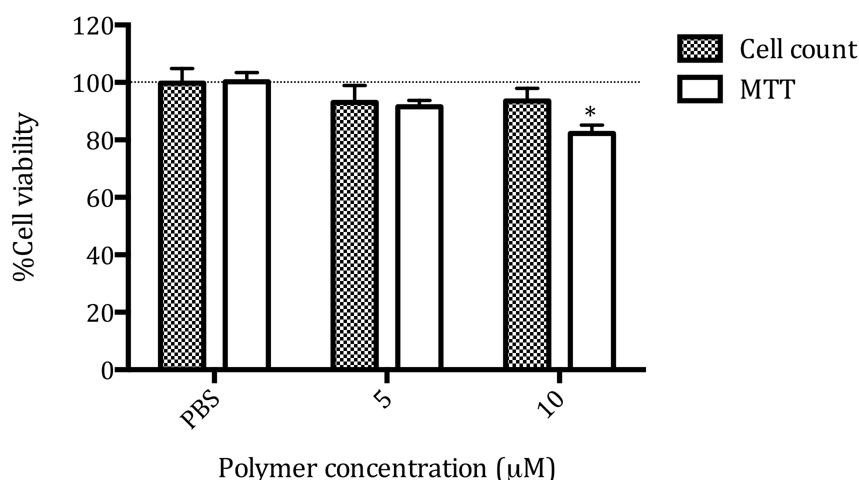

Figure 6.1: Cell viability of polymersomes treated cells. HeLa cells were cultured and treated with polymersomes at concentrations of 5 and 10 μM for 24 hours. After treatment, cells were trypsinised and counted under the light microscope. MTT assay was performed to test the cytotoxicity of the cells by comparing to the non-treated cells (white bar). Cell counting was achieved by direct counting the number of cells collected after trypsinisation on Haemocytometer (grey bar). The experiment was performed four repeats with three replicates. Statistical analysis was performed using 2-way ANOVA test, with Tukey's multiple comparison test (* p-value < 0.05).

MTT assay for polymersomes with and without siRNA was also performed. The results were plotted as shown in *Figure 6.2*, there is no significant difference between empty polymersomes and polymersomes encapsulated with siRNA.

However, there is also no change in cell viability tested in any treatment compared with untreated control cells. This suggests no effect of siRNA/polymersomes on HeLa cells.

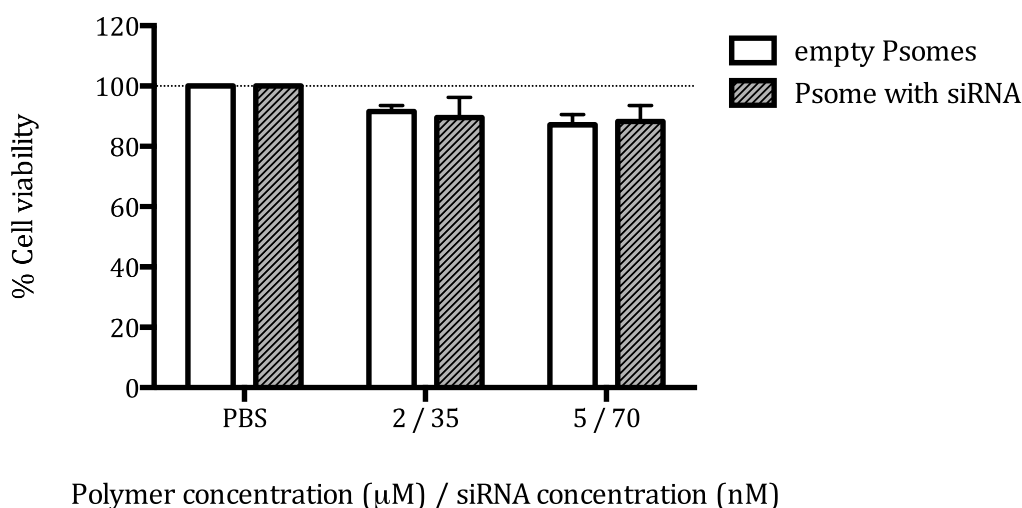

Figure 6.2: Cell viability of HeLa cells: effect of siRNA. HeLa cells were cultured and treated with polymersomes at concentrations of 2, and 5 μM for 24 hours with empty polymersomes (white bar) and polymersomes with negative control siRNA (grey bar). After treatment, cells were trypsinised. MTT assay was performed to test the cytotoxicity of the cells by comparing to the non-treated cells. The experiment was performed with three repeats. Statistical analysis was performed using 2-way ANOVA test, with Tukey's multiple comparison test.

The next experiment aims to test cell viability of cells treated with Lipofectamine™ 2000™, which will be used as transfection control. However, empty Lipofectamine™ 2000 cannot be used as the lipids work by condensing RNA and hence only RNA/ Lipofectamine complex are tested. From *Figure 6.3*, cells incubated with siRNA/ Lipofectamine are affected at 30 nM siRNA with 83% cell viability but statistical difference is observed at 50 nM siRNA with 70% cell viability at p-value = 0.0277. At similar siRNA concentration (35 nM), Lipofectamine™ 2000 system provides lower metabolic activity (80%) compared with polymersomes system (90%). This suggests higher cytotoxicity of Lipofectamine™ 2000 over polymersomes.

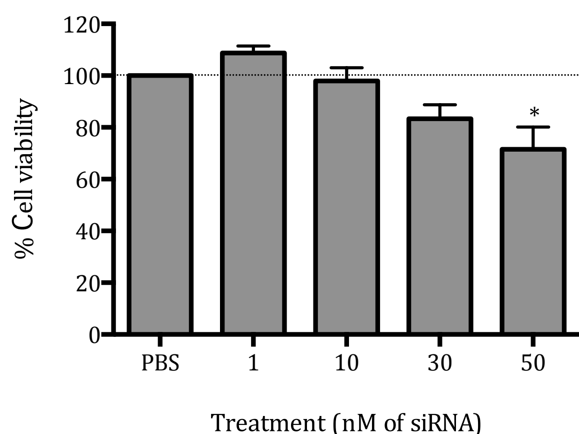

Figure 6.3: Cell viability of Lipofectamine™ 2000 treated cells. HeLa cells were cultured and treated with negative control siRNA/Lipofectamine at siRNA concentrations of 1, 10, 30, and 50 nM for 24 hours. After treatment, cells were trypsinised. MTT assay was performed to test the cytotoxicity by comparing to the non-treated cells. The experiment was performed with three repeats. Statistical analysis was performed using one-way ANOVA test followed by Dunnett's multiple comparison test comparing between each treatment with and untreated control (PBS treated cells) (* p-value < 0.05).

6.2.2. Knockdown of Lamin A/C by siRNA

Study on mRNA expression

LMNA gene and commonly used housekeeping genes for reference were amplified with specific primers designed for real-time PCR. The finding of suitable reference genes for real time PCR normalisation needs to be taken into account for assessing the actual knockdown. As some reports showed, the effect of unsuitable reference genes for real time PCR normalisation (Dheda et al. 2004, Silver et al. 2006, Curtis, Gomez et al. 2010, Sikand et al. 2012) can lead to misinterpretation of the mRNA expression level. In order to find the suitable reference genes for normalisation, 6 common housekeeping genes, including 18s ribosomal RNA (18s rRNA), β-actin, Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), Elongation translation Factor 1 alpha 1 (EEF1α1), Hypoxanthine phosphoribosyl transferase 1 (HPRT1), Ribosomal protein L13a (RPL13a) were investigated in HeLa cells treated with polymersomes containing negative control siRNA. Ideally, reference genes should have a stable and constant expression under the experimental condition. Here, Cycle Threshold (Ct) of genes in HeLa cells treated with negative control siRNA/ polymersomes were determined and shown in *Figure 6.1*. Normfinder analysis was also used to determine the stability value of each gene. Normfinder is the mathematical model (Andersen et al. 2004) designed to calculate the expression variation among samples to identify the most suitable gene for normalization. Mean Ct value and stability value of each gene were listed in *Table 6.1.*

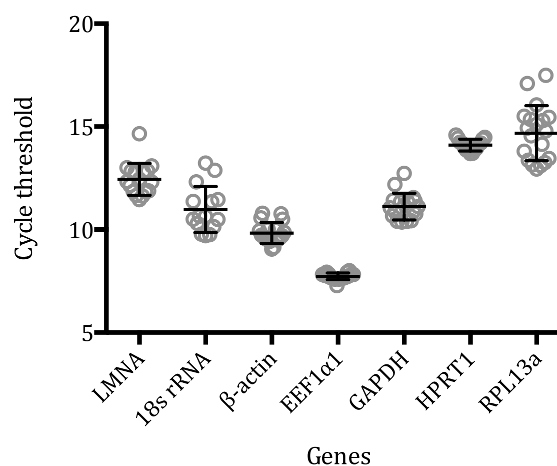

Figure 6.4: Cycle threshold (Ct) of reference genes among samples. qPCR of housekeeping genes were performed in HeLa cells treated with polymersomes containing negative control siRNA. Cycle threshold of 6 genes, including 18s ribosomal RNA (18s rRNA), β-actin, Elongation translation Factor 1 alpha 1 (EEF1α1), Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), Hypoxanthine phosphoribosyl transferase 1 (HPRT1), Ribosomal protein L13a (RPL13a) as well as Lamin A/C (LMNA) were analysed and compared.

Two criteria of chosen reference gene are (i) the low variation among tested conditions and (ii) similar cycle thresholds on the interest gene and reference gene. This indicates that the similar copy number of mRNA is used for tracking small changes in expression profile. According to the results in *Figure 6.4* and *Table 6.1*, three genes with the highest stability value are *GAPDH, HPRT1* and *β-actin*. The high stability of β-actin has, although, been questioned in previous reports (Selvey et al. 2001, Watson et al. 2007). As for the mean Ct value to *LMNA,* are *GAPDH, 18s rRNA,* and *HPRT1* have been observed to be most suitable.

Table 6.1: The PCR cycle threshold values of 3 reference genes, 18s rRNA, GAPDH, HPRT1 and LMN in polymersomes loaded with negative control siRNA treated cells at various concentrations.

Gene	Mean	SD	N	Stability value*
LMNA	12.45	0.77	17	4.95
18s rRNA	10.97	1.11	15	3.40
β-actin	9.86	0.51	18	2.06
EEF1α1	7.73	0.16	18	2.44
GAPDH	11.12	0.65	17	1.34
HPRT1	14.11	0.30	15	1.45
RPL13a	14.75	1.34	18	2.15

*calculated by Normfinder analysis (Andersen, Jensen et al. 2004)

18s rRNA or ribosomal RNA subunit 18 is an important subunit of the ribosome in eukaryotic cells. However *18s rRNA* is made of RNA and is not translated into protein. Unlike other genes, it can only be used as reference in mRNA expression, not in protein expression. *GAPDH* or glyceraldehyde 3-phosphate dehydrogenase (Mori et al. 2008a) is the key regulator enzyme in glycolysis, which is important for energy metabolism of cells. Whereas, HPRT1 or Hypoxanthine phosphoribosyl transferase I (Fu, Jia et al. 2009) is an enzyme involved in nucleotide synthesis pathway. As *18s rRNA, GAPDH* and *HPRT1* fall in the criteria for qPCR reference genes, these 3 housekeeping genes were selected as endogenous control on real time PCR experiments.

HeLa cells were incubated with 0.01-50 nM of siRNA with polymersomes and Lipofectamine™ 2000 as siRNA carrier. After 24 hours of treatment, the gene expression of *18s rRNA, GAPDH* and *HPRT1* and *LMN* were examined by real time PCR anaylsis.

The plots in *Figure 6.5* show the ΔCt of each gene which was calculated as follows:

$$\Delta Ct = \text{Ct of treated cells} - \text{Ct of untreated cells}$$

Overall, only small changes in the expression profile of each gene was observed in polymersomes treated cells. Empty polymersomes hardly affect the expression of all genes, with minor changes only visible at high polymersome concentration (3.5 μM). No changes in gene expression were observed at low siRNA-loaded polymersome concentration (0.01-1 nM). However, polymersome loaded with the scrambled LMNA sequence siRNA (scr) affected all tested genes at siRNA concentration of 10 nM.

On the other hand, Lipofectamine™ 2000 strongly affects gene expression, independently of RNA concentration and sequence. Only the 18s rRNA gene seems to be unaffected. Note that, as the effect from the Lipofectamine™ 2000 itself cannot be tested as empty, this system exists because of interaction between cationic lipid and anionic nucleotides. However, considering that genes were affected independently of sequence and concentration of RNA, it is very likely that the majority of the bioactivity arises from the carrier.

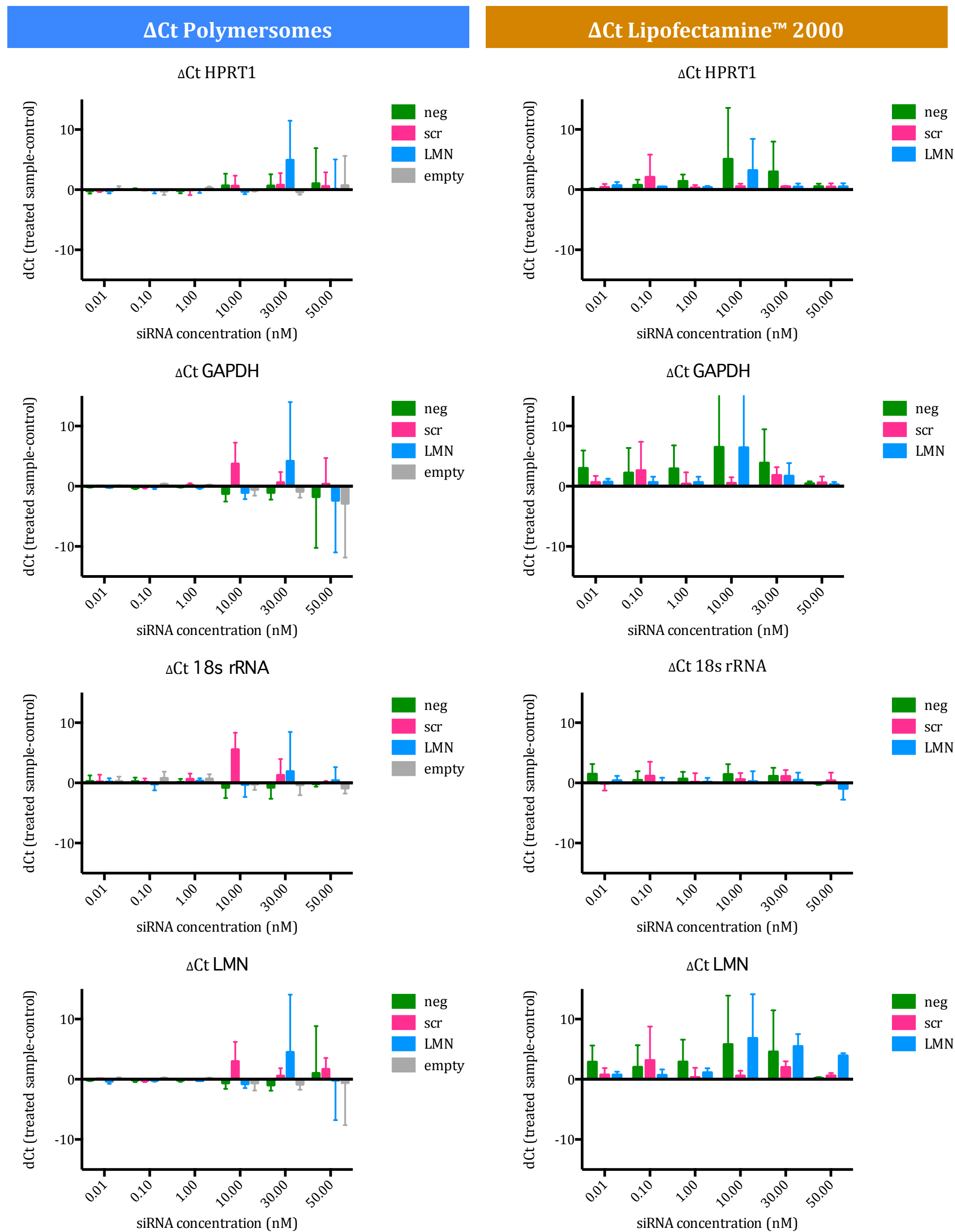

Figure 6.5: Difference in Cycle Threshold (ΔCt) values between treated samples and control samples. Real time PCR analysis of siRNA/polymersomes and siRNA/Lipofectamine treated samples were performed with 3 different siRNA including negative control (neg), anti-Lamin A/C (LMN) and scrambled (scr) sequences. The siRNA concentrations varied from 0.01 to 50 nM. The cycle thresholds (Ct) obtained from Lamin A/C and other 3 reference genes including HPRT1, GAPDH, and 18s rRNA were determined for each treated samples. Difference in cycle threshold (ΔCt) was performed based on the untreated control sample. Three sets of experiments was performed.

Knockdown ability of siRNA/polymersomes has been shown in *Figure 6.6*, with the plot of relative gene expression of Lamin A/C mRNA across various siRNA concentrations ranging from 0.01-50 nM. The relative gene expression was analysed

by normalisation with reference genes (*GAPDH, 18s rRNA, and HPRT1*) and comparison with the non-treated cells which defined as $2^{-\Delta\Delta Ct}$, where

$$\Delta\Delta Ct = \Delta Ct \text{ of gene of interest} - \Delta Ct \text{ of reference gene}$$

The relative gene expression value closed to 1 refers to no change in mRNA expression, lower and higher value refers to reduction in gene expression and overexpression, respectively. Three different siRNA sequences, negative control (neg), scrambled (scr) and anti-Lamin A/C (LMN), were compared.

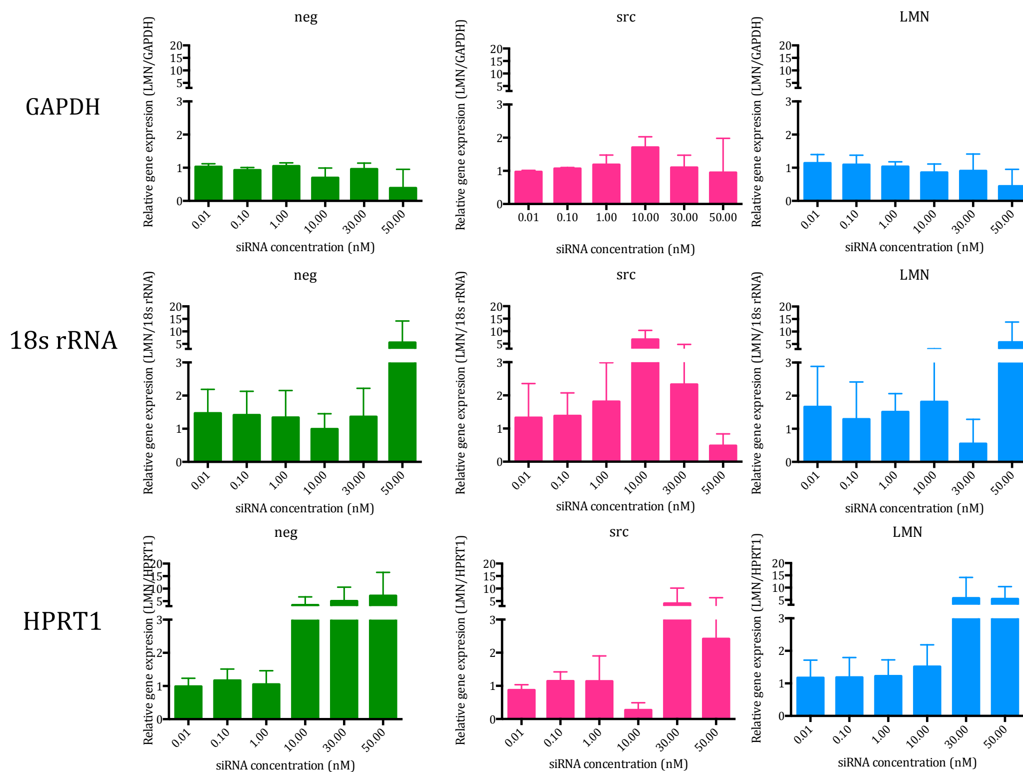

Figure 6.6: mRNA expression on Lamin A/C in HeLa cells treated with siRNA/ polymersomes. HeLa cells were incubated with polymersomes in the presence of negative control, scrambled and anti-lamin A/C siRNA at different siRNA concentrations (0.01, 0.1, 1, 10, 30, and 50 nM). After treatment, the HeLa cells were collected to perform RNA extraction followed by cDNA synthesis and real-time PCR. The relative lamin A/C gene expression was normalised with the untreated cells. mRNA expression of GAPDH, 18s rRNA and HPRT1 were used as internal control. Three sets of experiments were performed. The statistical analysis used in this study was one-way ANOVA with Dunnett's multiple comparison test.

According to the data, no significant differences in gene expression were observed in polymersomes as a carrier, suggesting low knockdown ability of siRNA loaded in polymersomes. On the contrary, overexpression of LMN mRNA occurred in high siRNA concentration (10 nM in neg siRNA and 30 nM in LMN siRNA). This might result from an off-target effect from overloaded siRNA inside the cells.

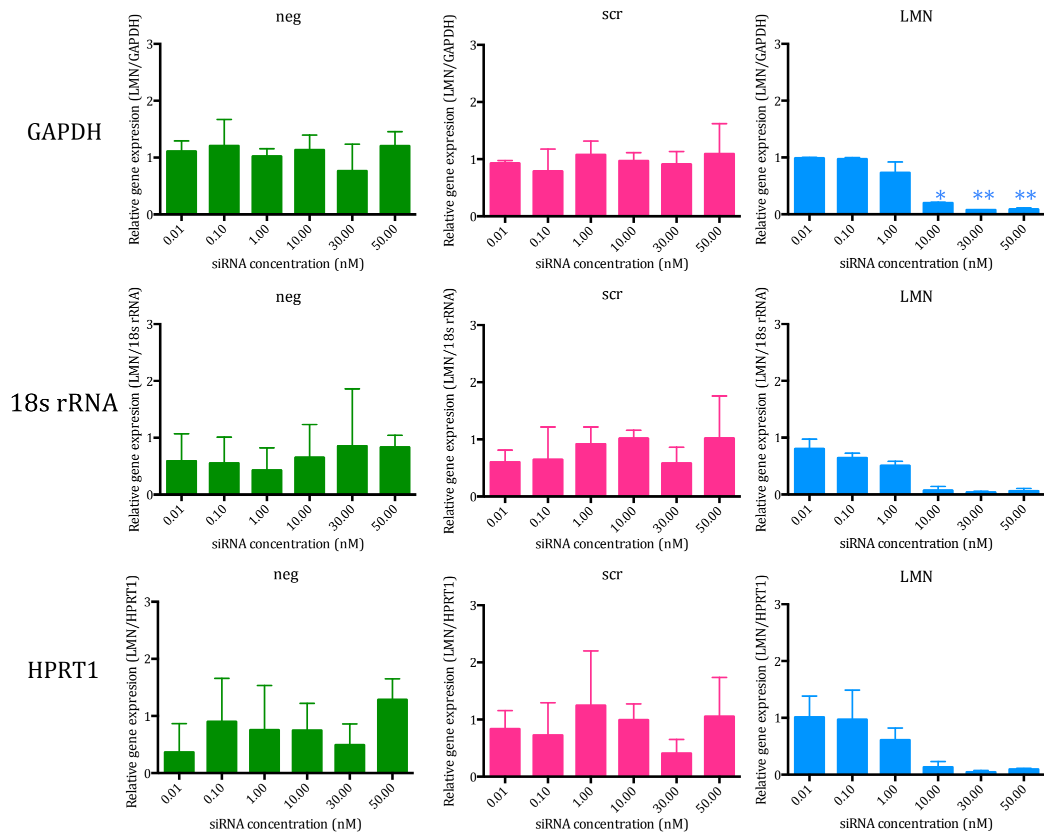

Figure 6.7: mRNA expression on Lamin A/C in HeLa cells treated with siRNA/ Lipofectamine. HeLa cells were incubated with Lipofectamine™ 2000 in the presence of negative control, scrambled and anti-lamin A/C siRNA at different siRNA concentrations (0.01, 0.1, 1, 10, 30, and 50 nM). After treatment, the HeLa cells were collected in order to perform RNA extraction followed by cDNA synthesis and real-time PCR. The relative lamin A/C gene expression was normalised with the untreated cells. mRNA expression of GAPDH, 18s rRNA and HPRT1 were used as internal control. Three sets of experiments were performed. The statistical analysis used in this study was one-way ANOVA with Dunnett's multiple comparison test comparing between siRNA/Lipofectamine treated cells and untreated control (PBS treated cells) (p-value: *p<0.05, **p<0.01).

Results shown in *Figure 6.7* suggest the knockdown ability of siRNA/Lipofectamine as Lamin A/C mRNA expression was decreased in anti-lamin siRNA/Lipofectamine treated cells, in normalisation with all three reference genes. However, significant knockdown of Lamin A/C can be seen at siRNA concentration of 10-50 nM when normalised with GAPDH, whereas no significant difference was observed when normalised with 18s rRNA and HPRT1. Moreover, the variation on Lamin A/C mRNA expression can be observed in the cells treated with negative control sequence siRNA. This might suggest an effect from the siRNA carrier system as no such change was observed in the polymersomes system.

Change in expression of reference gene might enhance the difference in relative gene expression of gene of interest, causing the misinterpretation of actual gene expression. An example can be seen from different relative gene expression of

Lamin A/C in 30 nM siRNA/polymersomes treated cells. When normalised with GAPDH, there is no change in Lamin A/C expression as the similar expression pattern was found in Lamin A/C and GAPDH. On the other hand, when normalised with 18s rRNA which was lower expressed, the relative expression of Lamin A/C become smaller than that normalised by GAPDH. The results confirm the importance of selection of the reference gene, as relative expression of the interest gene relies on the used for normalisation by reference gene. Moreover, the non-significance knockdown of 30 nM anti-LMN siRNA/polymersomes is a consequence of the large error obtained from the raw data.

Study on protein expression by automated Western analysis

The Lamin A/C protein expression was determined by automated Western blot machine (Simon, Protein Simple). In this work, HeLa cells were treated with siRNA/ polymersomes or siRNA/Lipofectamine at different siRNA concentrations for 48 hours, followed by protein extraction. The results of cells treated with siRNA/ polymersomes are shown in *Figure 6.8*, and siRNA/Lipofectamine shown in *Figure 6.9*. According to the mRNA expression experiment, the cells were treated with 1, 10, and 30 nM siRNA. No silencing activity was observed in these tested conditions, which confirms the previous mRNA expression. However, the scrambled sequence siRNA shows upregulation at 1 nM and 10 nM siRNA, which might be the off-target effect from the non specificity of this sequence. An absence of knockdown activity detected in siRNA/polymersomes treated cells suggests that the siRNA delivered by polymersomes cannot perform its function in HeLa cells.

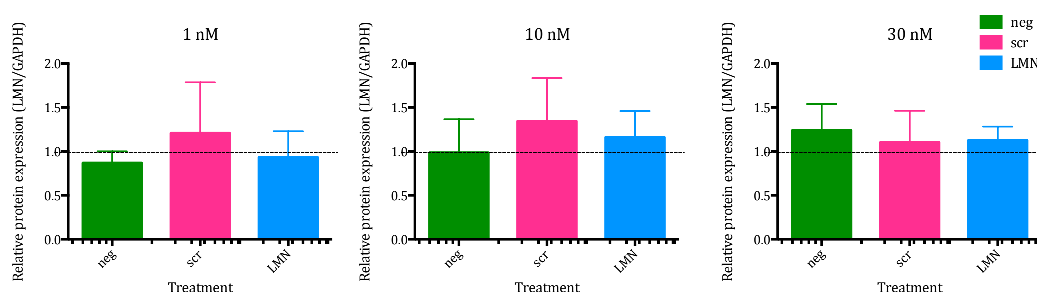

Figure 6.8: Lamin A/C expression at protein level of polymersomes treated cells. HeLa cells were incubated with polymersomes in the presence of negative control, scrambled and anti-lamin A/C siRNA at different siRNA concentrations (1, 10, and 30 nM). After treatment, the HeLa cells were lysed and prepared for western blotting using automated western blot machine (Simon, Protein Simple). The relative lamin A/C gene expression was normalised with the untreated cells and GAPDH was used as internal control. Three sets of experiments were performed. The statistical analysis used in this study was one-way ANOVA with Dunnett's multiple comparison by comparing all treated cells with PBS treated cells.

siRNA/Lipofectamine treated cells show silencing activity on Lamin A/C protein in all tested siRNA concentration, 10, 30 and 50 nM. Although these siRNA

concentrations show significant knockdown activity in mRNA expression level, no significant difference was observed in the protein level. The difference in mRNA and protein level might be the consequence of mRNA stability of Lamin A/C which could retained for a period of time, in order to repeat the translation. In addition, unexpected silencing activity was observed in 30 and 50 nM negative control siRNA. This suggests off-target effect from high siRNA concentration which might lead to false positive knockdown activity in all siRNA sequences.

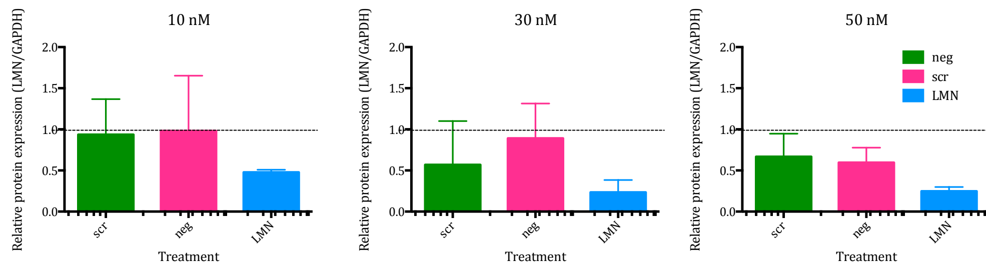

Figure 6.9: Lamin A/C expression at protein level of Lipofectamine™ 2000 treated cells. HeLa cells were incubated with Lipofectamine™ 2000 in the presence of negative control, scrambled and anti-Lamin A/C siRNA at different siRNA concentrations (10, 30, and 50 nM). After treatment, the HeLa cells were lysed and prepared for western blotting using automated western blot machine (Simon, Protein Simple). The relative lamin A/C gene expression was normalised with the untreated cells and GAPDH was used as internal control. Three sets of experiments were performed. The statistical analysis used in this study was one-way ANOVA with Dunnett's multiple comparison by comparing all treated cells with PBS treated cells.

Beside the delivery system, low activity might be a side effect of the method of detection. Normally, the protein expression is observed with traditional Western blot technique as the qualitative result, quantitative analysis can be determined indirectly by measuring the band intensity. Whereas, in automated Western blot, the results are observed directly as a chemiluminescent signal and presents as quantitative electropherogram. Moreover, normalisation was performed as both Lamin A/C and GAPDH expressions were examined in the same sample by probing both proteins at the same time. This is different from conventional western blotting which normally evaluates the control for protein expression by total protein on the gel before performing membrane transfer, or re-probing the same membrane with different protein probe. The total protein is not the ideal internal control for protein expression as it is a different method of detection, and the protein are shown in large quantity, therefore small differences in protein expression might not be recognised. Re-probing on the same membrane might be better in terms of similar methods of detection, but a different experiment on developing the signal might not reflect the exact expression level.

6.2.3. Pro-inflammatory response

Apart from cytotoxicity test (in section 6.2.1), cellular responses have to be considered to determine the possible effect of carriers on tested cells which might interfere with knockdown efficiency. In order to protect themselves, cells can respond to foreign proteins or viruses by activation of several pathways. Two typical cellular responses have been evaluated in this work including interferon response NF-κB translocation.

NF-κB activation

Nuclear Factor-κB (NF-κB) are the complex proteins which function as transcription factor (Sen et al. 1986a) involved in control of cell growth (Chen et al. 2001), differentiation (Guttridge et al. 1999) and immune system (Li et al. 2002). The inactive NF-κB consists of three subunits (i) DNA binding p50, (ii) p65 and (iii) inhibitory subunit IκB which forms complex protein, and it is typically located in the cytoplasm (Sen et al. 1986b). With activation by cell stress, pro-inflammatory cytokines or other stimuli (Baldwin 1996), IκB will be phosphorylated and released from the complex. This generates the translocation of NF-κB into the nucleus, allowing activation of regulated genes.

In this work, translocation of NF-κB has been observed to confirm the pro-inflammatory response of siRNA/polymersomes and siRNA/Lipofectamine treated cells. HeLa cells have been exposed to Tumor Necrosis Factor-alpha (TNF-α) as a positive control to compare with polymersomes or Lipofectamine™ 2000 treatment. TNF-α activates cellular responses including cell apoptosis and cell proliferation by binding to a TNF receptor on the cell membrane, resulting in recruitment of downstream transducer to activate NF-κB translocation (Baud et al. 2001). The cells were assigned the rate of NF-κB activation by observing the localisation of NF-κB under microscope. The scores, shown in *Figure 6.10*, were obtained from the presence of anti NF-κB signal, ranging from 0, 0.25, 0.5 and 1. The cells with no signal inside their nucleus were assigned as 0. The cells with higher signal in the cytoplasm compared with their nucleus were scored as 0.25. The cells with equal signal from both cytoplasm and nucleus were scored as 0.5, and cells with signal only from nucleus were scored as 1. In this case, positive cells were those with NF-κB located in the nuclear region. All cells in the image were scored and divided by the total number of cells presented and used to calculate, the percentage of positive cells. In this case, if the NF-κB translocation occurred in all cells, the percentage of positive cells equalled 1. The quantity of cells were obtained from Hoechst 33342 staining, and compared with non-treated cells as negative control.

TNF-α treated cells responsive curve has been plotted and shown in *Figure 6.11* as the percentage of positive cells versus TNF-α concentration. HeLa cells response to TNF-α at tested concentrations and show more NF-κB activation at high concentration of TNF-α. In this experiment, the minimum TNF-α concentration to activate all cells is 10 ng/ml.

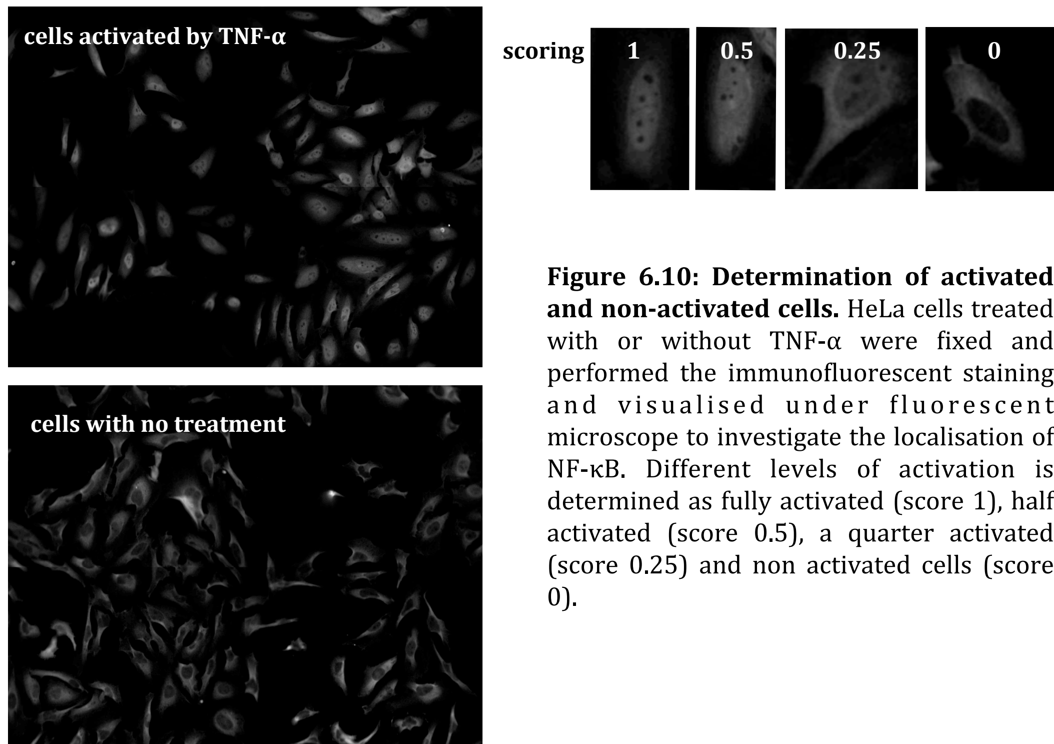

Figure 6.10: Determination of activated and non-activated cells. HeLa cells treated with or without TNF-α were fixed and performed the immunofluorescent staining and visualised under fluorescent microscope to investigate the localisation of NF-κB. Different levels of activation is determined as fully activated (score 1), half activated (score 0.5), a quarter activated (score 0.25) and non activated cells (score 0).

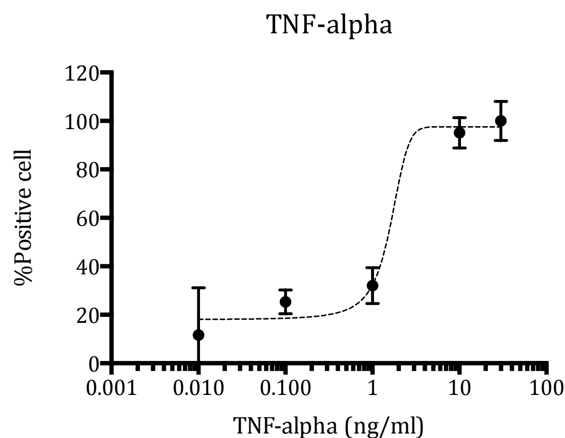

Figure 6.11: Dose response of TNF-alpha treated cells. HeLa cells were treated with various TNF-alpha concentrations and the localisation of NF-κB compared with the criteria described in *Figure 6.10*.

HeLa cells were treated with empty polymersomes to investigate NF-κB activation. Polymersomes concentrations were found ranging from 0.05-1 mg/ml. The results were shown in *Figure 6.12*, with very low NF-κB signal found in the nucleus of

polymersomes treated cells. No significant difference in the number of positive cells was observed when compared with negative control (non-treated cells). This suggests treatment of empty polymersomes on HeLa cells cause no NF-κB activation.

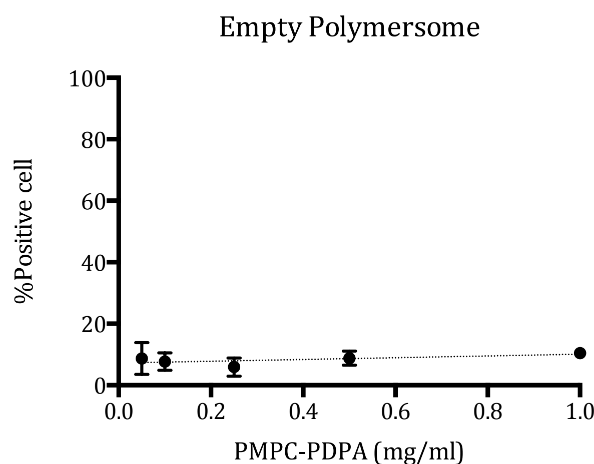

Figure 6.12: Dose response of polymersomes treated cells. HeLa cells were treated with various polymer concentrations and the localisation of NF-κB compared with the criteria shown in *Figure 6.10.*

NF-κB activation was investigated further in HeLa cells incubated with siRNA/ polymersomes and siRNA/Lipofectamine. Two siRNAs were used in this study, negative control siRNA and anti-Lamin A/C siRNA at siRNA concentrations of 1, 3 and 30 nM. The results are shown in *Figure 6.13*, and provide information on the effect of treatment on HeLa cells. No difference in the percentage of positive cell has been observed between two siRNA sequences in both polymersomes and Lipofectamine™ 2000, suggesting that siRNA sequence has no effect on NF-κB activation. However, the effect from transfection carrier was found in cells treated with Lipofectamine™ 2000 at 3 and 30 nM. *p*-value of negative control siRNA at 3 nM and 30 nM were 0.0190 and 0.0008, respectively. Whereas the *p*-value of anti-Lamin A/C siRNA at 3 nM and 30 nM were 0.0035 and 0.0054, respectively. Slight NF-κB activation has been observed in siRNA/polymersomes treated cells but was not significantly different from non-treated control. Moreover, the percentage of positive cells found in polymersomes treated cells is lower than that of siRNA/ Lipofectamine. Activation of negative control siRNA/polymersomes is less than Lipofectamine™ 2000 at 3 nM (*p*-value = 0.0261) and anti-Lamin A/C siRNA at 30 nM (*p*-value = 0.0002). This suggests NF-κB activation is caused by Lipofectamine™ 2000 which might lead to other inflammatory responses, and it is confirmed in previous study using polymersomes (Canton, Massignani et al. 2013) that no detectable NF-κB translocation was observed with treatment of polymersomes.

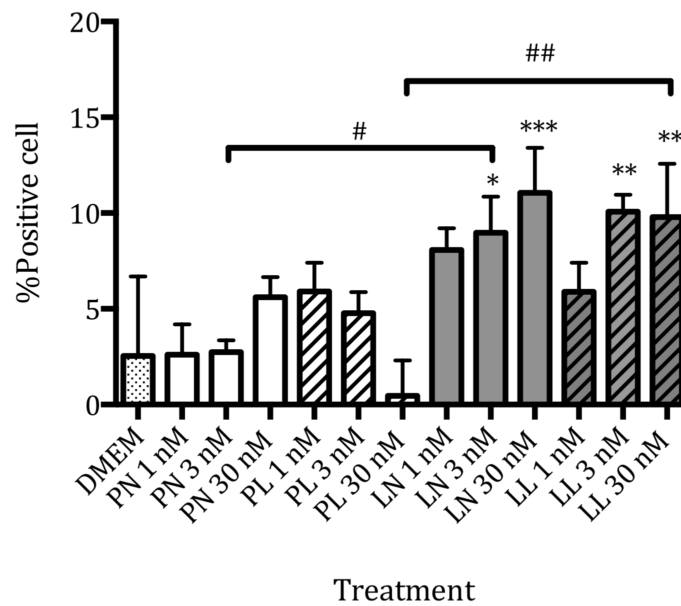

Figure 6.13: Pro-inflammatory response of HeLa cells via NF-κB translocation study. NF-κB translocation in various concentration of siRNA delivered by polymersomes and Lipofectamine™ 2000 were analysed and compared according to the criteria in figure 6.5. (PN=negative control siRNA/polymersomes, PL=anti-Lamin A/C siRNA/polymersomes, LN=negative control siRNA/Lipofectamine, LL=anti-Lamin A/C siRNA/Lipofectamine). The experiment was performed with three repeats. Statistical analysis was performed using One way-ANOVA with Tukey's multiple comparison test (p-value: *or # $p < 0.05$, ** or ## $p < 0.01$, ***$p < 0.005$ when * is compared with non-treated cells and # is comparing between different siRNA carriers).

IL-6 production

NF-κB translocation is one of an intermediate pathway of Interleukin-6 (IL-6) production in cells, as the NF-κB binding site is found in the IL-6 promoter region (Libermann et al. 1990). IL-6 expression in response to NF-κB activation is directly involved with infammatory and infection response. It has pro- or anti-inflammatory function by responding to different receptors located in membrane or cytoplasm (Scheller et al. 2011). IL-6 can be enhanced by x-ray (Beetz et al. 2000), hydrogen peroxide (Zhang et al. 2001) and cationic liposomes (Sioud et al. 2003). Following from previous experiment, IL-6 expression was observed to confirm the NF-κB translocation as exhibiting pro-inflammatory response against Lipofectamine™ 2000 or polymersomes treatment.

ELISA was used to examine the IL-6 expression in HeLa cells that were incubated with siRNA/polymersomes and siRNA/Lipofectamine for 2, 6, and 24 hours. The culture media were collected in order to perform ELISA assay. TNF-α was used as positive control which induce releasing of IL-6 at concentration of 10 pg/ml. PBS treated cells were used as negative control which caused low effect on cellular response. Human IL-6 was prepared at different concentrations ranging from 0-500

treated cells in tested incubation periods. While IL-6 expression in Lipofectamine™ 2000 treated cell was higher than PBS treated cells after 24 hours (*p*-value = 0.0362) but not as high as the positive control cells. This data confirms the results of previous experiments and suggests that the pro-inflammatory response was induced by Lipofectamine™ 2000 but not with polymersomes. There is no significant increase in IL-6 expression with short incubation time (2 and 6 hours), but it does not confirm that there is no effect on cellular response to cells. However, the negative effect of pro-inflammatory response can be avoided by lessening the incubation time during which cells are exposed to Lipofectamine™ 2000.

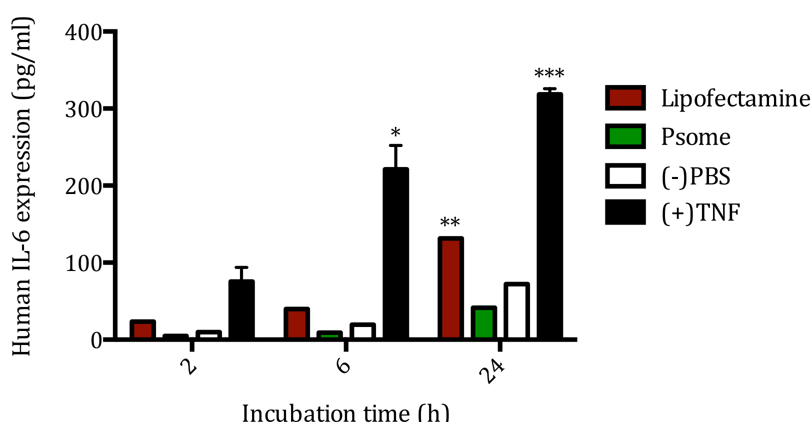

Figure 6.14: Pro-inflammatory response of HeLa cells via Human IL-6 expression. HeLa cells were incubated with Lipofectamine™ 2000 or polymersomes for 2, 6 and 24 hours. The culture media were collected to examine the release of IL-6, as pro-inflammatory cytokine. The experiment was performed with three repeats. Statistical analysis was performed using One way-ANOVA with Dunnette's multiple comparison test, comparing between all treatments with the untreated control (PBS treated cells represented as white bar) (*p*-Value: **p<0.01)

6.2.4. Type I Interferon response

Interferons (IFNs) are the group of secreted cytokine which are important in invader-response by the activation of signal-transduction pathway. Stimulation of IFN results in an induction of IFN-stimulated genes (ISGs) (76) that transcribes into specific enzymes or signals in order to respond to the infection. IFNs can be divided into type I, II and III according to their receptors. However, type I and II IFNs are the most typical IFNs in responsible to virus (type I IFN) or virus and microbes (type II IFN).

To study the effect of siRNA/polymersomes delivery involved with immune response, type I IFN are considered as it reflects the cellular response to viral transfection system. Type I interferon response have been analysed in cells treated

with empty polymersomes, polymersomes containing siRNA, and siRNA/ Lipofectamine at 2 and 20 hours of incubation. All genes are listed in *Appendix E.*

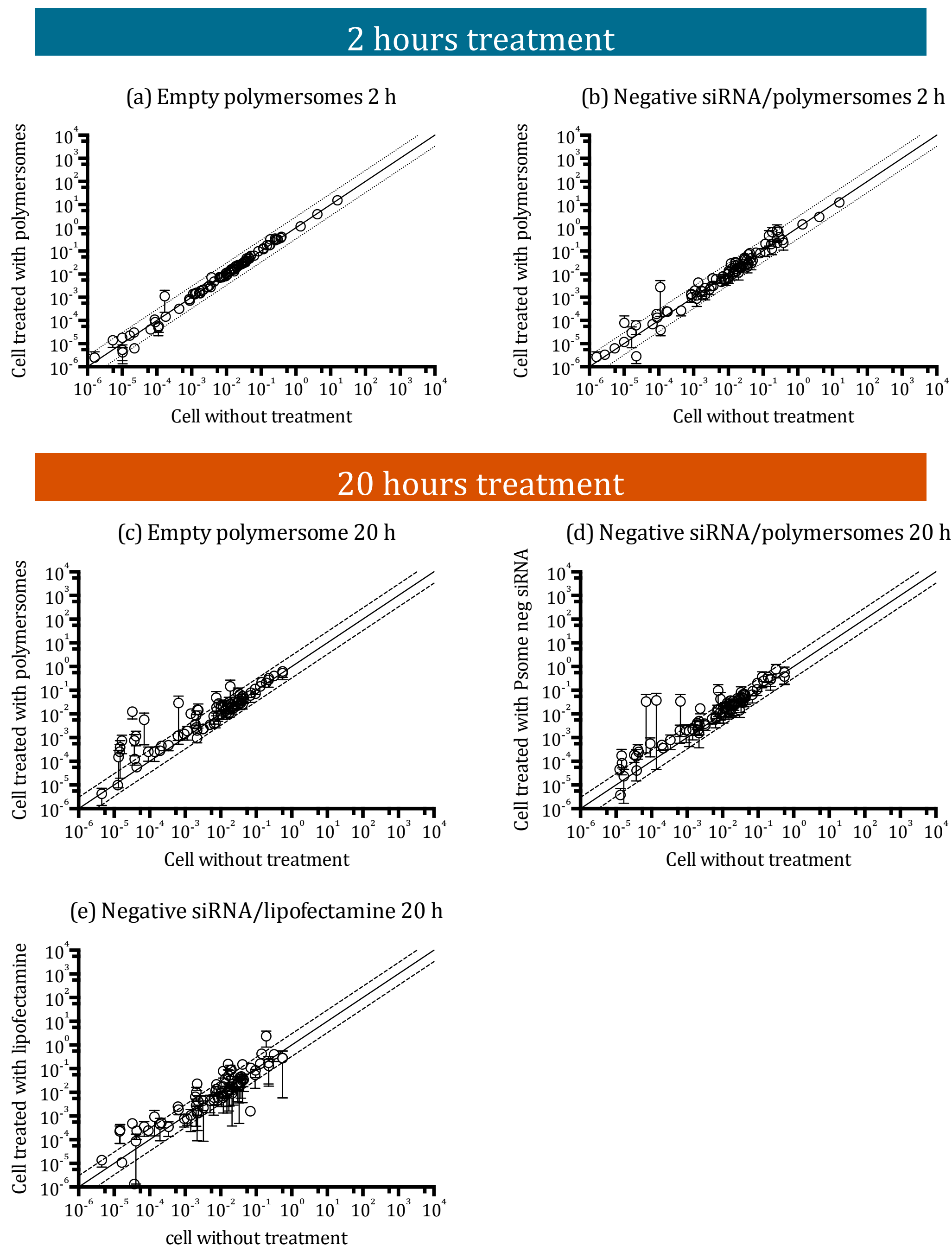

Figure 6.15: Type-I interferon response in HeLa cells. Real time PCR of 84 genes involved in type-I interferon response was performed in the cells treated with Lipofectamine™ 2000 and polymersomes. The plot showed the $2^{-\Delta Ct}$ value between treated cells and non-treated cells at 2 hours incubation;(a) empty polymersomes, (b) polymersomes containing negative control siRNA, and 20 hours incubation; (c) empty polymersomes, (d) polymersomes containing negative control siRNA, and (e) Lipofectamine™ 2000 containing negative control siRNA. The experiment was performed in triplicate with three repeats.

The results were shown as scatter plots in *Figure 6.15*, each empty circles represent the $2^{-\Delta ct}$ value between control cells (X-axis) and treated cells (Y-axis). The smooth line in the middle represents the equal $2^{-\Delta ct}$ value between control and tested samples, while dash lines set as the upper and lower bound for 3-fold change in gene expression (this value is suggested as the protocol given by Arikawa, et al. (Arikawa et al. 2011)). All data were also presented as heat map diagrams in *Figure 6.16* (2 h incubation), and *Figure 6.17* (20 h incubation) or Venn diagram in *Figure 6.18*. The genes with changes in the level of expression were presented on the right. The level of expression can be divided into gene with upregulation (*in green*) and downregulation (*in red*) compared with untreated control.

For 2 hours of incubation, only *IFNA1* was upregulated in empty polymersome treated cells, whereas *HLA-C, HLA-G, IFNA2,* and *MAL* were downregulated. In polymersomes containing negative control siRNA, 3 genes display upregulation including *HLA-DQA1, HLA-G, IFIH1,* and *PTTG1* while *IFNA2, MAL* and *MX2* were downregulated. For 20 hours of incubation, in empty polymersome treated cells, 16 genes were upregulated but only 1 gene was downregulated. While in negative control siRNA/polymersomes, there are 15 upregulated genes and 2 downregulated genes. 10 genes in both empty polymersomes and negative control siRNA/ polymersomes treated cells show the same trend on their expression, including *CXCL10, HLA-C, HLA-DQA1, HLA-F, IFNB1, IRF5, IRF7, IRF9, ISG20,* and *MAL*. It can be claimed that expression of those genes was the effect of the polymersomes themselves.

24 out of 84 genes were changed in negative control siRNA/Lipofectamine treated cells. Only 8 genes were similar to those in negative control siRNA/polymersomes treated cells including *CXCL10, HLA-G, IFNB1, IRF5, MAL, MX2, NRG1,* and *SAMSN1*. Whereas 15 genes were not matched with negative control siRNA/polymersomes, which can be ascribed to Lipofectamine™ 2000.

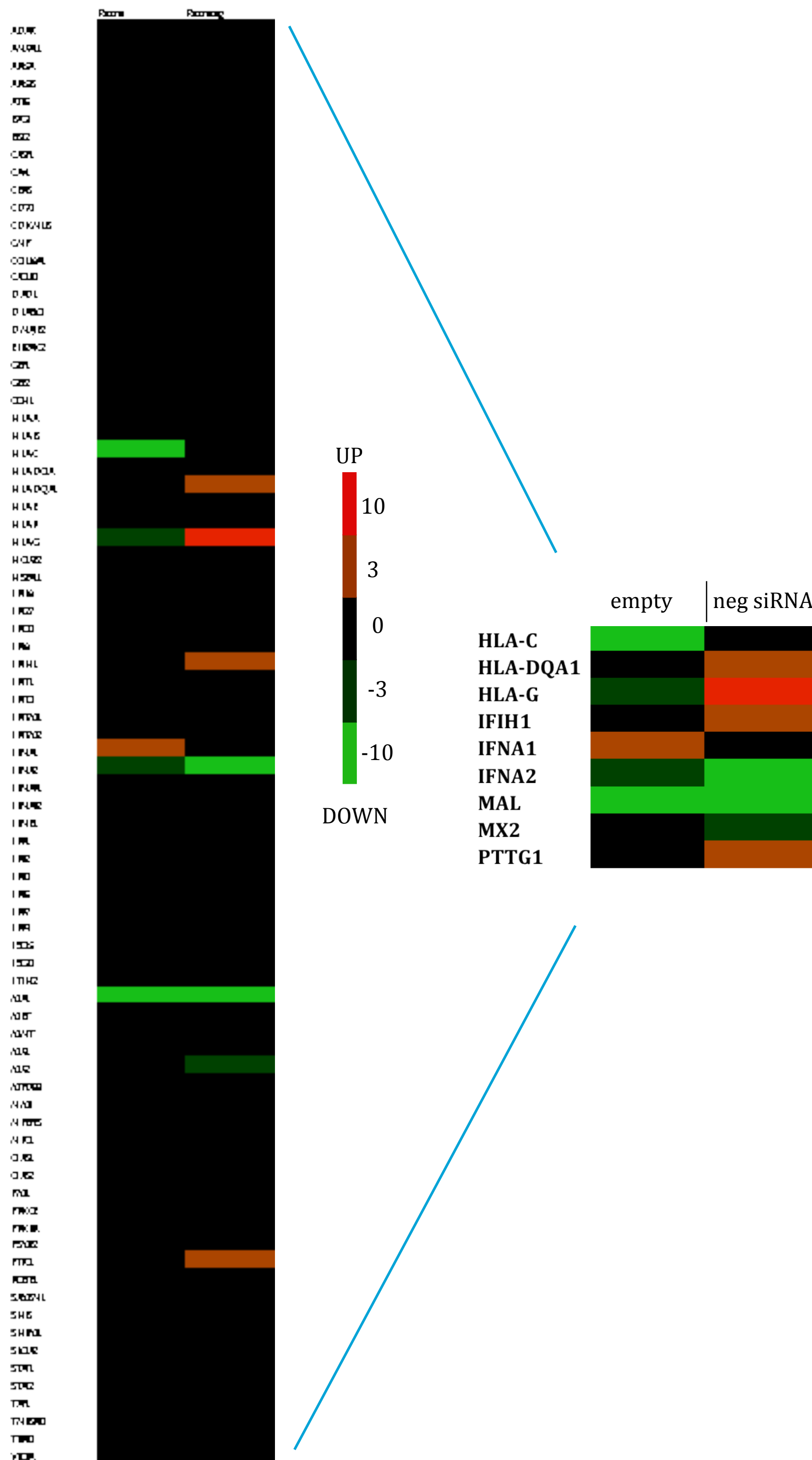

Figure 6.16: Heat map of type-I interferon response in 2 hours PMPC-PDPA polymersome treated HeLa cells. Real time PCR of 84 genes involved in type-I interferon response was performed in the cells treated with siRNA/Lipofectamine, empty polymersome and siRNA/polymersomes. The Ct value was normalised with the average Ct of 5 housekeeping genes including B2M, HPRT1, RPL13A, GAPDH and β-actin. The heat map showed the overall fold change in mRNA expression of cells incubated with polymersomes compared with non-treated cells.

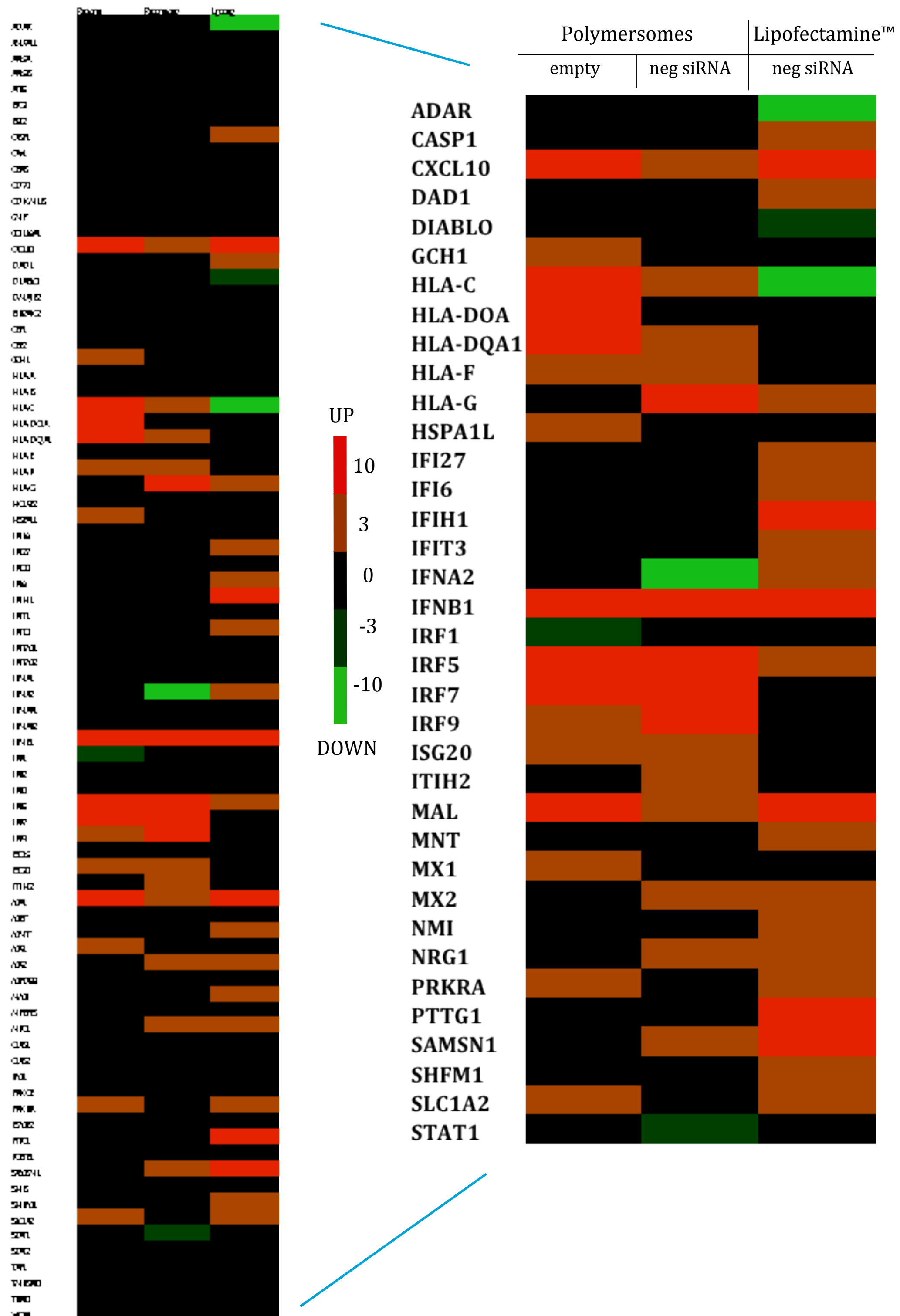

Figure 6.17: Heat map of type-I interferon response in overnight PMPC-PDPA polymersome treated HeLa cells. Real time PCR of 84 genes involved in type-I interferon response was performed in the cells treated with siRNA/Lipofectamine, empty polymersome and siRNA/polymersomes. The Ct value was normalised with the average Ct of 5 housekeeping genes including B2M, HPRT1, RPL13A, GAPDH and β-actin. The heat map showed the overall fold change in mRNA expression of cells incubated with polymersomes compared with non-treated cells.

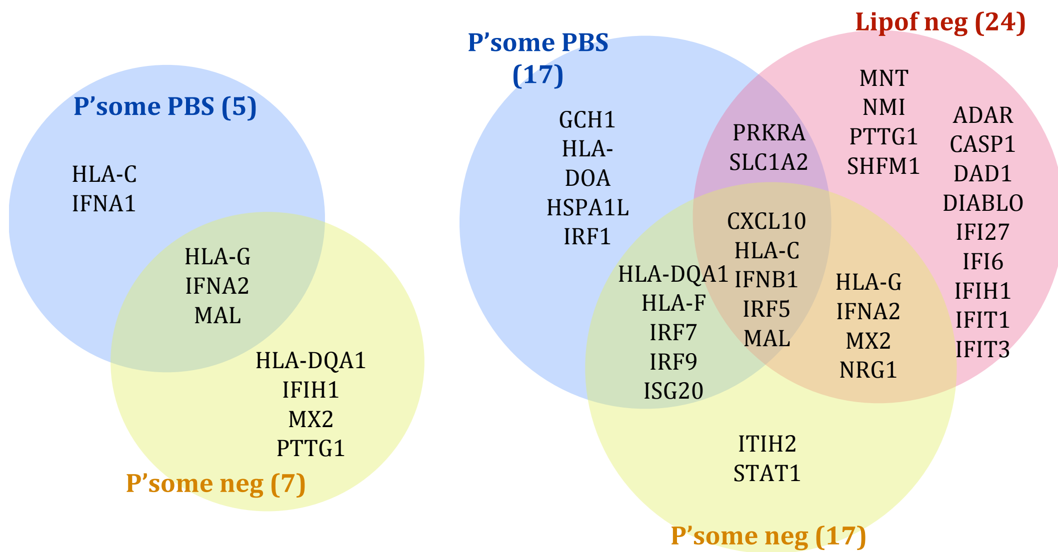

Figure 6.18: Venn diagram for overview of gene expression. Change in gene expression involved in type-I interferon response in PMPC-PDPA polymersome treated HeLa cells for 2 hours (Left) and 20 hours (Right) of incubation.

Details in genes induced by polymersomes or Lipofectamine™ 2000

All genes that show change in their expression were listed with details provided in *Table 6.2*.

Table 6.2: The details of genes used in interferon response study.

Gene	**Functions**
Membrane bound proteins	
MHC genes including HLA-C, HLA-DOA, HLA-DQA1, HLA-G, and HLA-F	Major Histocompatibility Complex (MHC) (Gorer et al. 1948) is the group of cell membrane glycoprotein, located on the cell surface to present self or non-self peptide in order to interact and be recognised by immune cells (Abbas et al. 2010). MHC genes can be divided into 2 classes, I, and II. Class I MHC is including Human leukocyte antigen (HLA) - A, -B, -C, -E, -F and -G which can be found in all nucleated cells and inducible by IFN-α, -β and -ϒ. Class II MHC is including HLA-DO, -DP, -DQ and -DR which can be expressed only in some few cell types (such as antigen-presenting cells) and stimulated by IFN-ϒ. s. The expression of class I MHC at the cell surface is important as it represents the “self” recognition signal to lymphocyte, so it will not be recognised as foreign cells (Karre 1995).
Genes involved in apoptosis	
CXCL10: C-X-C motif chemokine Ligand 10 or IP-10, IFN-γ inducible cytokine (NM_001565)	This protein is one of the pro-inflammatory chemokine in the CXC subfamily responded to interferon signaling. It is involved with cytosolic DNA-sensing pathway which is activated by NF-κB, also mediates apoptosis (Zhang et al. 2005).
DAD1: Defender Against cell Death 1 (NM_003144)	DAD1 belongs to the oligosaccharyltransferase (OST) (Kelleher et al. 1997) which catalyses the N-link glycosylation. It acts as a negative regulator of apoptosis by binding to MCL1 (Makishima et al. 2000). It has been proven that reduction of DAD1 occurs prior to apoptosis (Nakashima et al. 1993).

Gene	Functions
DIABLO: Direct Inhibitor of Apoptosis protein Binding protein with a Low pI (NM_019887)	DIABLO is mitochondrial pro-apoptotic protein, which is involved with apoptosis by interacting with the inhibitor of apoptosis protein (IAP). DIABLO prevents the IAP from binding with pro-caspase3, leads to progress in an apoptosis cascade (Verhagen et al. 2000).
NRG1, Neuregulin 1 (NM_013957)	This gene encodes the ligands for ERBB receptor including Neu Differentiation Factor (NDF), Heregulin (HRG), Glial Growth Factors (GGFs), and Acetylcholine Receptor Inducing Activity (ARIA) which are involved with the tumor signaling pathway and also induce apoptosis in human kidney cells (Grimm et al. 1997) and human breast cancer cells (Weinstein et al. 1998).
Casp1, Caspase 1, apoptosis-related cysteine peptidase (NM_033292)	Caspase-1 is the prototypic member of a family of inflammatory caspases (including human caspase-4 and -5, and mouse caspase-11 and -12) which all contain an N-terminal caspase recruitment domain (CARD). Increases in caspase-1 activity in adipose tissue leads to the production of pro-inflammatory cytokines IL-1b, IL-6, IL-8 and IL-18. (Koenen et al. 2011).
PTTG1, pituitary tumor-transforming 1 (NM_004219)	This gene can protect HeLa from DNA-damaged induced apoptosis (Lai et al. 2007).
Genes involved in signalling pathway	
Interferon Regulatory Factors (IRFs) including IRF1, IRF3, IRF5, IRF7, and IRF9	Interferon Regulatory Factors are the transcription factors which contain well-conserved DNA-binding domain located at the amino terminus and forms a helix–turn–helix motif. This DNA-binding domain recognises a consensus DNA sequence that is known as the IFN-stimulated response element (ISRE). IRFs have different roles in the development and function of immune cells. IRFs function to promote antibacterial and antiviral innate immunity by activating transcription of IFNB and IFNA after infection. All of IRFs are constitutively expressed and some also can be inducible. For example, IRF5, and IRF7 can be stimulated by type I IFN. IRF5 mediates NF-κB and IL6 expression and promote inflammatory response (Takaoka et al. 2005). IRF7 enhances IFNA and IFNB for antiviral activity (Honda et al. 2005). IRF1 is also inducible by IFN-γ, it can function to response to viral response (Kimura et al. 1994), bacterial infection (Ko et al. 2002), as well as DNA damage response (Pamment et al. 2002). IRF9 functioned as type I IFN mediator especially for anti-proliferation (Tsuno et al. 2009).

Gene	Functions
STAT1, Signal transducer and activator of transcription 1, (NM_007315)	STAT1 is the cytoplasmic protein that can translocate into the nucleus to act as the transcription mediator of IFN-induced signaling (Takeda et al. 2000). Production of STAT1 is inducible by IFN-β (Gough et al. 2010), in order to balance the level of type I and II interferon. In addition, STAT1 can function as tumor suppressor (Huang et al. 2002), overexpression of STAT1 activates the subset of ISGs which can promote resistance to tumor toxic stress (Dunn et al. 2006).
Genes involved with membrane trafficking	
MAL, Myelin And Lymphocyte protein or T-cell differentiation proteins, (NM_002371)	MAL was first identified as hydrophobic protein in T-cells (Alonso et al. 1987). It shows to be important in malignancy as it shows downregulation in oral squamous cell carcinoma compared with normal cells (Pal et al. 2012). Apart from its role in T-cells, this protein has been studied in Madin-Darby Canine Kidney Epithelial (MDCK) cells for its function in apical sorting machinery between Golgi and membrane (Puertollano and Alonso 1999, Puertollano, Martin-Belmonte, et al. 1999). Overexpression of this protein is found to enhance exocytosis and endocytosis in urothelial (transitional epithelial) cells (Zhou, Liang, et al. 2012).
SHFM1: split hand/foot malformation type 1 (NM_004171)	In yeast, the homologue of this gene can regulate exocytosis (Jantti et al. 1999).
Gene involved in antiviral response	
IFIH1, Interferon-Induced Helicase 1 (NM_022168)	Code for MDA5 (melanoma-differentiation-associated gene 5) protein which has two domain, RNA helicase and CARD (caspase recruitment domain), function as sensor for dsRNA and essential for anti-viral responses also proapoptotic role. It is considered as an early response gene, which induced by IFN-β after 6 h and maintain for 96 h in melanoma cell (Kang et al. 2002).
IFIT3, Interferon-induced protein with tetratricopeptide repeats 3 (NM_001549)	This protein can be induced by IFN-α and perform antiviral activity, overexpression of this gene leads to decrease in viral titer (Schmeisser et al. 2010).

Gene	Functions
ISG20, Interferon stimulated exonuclease gene 20kDa, (NM_002201)	This protein function as antiviral with 3' to 5' exoribonuclease which can cleave to both single-(Espert et al. 2003) and double-stranded (Espert et al. 2004) RNA.
Other genes	
ADAR1, Adenosine deaminase, RNA-specific (NM_001111)	ADAR1 converts adenosines to inosines (A→I editing) in double-stranded RNA (dsRNA) substrates which might change the codon and leads to the protein diversification. It acts as a suppressor of interferon (IFN) signaling in stem cell *in vivo.* (Hartner et al. 2009) ADAR1 also related to function of siRNA as it can binds strongly to siRNA, resulting in lower concentration of siRNA to bind the target mRNA (Nishikura 2010).
Mx1, Myxovirus Myxovirus resistance 1, (NM_002462)	Mx1 belongs to the dynamin-like GTP-binding protein inducible by type I interferon (Haller et al. 2002). It can function as effector molecule to protect the cells from influenza virus (Grimm et al. 2007) and other virus by blocking the transportation of nucleocapsid to the nucleus (Kochs et al. 1999). Moreover, overexpression of MxA (homologue of Mx1 in mouse) affects endocytosis trafficking (Jatiani et al. 2004) by blocking at the early stage of endocytosis (Palm et al. 2010).
MX2, Myxovirus resistance 2, (NM_002463)	MX2 was first discovered in mouse (Staeheli et al. 1988) as interferon-inducible protein. In human, the MX2 homologue has been identified as MXB (Aebi et al. 1989). This protein belongs to Mx family with strong induction against IFN. MX2 shows no antiviral activity and its function remains unclear in mouse and human.
PRKRA, protein kinase, interferon-inducible double stranded RNA dependent activator (PRKRA) or RAX, PACT (NM_003690)	This gene can be translated as PACT (in human) (Patel et al. 1998) or RAX (in mice) (Ito et al. 1999). This protein is stress (such as ER stress) inducible which leads to further activation of interferon inducible, double-stranded RNA dependent protein kinase (PKR) to inhibit the protein synthesis (Hershey 1989).

Difference in gene expression between polymersomes and Lipofectamine™ 2000 treated cells were described below.

Membrane bound proteins

The result shows that upregulation can be found in both MHC class I (HLA-C, -G, and -F) and MHC class II (HLA-DOA and -DQA1). As previous work shows, the expression of *HLA-C* on the cell surface is relatively low compared with *HLA-A and -B* (Snary et al. 1977), which is caused by low mRNA stability (McCutcheon et al. 1995). The possible explanation is that these HLA proteins might be internalised together with polymersomes or Lipofectamine™ 2000, leading to reduction in the HLA level on the cell surface, together with less mRNA stability of some HLA proteins. The mRNA production of HLA is required to maintain its normal level on the cell surface.

Genes involved in apoptosis

Apoptosis is the mechanism of cells to protect the adjacent cells from viral infection by programming themselves to die. Some interferon responsive genes can function as apoptotic genes. Upregulation of genes in this group suggests the induction of apoptosis in Lipofectamine™ 2000 treated cells. Although upregulation of *CXCL10* and *NRG1* can be observed in negative control siRNA/polymersomes, other apoptosis associated genes were not observed. In contrast, negative control siRNA/ Lipofectamine™ 2000 treated cells cause induction of apoptotic genes, including *CXCL10, NRG1*, and *DAD1*, and the suppression of *DIABLO. CXCL10* and *NRG1* expression leads to progress in apoptosis. However, study on *DAD1* suggest its role in apoptosis that reduced in this gene occurs prior to apoptosis. Overexpression of DAD1 leads to cell rescue from apoptosis in yeast (Sugimoto et al. 1995), but not in human (Hong et al. 1999). High expression of *Casp1* and *IFI16* was observed only in Lipofectamine™ 2000 treated cells, suggesting that the activation of apoptosis occurs in Lipofectamine™ 2000 treatment. *Casp1* upregulation is supported by an increased in IL-6 (in IL-6 expression, *Figure 6.14*). HeLa cells also express the recovery protein, PTTG1 as a protection in early polymersomes treatment (2 hours).

Genes involved in signaling pathway

IRFs genes (*IRF1, IRF5, IRF7,* and *IRF9*) were upregulated and presented in both polymersomes and Lipofectamine™ 2000 as they are intermediates of activation of interferons at the long incubation period but not in early incubation. This suggests the activation of interferon as a result of mild stress response. Similarly, increase in *STAT1* level in neg siRNA/polymersomes treated cells might be the consequence of high level of *IFNA,* which lead to STAT1 production. This, again, confirms the activation of interferon to enhance anti-viral response.

Genes involved with membrane trafficking

Upregulation of *MAL* suggests an enhancing of polymersomes or Lipofectamine™ 2000 uptake according to an increase in uptake (endocytosis) or clearance (exocytosis). In addition, upregulation of *SHFM1* was observed in Lipofectamine™ 2000 treated cells, suggesting a possible exocytosis occurrence in Lipofectamine™ 2000 treated cells.

Gene involved in antiviral response

High expression of *IFIH1* and *IFIT3* were observed only in Lipofectamine™ 2000 treated cells, suggesting higher antiviral activity was induced. Only polymersomes show the upregulation of *ISG20*, perhaps lowering the amount of siRNA in the cells delivered by polymersomes.

Other genes

Downregulation of *ADAR* in Lipofectamine™ 2000 treated cells might enhance siRNA activity, leading to improvement in silencing activity. *Mx1* shows upregulation only in empty polymersomes treated cells, suggesting some blocking of endocytosis trafficking occurs. Upregulation of *Mx2* was found in the presence of siRNA treatment, suggesting the response of this gene is directly involved with siRNA, not the carriers. *PRKRA* was upregulated in both Lipofectamine™ 2000 and polymersomes treatment, suggesting stress was induced by these treatments.
According to the information on interferon response from PCR array, polymersomes treatment provides induction of interferon response, anti-viral response with no strong activation of apoptosis. On the contrary, Lipofectamine™ 2000 treatment seems to cause stronger stress as well as apoptotic induction. However, the results obtained from PCR array are in mRNA level, not in protein level. These results can be claimed as preliminary study which require confirmation of actual induction of these interferon signals.

6.2.5. Stress induced silencing activity

Difference in cellular response between cells treated with siRNA/Lipofectamine and siRNA/polymersomes provides a clue to specific stress induction patterns which are involved in the siRNA knockdown activity. The following experiments were performed to investigate whether cellular stress is required for the silencing activity of siRNA or not.

Polymersomes plus TNF-α

According to evidence of pro-inflammatory response from siRNA/Lipofectamine treated cells (as shown in NF-κB translocation and IL-6 expression), this experiment

was performed to investigate the involvement of pro-inflammatory response and silencing ability of siRNA. Polymersomes loaded with anti-Lamin A/C siRNA were incubated with HeLa cells in the presence of TNF-α as a IL-6 inducer, and compared the knockdown activity with cells without TNF-α. The results of protein expression were presented as shown in *Figure 6.19.* No silencing was observed in all conditions. This suggests that TNF-α alone cannot enhance siRNA activity.

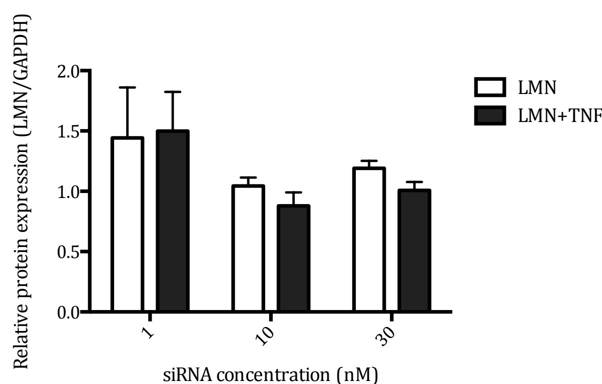

Figure 6.19: Knockdown of Lamin A/C with polymersomes in the presence of TNF-alpha. HeLa cells were incubated with anti-LMN siRNA/polymersomes at different siRNA concentrations (1, 10, and 30 nM) in the presence of TNF-α. After treatment, the HeLa cells were lysed and prepared for western blotting using automated western blot machine (Simon, Protein Simple). The relative lamin A/C gene expression was normalised with the untreated cells and GAPDH was used as internal control. The experiment was performed with three repeats. Statistical analysis was performed using One way-ANOVA with Tukey's multiple comparison test.

Polymersomes plus Lipofectamine™ 2000

According to the previous experiment, addition of TNF-α alone is insufficient to increase siRNA activity of polymersomes. As siRNA/Lipofectamine treated cells show induction of some interferon responsive genes as well as apoptotic related genes, whereas no such cellular response was observed in siRNA/polymersomes. Those cellular responses could be the factor which enhances the knockdown activity of siRNA delivered by Lipofectamine. In this experiment, HeLa cells were incubated with both siRNA/polymersomes and siRNA/Lipofectamine in order to observe the effect of Lipofectamine on the siRNA knockdown activity. Delivery of negative control siRNA was performed to observe the effect of Lipofectamine alone. The knockdown activity of anti-lamin siRNA was examined by real-time PCR.

mRNA expression is shown in *Figure 6.20*, no decrease in mRNA level was observed in cells treated with anti-lamin siRNA/polymersomes plus negative control siRNA/ Lipofectamine. This result indicates that cellular response from neg siRNA/ Lipofectamine cannot induce the knockdown ability of anti-lamin siRNA/ polymersomes. Knockdown activity can be detected in the cells treated with anti-

lamin siRNA/Lipofectamine in all conditions. However, with the presence of anti-lamin/polymersomes, the knockdown efficiency is not as high as of anti-lamin/ Lipofectamine alone. This observation suggests a possible inhibitory effect on Lipofectamine™ 2000 activity by the polymersomes. This might result from the induction of viral response genes in polymersomes treated cells such as ISG20, that can digest exogenous dsRNA, resulting in lower amount of siRNA to perform silencing activity.

However, this experiment cannot claim that activity of siRNA requires cellular stress induced by Lipofectamine™ 2000 system, but it suggests an involvement of the cellular response from delivery system and silencing ability.

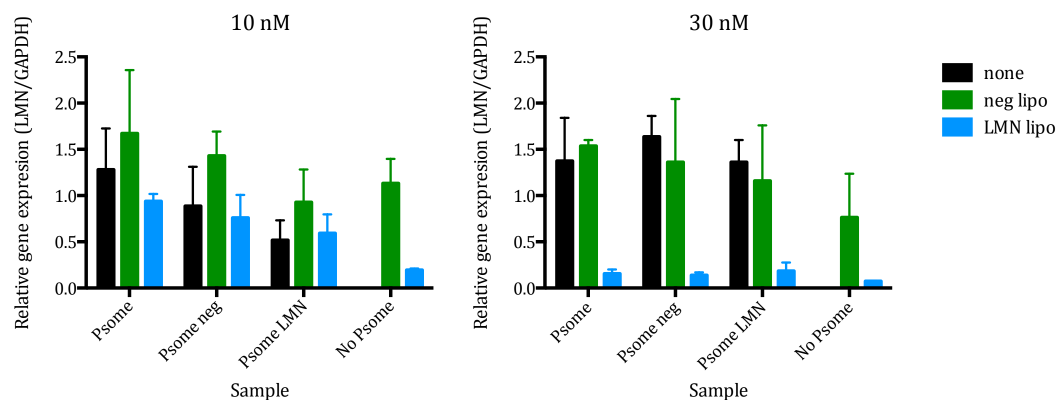

Figure 6.20: Knockdown of Lamin A/C with polymersomes in the presence of Lipofectamine™ 2000. HeLa cells were incubated with siRNA/polymersomes with or without Lipofectamine™ 2000 with two different sequence of siRNA (neg: negative control, LMN: Anti-lamin A/C siRNA). After treatment, the HeLa cells were collected to performed RNA extraction followed by cDNA synthesis and real-time PCR. The relative lamin A/C gene expression was normalised with the untreated cells and GAPDH was used as internal contro The experiment was performed with three repeats. Statistical analysis was performed using One way-ANOVA with Tukey's multiple comparison test.

6.3. Summary

Knockdown Lamin A/C by siRNA was performed with polymersomes as well as Lipofectamine™ 2000 as the control system. No silencing activity was observed in cells treated with siRNA delivered by polymersomes, at both the mRNA and protein level. Lipofectamine™ 2000 system showed silencing activity of mRNA and protein expression, however, the knockdown was statistically significant only at the mRNA level. This could be because the relative gene expression relies on normalisation with reference genes. In addition, the gene expression can be varied according to the experimental condition, in this case, different carriers can also affect the non-target gene expression which might realted to the gene of interest.

Based on our data, Lipofectamine™ 2000, when compared to polymersomes, shows stronger cellular response. Lipofectamine™ 2000 causes more cytotoxicity, activates NF-κB translocation, and causes IL-6 pro-inflammatory cytokine production. On the other hand, low cytotoxicity and no pro-inflammatory responses were observed in cells treated with polymersomes. The effect of carrier, especially for Lipofectamine™ 2000, on gene expression profile has also been observed and compared with other methods. When compared to electraoporation, Lipofectamine™ 2000 treatment induced in the upregulation of 65 genes in HeLa cells (no data about function of the genes), with 36 out of 65 genes were changed according to the increase in Lipofectamine™ 2000 concentration (Fedorov et al. 2005). A recent study on the Lipofectamine ™ 2000 compared with polymeric carrier TransI$^{T®}$ system (Mirus Bio LLC, Wisconsin, USA) showed that Lipofectamine ™ 2000 has a stronger effect on the gene expression of many pathways, including cell cycle, p53 signaling, acute phase response, mitochondria dysfunction, and also apoptosis signaling (Khodthong et al. 2013).

Proposed diagram of interferon response of cells treated with polymersomes containing negative control siRNA is shown in *Figure 6.21*. The response is drawn according to the expression of genes in the Type I interferon pathway and divided into three groups including genes involved with internalisation, genes that act as mediator of the responsive pathway, and genes with function to protect the cells from infection.

For cells treated with Lipofectamine™ 2000 containing negative control siRNA, the interferon response is shown in *Figure 6.22.* Cellular responses are similar to those of polymersomes treated cells such as genes involved with internalisation, and signaling molecules. However, strong activation of genes involved with apoptosis response is also observed.

The evidence of a specific stress pattern in Lipofectamine™ 2000 treated cells, such as cytotoxicity, pro-inflammatory response and activation of apoptotic genes, can be considered in two aspects (i) these cellular responses play an important role to

assist the knockdown or (ii) these off-target effects might change the cell activity, and counterfeit silencing activity of siRNA. In this study, Lipofectamine™ 2000 shows an effect on the cell viability as well as causing cellular response, and this might cause changes in expression of genes which are directly or indirectly involved with the reference genes.

In order to understand more about the failure of silencing activity in siRNA/ polymersomes treated cells, the assumption based on different cellular responses between polymersomes and Lipofectamine™ 2000 system was tested. The pro-inflammatory responses and induction of interferon response of Lipofectamine™ 2000 system were dissimilar to polymersomes system. Induction of cellular response (presence of TNF-α or Lipofectamine™ 2000) in polymersomes treated cells showed no increase the silencing ability, which confirms that the cellular response pattern in Lipofectamine™ 2000 treated cells does not promote silencing activity. However, what it is very interesting is that co-treatment with polymersomes hinders Lipofectamine™ 2000 silencing. This can suggest two possible scenarios: (i) the silencing is somehow related to cellular stress and the polymersomes act as a rescue agent or (ii) the polymersomes induced the upregulation of specific RNase enzyme that can interfere with the Lipofectamine™ 2000 delivered siRNA. Indeed we show here that polymersomes upregulate ISG20 which controls the expression of 3’ to 5’ exoribonuclease able to cleave both single- (Espert et al. 2003) and double-stranded (Espert et al. 2004) exogenous RNA. This would suggests that such a gene/protein will result in insufficient siRNA-induced knockdown.

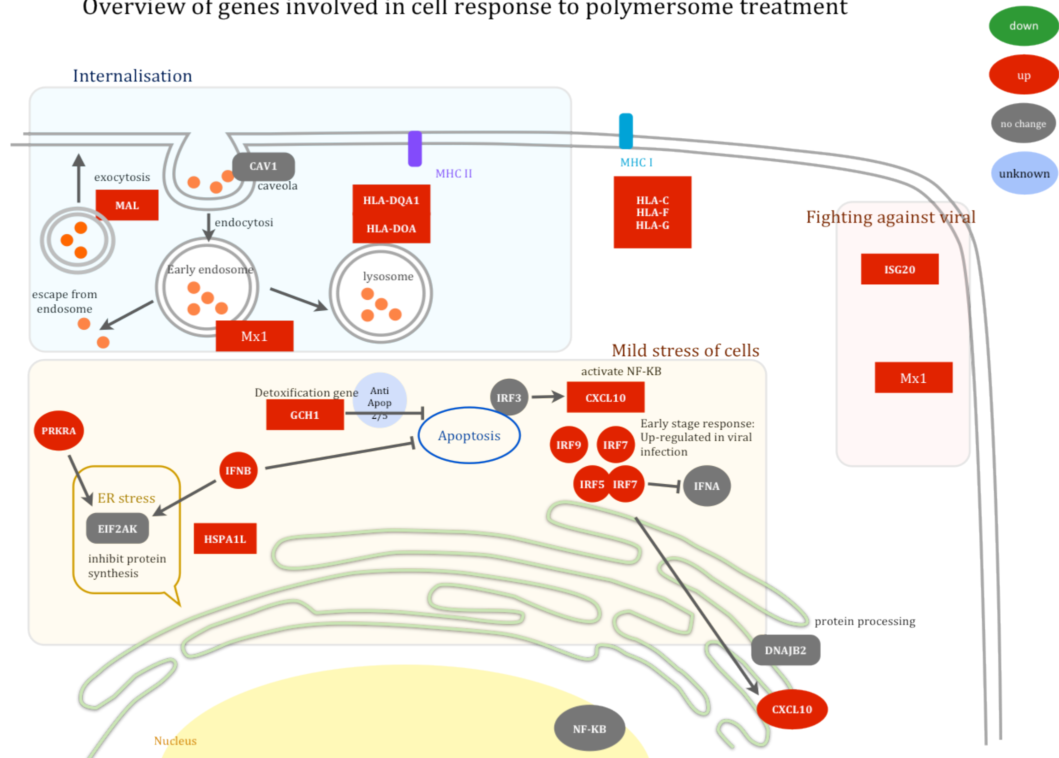

Figure 6.21: Summary of cellular activity in response to siRNA/polymersomes treatment.

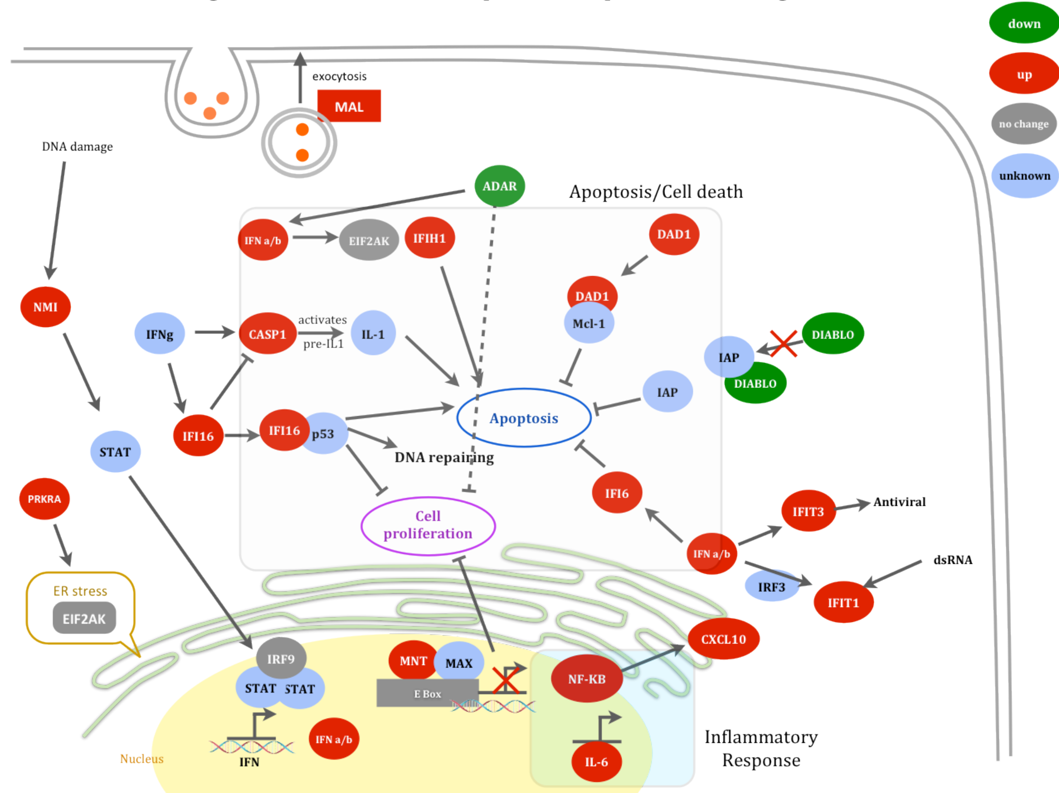

Figure 6.22: Summary of cellular activity in response to siRNA/Lipofectamine treatment.