Chapter 3

Materials and Methods

3.1. Materials

3.1.1. Chemicals

Methanol, Chloroform, Ethanol, Sodium hydroxide, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Sepharose® 4B, RNaseZAP™, Diethylpyrocarbonate (DEPC), Triton™ X-100, Tween® 20, Tris Acetate-EDTA buffer (TAE), L-glutamine, penicillin/streptomycin and trypsin-EDTA solution were purchased from Sigma-Aldrich. RNeasy mini kit, QuantiTect® Reverse Transcription Kit, Rotor-Gene SYBR Green PCR Kit, RT^2 First strand kit, RT^2 SYBR Green ROX Fast mastermix, RT^2 Profiler PCR array and Rotor-Disc heat sealing film were obtained from Qiagen (UK). Ethidium Bromide Solution was bought from Bio-Rad (USA). Phosphate buffered saline (PBS) tablet was purchased from Oxoid (Pittsburgh, PA, USA). Hydrochloric acid was obtained from BDH AnalaR. Dulbecco's Modified Eagle Medium (DMEM) and Fetal Bovine serum (FBS) were bought from Biosera (East Sussex, UK). DMEM without phenol red and amphotericin B was purchased from Gibco (UK). Recombinant human TNF-α (catalog no. 654205) was obtained from EMD Millipore (San Diego, CA, USA). Phosphate buffer and distilled water were treated with 0.1% (v/v) DEPC to inactivate RNase before using in the polymersomes preparation.

3.1.2. siRNA

siRNA used in this study were listed as following table.

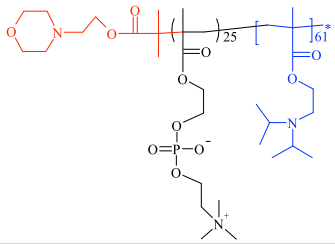
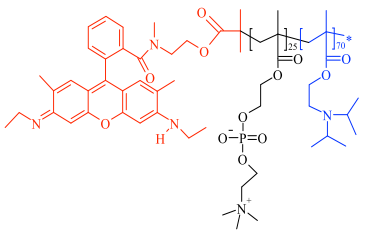
Table 3.1: siRNA sequences used in this study.

Name	Abbreviation	Sequence (5'-3') or Catalog number	Purchased from
Lamin A/C (NM_005572) (Elbashir, Harborth et al. 2002)	LMN	Sense CUGGACUUCCAGAAGAACAtt Antisense UGUUCUUCUGGAAGUCCAGtt	Ambion, UK
Silencer Select Negative Control#1 siRNA	neg	AM4635	Ambion, UK
scrambled	scr	Sense GAGAAACUCGGCCUACUAAtt Antisense UUAGUAGGCCGAGUUUCUCtt	Ambion, UK
Silencer® Cy™ Labeled Negative control siRNA #1	Cy3	AM4621	Ambion, UK
AllStars Negative siRNA Alexa Fluor® 647	647	1027295	Qiagen, UK

3.1.3. Polymers used in this study

The block copolymer used in this study is the poly(2-(methacryloyloxy)ethyl phosphorylcholine)-*b*-poly(2-(diisopropylamino)ethyl methacrylate) (PMPC-PDPA). $PMPC_{25}$-$PDPA_{70}$ and rhodamine 6G labeled $PMPC_{25}$-$PDPA_{70}$ (Rho-$PMPC_{25}$-$PDPA_{70}$) copolymers were synthesised and characterised by our colleague, Dr. Jeppe P. Madsen in Professor Steven P. Armes' group (Department of Chemistry, University of Sheffield) using the previously described procedure (Du, Tang et al. 2005, Massignani, LoPresti et al. 2009, Madsen et al. 2011). The characteristics of $PMPC_{25}$-$PDPA_{70}$ and Rho-$PMPC_{25}$-$PDPA_{70}$ are as shown in *Table 3.2.*

Table 3.2: Block copolymer used in this study.

Property	$PMPC_{25}$-$PDPA_{70}$	Rho-$PMPC_{25}$-$PDPA_{70}$
Structure		
Code	JPD70	JPD255
Target composition	PMPC25-PDPA70	$PMPC_{25}$-$PDPA_{70}$
NMR composition	ME-$PMPC_{25}$-$PDPA_{61}$	RH-$PMPC_{25}$-$PDPA_{70}$
^{1}H NMR MW (g/mol)	~22,300	~22,500
GPC ($CHCl_3$:CH_3OH 3:1)	M_n = 21,700 M_w/M_n = 1.22	M_n = 29,000 M_w/M_n = 1.27
GPC (Aqueous pH 3)	M_n = 28,200 M_w/M_n = 1.44	-

3.2. Methods

3.2.1. Polymersomes preparation

Polymersomes were prepared based on previous work (Lomas, Canton et al. 2007, Canton, Massignani et al. 2013) with some modification. $PMPC_{25}$-$PDPA_{70}$ powder was dissolved with chloroform:methanol solution (at ratio of 2:1) in a glass vial at the concentration of 2 mg/ml. The copolymer solution was placed in the desiccator chamber and left under vacuum condition for 4 hours to evaporate the solvent in order to obtain the copolymer film. The following steps were carried out in RNase-free conditions and with RNase-free solution. The copolymer film was then rehydrated with 100 mM acidified PBS buffer pH 2.0 to make the final concentration of 10 mg/ml of $PMPC_{25}$-$PDPA_{70}$ solution. The polymer solution was sterilised by passing through 200 nm-membrane filter before adjusted the pH to 6.0 by adding 0.5 M NaOH. siRNA encapsulation with pH switch method can be performed by mixing siRNA into the polymer solution at pH 6.0 before continuing the polymersome formation. Polymersomes were formed by increasing the pH to 7.0-7.4 with the addition of 0.5 M NaOH to the polymer solution.

3.2.2. Preparation of polymersomes in different size

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. The polymersomes were prepared by using the syringe pump in order to control the speed of NaOH adding to the polymer solution.

3.2.3. Encapsulation of siRNA

pH switch

In order to encapsulate siRNA into polymersomes, siRNA was added to the polymer solution at pH 6.0 in the polymersomes preparation step described in the previous section. After incubation for 5 minutes, 0.5 M NaOH was added to adjust pH to 7.4. The solution was mixed with magnetic stirring while adjusting pH.

Electroporation

The method was established during this work and published previously (Wang, Chierico et al. 2012b). Polymersomes were prepared with the same method as pH switch but without addition of siRNA. The empty polymersomes were incubated with siRNA for 5 minutes before being transferred into the 2 mm gap width electroporation cuvettes (Eppendorf). Electroporation was done with Electroporator 2510 (Eppendorf) for 1 pulsing time with 2500 volts. All the steps were performed on ice to avoid RNA degradation, and with a 20 seconds resting stage between pulsing time.

3.2.4. Purification of polymersomes

After polymersomes preparation and siRNA encapsulation by either pH switch or electroporation, Gel Permeation Chromatography (GPC) was used to separate the polymersomes and free siRNA according to their sizes. This purification method was validated in previous work (Canton, Massignani et al. 2013). Sepharose® 4B with the bead size of 45-165 μm in diameter was used to make the GPC column. The Sepharose® 4B was sterilised by soaking in RNase-free 70% ethanol at least for one hour before washing with 100 mM PBS buffer at pH 7.4. Sepharose® 4B was packed into the glass column and soaked with PBS solution. The GPC column placed vertically allowing packing to occur by gravitational force. The sample solution was added onto the GPC column enable separation of polymersomes, micelles, unimers, and unencapsulated siRNA according to the size of those molecules. The elution fraction was collected at the bottom of the column with the volume of 250 μl for each fraction. All elution fractions were kept for further analysis to determine the size, polymer concentration and siRNA encapsulation efficiency.

3.2.5. Micelles formation and siRNA encapsulation

Micelles were prepared according to the protocol from previous work (Pearson et al. 2013). $PMPC_{25}$-$PDPA_{70}$ was processed as described in polymersomes preparation section, except for the temperature. The rehydration step and pH switch were performed at 50°C by using heat plate to control the experimental condition. Similar to the polymersomes preparation, the siRNA encapsulation can be performed by addition of siRNA into the polymer solution at pH 6.4 before the pH was adjusted to 7.4 by 0.5 M NaOH.

3.2.6. Transmission Electron Microscopy (TEM)

Samples were prepared with the method described previously (Lomas, Du et al. 2010, Wang, Chierico et al. 2012b). Briefly, the copper grids were coated with carbon (10-20 nm thick) using Carbon coater before being submerged in the polymersome samples for one minute. The grids were dried and stained with Phosphotungstic acid (PTA) solution (at 0.75% w/w) for 5 seconds and blotted dry. The grids were imaged using a FEI Technai G2 Spirit TEM, with the operating voltage at 120 kV, equipped with an Orius SC1000A CCD camera.

3.2.7. Particle size and zeta potential

For the particle size measurement, 50 µl of each of the elution fractions obtained from GPC column plus 750 µl of 100 mM PBS buffer (pH 7.4) were mixed and loaded into the disposable polystyrene cuvettes (DTS0012 or ZEN1060C, Malvern). For the surface charge analysis, the samples were prepared in water at pH of 2.0 and 7.4. HCl and NaOH were used to adjust the pH of water to pH 2.0 and 7.4 (with ionic strength of 10^{-2} and 10^{-7} mol/l, respectively). Each sample was placed into the disposable capillary cuvette (DTS1070, Malvern). The particle size of polymersome samples was determined by dynamic light scattering technique and the surface charge of particles was measured with zeta potential by using Zetasizer Nano ZS (Malvern) supplied with 4mW He-Ne, 632.8 nm laser and analysed with the software provided by the manufacturer.

3.2.8. Polymer concentration

The sample was diluted in 100 mM PBS buffer at pH 2.0 to deform the self-assembly and allow only unimers in the solution. $PMPC_{25}$-$PDPA_{70}$ polymer powder was dissolved in 100 mM PBS buffer at pH 2.0 at the concentration of 0.625, 1.25, 2.50, and 5 mg/ml were used as a standard concentration for the calibration curve. The 100 mM PBS buffer at pH 2.0 was used as the blank control. Each sample was transferred to the quartz cuvette. The polymer concentration was measured at a wavelength of 220 nm by the V-630 UV-Vis spectrophotometer (Jasco, Japan).

3.2.9. siRNA concentration

Measurement of siRNA concentration inside polymersomes was performed by Quant-it™ Picogreen® dsDNA assay kit according to the product's manual. Briefly, the Picogreen reagent was diluted 200-fold in TE buffer pH 7.4 to make the working solution. The siRNA samples were added into the working solution at a ratio of 1:1 and incubated at room temperature for 30 minutes. with protection from light. The fluorescence of samples was measured with Cary Eclipse fluorescent spectrophotometer (Varian, Inc.) at excitation of 480 nm, and emission of 520 nm.

3.2.10. Gel electrophoresis

The siRNA integrity was determined by gel electrophoresis. siRNAs, after performance of electroporation in different conditions, were loaded into the 5% (w/v) agarose gel (Hi-Pure low EEO agarose, Biogene, UK) containing 0.3 μg/ml ethidium bromide. The gel was placed into the gel tank containing Tris Acetate-EDTA buffer, (40 mM Tris-acetate and 1 mM EDTA, pH 8.3) and supplied with 80 volts, 3 A, 300 W by PowerPac™ HC High-Current Power Supply (Bio-Rad, USA) for one hour. The image was obtained from gel imaging system (Syngene, UK).

3.2.11. Encapsulation efficiency

After preparation and purification of polymersomes/siRNA, the study of siRNA encapsulation was performed. Encapsulation efficiency of siRNA was analysed as a percentage of retention efficiency and loading efficiency obtained from the following formulas.

$$\%\text{Retention efficiency} = (\text{Final siRNA mass}/\text{Initial siRNA mass}) \times 100$$

$$\%\text{Loading efficiency} = (\text{Final siRNA mass}/\text{Theoretical loading}) \times 100$$

Retention efficiency defined as the ratio of final siRNA mass, and initial siRNA mass, respectively. For loading efficiency, the internal volume of polymersomes has been used as it is the actual volume which siRNA can be encapsulated, not the total volume of the solution. The internal volume of polymersomes were calculated according to their size determined by DLS. As shown in *Figure 3.1,* the cartoon shows the cross section of polymersomes with PMPC in blue colour and PDPA in red colour. The membrane thickness (t) is obtained from previous work (Pearson, Warren et al. 2013). The definition of each parameter is described in Table 3.3, according to previous work (Wang, Chierico et al. 2012b), and support from Prof. Giuseppe Battaglia and Adrian Steve Joseph.

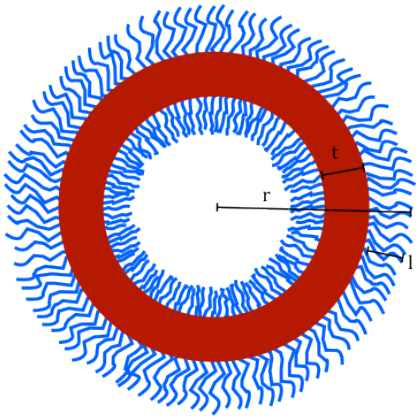


Figure 3.1: Cross section of polymersome.

This cartoon represents the cross section of polymersome, the blue lines represent the hydrophillic part (PMPC) and the red area shows the hydrophobic region (PDPA). The radius is counted from the center of polymer to the hydrophillic brushes outside. The radius of polymersome was measured by DLS and be calculated by the Zetasizer software (Malvern).

Table 3.3: List of parameters used in calculation of encapsulation efficiency.

Name	Abbreviation	Definition/value	Unit
Initial siRNA concentration	C'_{si}	-	$g.l^{-1}$
Final siRNA concentration	C_{si}	-	$g.l^{-1}$
Initial polymer mass	g'_{Pmer}	-	mg
Final polymer mass	g_{Pmer}	-	mg
Initial volume	V'	-	ml
Final volume	V	-	ml
Initial siRNA mass	g'_{si}	-	mg
Final siRNA mass	g_{si}	-	mg
Initial siRNA	mol'_{si}	$\frac{(g'_{si})(10^{-3})}{MW_{si}}$	mol
Initial polymer	mol'_{Pmer}	$\frac{(g'_{Pmer})(10^{-3})}{MW_{PMPC-PDPA}}$	mol
Final siRNA	mol_{si}	$\frac{(g_{si})(10^{-3})}{MW_{si}}$	mol
Final polymer	mol_{Pmer}	$\frac{(g_{Pmer})(10^{-3})}{MW_{PMPC-PDPA}}$	mol
MPC molecular weight	MW_{MPC}	293.5	$g.mol^{-1}$
Hydrophilic block length	m	-	-
Hydrophilic density	D_{PMPC}	1.05	pg/l
Hydrophilic brush length	l	6.39	nm
DPA molecular weight	MW_{DPA}	213.3	$g.mol^{-1}$
Hydrophobic block length	n	-	-
Hydrophobic density	D_{PDPA}	1	pg/l
Membrane thickness	t	6.22	nm
PMPC-PDPA molecular weight	$MW_{PMPC-PDPA}$	$(MW_{MPC})(m)+(MW_{DPA})(n)$	$g.mol^{-1}$
Avogadro's number	N_{Avo}	6.02×10^{23}	mol^{-1}
Hydrophobic molecular volume	V_{PDPA}	$\frac{MW_{PDPA}}{(N_{Avo})(D_{PDPA})}$	l
siRNA molecular weight	MW_{si}	13300	$g.mol^{-1}$
Number of polymersomes	N_{Psome}	$\frac{(mol_{Pmer})(N_{Avo})(\frac{DLS\ number}{100})}{N_{Agg}}$	-
Internal volume of polymersomes	V_{Psome}	$\frac{4}{3}\pi(r-l-t)^3(N_{Psome})x10^{-18}$	l
Number of aggregates	N_{Agg}	$\frac{\frac{4}{3}\pi[(r-l)^3-(r-l-t)^3]}{V_{PDPA}}$	-
Theoretical loading	-	$\frac{(g'_{si})(\sum V_{Psome})}{V'}$	mg

3.2.12. Cell culture

Cells used in this study were Human cervix adenocarcinoma cell, HeLa cell (CCL-2™), from the American Type Culture Collection (ATCC; Manassas, VA, USA). HeLa cells were grown in DMEM with 4.5 g/l glucose supplemented with 10% FBS, 2 mM L-glutamine (Sigma-Aldrich, UK), 100 U/ml penicillin, 100 µg/ml streptomycin (Sigma-Aldrich, UK) and 625 µg/ml amphotericin B (Gibco, UK). HeLa cells were maintained at 37°C under an atmosphere of 5% CO_2. The seeding density was $5x10^3$, $5x10^4$, $1x10^5$, $3x10^5$ and $4x10^6$ cells per well in 96-, 24-, 12-, 6-well plate and T-75 flask, respectively. The cells were provided with fresh culture medium on the day after seeding and every two days thereafter. In order to collect the cell pellet for further analysis, the cells were washed twice with PBS to remove the remaining culture medium before being trypsinised with trypsin-EDTA solution (0.02% (w/v) trypsin and 0.01% (w/v) EDTA) and incubated for 5 minutes. The cells were centrifuged at 2000 rpm for 5 minutes and the cell pellets were used for further study.

3.2.13. Transfection

Transfection with polymersomes

HeLa cells were prepared in 24-well plate with a seeding density of $5x10^4$ cells per well, and cultured overnight in normal growth medium. When the cells reached to the 70% confluent, the culture medium were removed and replaced with fresh culture medium containing empty polymersomes or polymersomes with siRNA at 0.01, 0.1, 1, 10, 30 and 50 nM siRNA as the final concentration in the culture media (according to the experimental design). The culture media were removed and the cells were washed with PBS buffer after 24 hours treatment before replacing with the fresh media. The cells were collected at 24 hour (for real time PCR analysis) and 48 hours (for western blot analysis) after treatment with the replacement of fresh culture media after 24 hours, before further analysis.

Transfection with Lipofectamine™ 2000

Lipofectamine™ 2000 reagent was purchased from Invitrogen to use as a transfection control for siRNA. The protocol of transfection was carried out as described in the product manual. HeLa cells were cultured in 24-well plate at the seeding density of $5x10^4$ cells per well in normal growth medium. At the transfection, the culture media was changed to anti-biotic free media to reduce the number of cell deaths. The siRNA and Lipofectamine™ 2000 was diluted in the Opti-MEM® I reduced serum medium with the recommended concentration as in the product guideline. After that, the siRNA and Lipofectamine™ 2000 were mixed together and incubated at room temperature for 20 minutes to allow the complex

formation of siRNA and Lipofectamine™ 2000. 100 µl of the complex mixture was added into the HeLa cell in each well. The HeLa cells were incubated for 6 hours before washing with PBS buffer and replacing with the fresh culture media. The cells were collected at 24 hour (for real time PCR analysis) and 48 hours (for western blot analysis) after treatment with the replacement of fresh culture media after 24 hours, before further analysis.

Polymersome and Lipofectamine™ 2000 containing siRNA were transfected into the cells according to siRNA concentration, the concentration of polymer and Lipofectamine™ 2000 are varied as shown in *Table 3.4.*

Table 3.4: $PMPC_{25}$-$PDPA_{70}$ polymer and Lipofectamine™ 2000 concentration used in the transfection experiments (according to tested siRNA concentration).

siRNA (nM)	siRNA (mg/l)	Pmer (mg/l)	Lipofectamine™ 2000 (mg/l)
0.01	$1.33x10^{-4}$	$3.58x10^{-6}$	$5.05x10^{-4}$
0.10	$1.33x10^{-3}$	$3.58x10^{-5}$	$5.05x10^{-3}$
1	$1.33x10^{-2}$	$3.58x10^{-4}$	$5.05x10^{-2}$
10	0.13	$3.58x10^{-3}$	0.51
30	0.40	$1.08x10^{-2}$	1.52
35	0.47	$1.25x10^{-2}$	-
50	0.67	$1.79x10^{-2}$	2.53
70	0.93	$2.51x10^{-2}$	-

3.2.14. Flow cytometry analysis

Polymersomes uptake

Rhodamine-labelled $PMPC_{25}$-$PDPA_{70}$ (at ratio of Rho-$PMPC_{25}$-$PDPA_{70}$: $PMPC_{25}$-$PDPA_{70}$ as 1:9) polymersomes were incubated with HeLa cells at a concentration of 0.5 mg/ml for 10, 60, 360, and 1200 minutes (in experiments with short incubation times) or 60, 1200, 2880 and 4320 minutes (in experiments with long incubation times).

siRNA uptake

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.

After incubation with Rho-$PMPC_{25}$-$PDPA_{70}$ polymersomes or Alexafluor® 647-siRNA/polymersomes, for the experimental time point, the culture media was removed and HeLa cells were thoroughly washed with PBS solution and detached using trypsin. Then the cells were centrifuged at 2000 rpm for 5 minutes and the cell pellets were resuspended in cold PBS before being loaded into the 96-well plates for analysis by BD FACSArray™ Bioanalyzer (BD Biosciences, San Jose, CA, USA). The machine was equipped with 532 nm (for detection of rhodamine-labeled polymersomes) and 635 nm laser (for detection of Alexafluor®647 siRNA signal). The procedure was performed according to the instrument's handbook. The fluorescent signal was analysed based on 5000 events per sample. The BD FACSArray™ software was used to operate the machine and FlowJo 8.7 Flow cytometry analysis software (Tree Star Inc., Ashland, OR, USA) was used to analyse the data.

3.2.15. Fixed cell imaging

HeLa cells were cultured in a BD Falcon™ 96-well imaging plate with black/clear bottom (BD Biosciences) until they reached about 70% confluent. Cells were treated with the 0.5 mg/ml Rho-$PMPC_{25}$-$PDPA_{70}$ polymersome for 10, 60, 360 and 1200 minutes before washed and fixed with 3.7% paraformaldehyde at 37°C for 15 minutes. The cells were washed again and soaked with 0.01% Triton X-100 to permeabilise cell membrane for 10 minutes. The cells were washed and stained with SYTO® 9 (Molecular probes®) or Hoechst 33342 (Molecular probes®) after rinsed with PBS. The cells were filled with PBS buffer, cells can be stored at 4°C. The microscopes used in this study were the laser scanning microscope BD Pathway™ 855 system (BD Biosciences, San Jose, CA, USA) or the Olympus FV1000 confocal with SIM-scanner on a BX61 upright microscope equipped with four lasers (405nm, 488nm (argon), 561nm and 640nm).

3.2.16. Live cell imaging

HeLa cells were grown in a BD Falcon™ 96-well imaging plate with black/clear bottom (BD Biosciences). The cells were incubated with the CellLight® Early Endosomes-GFP (Invitrogen) for 10 hours as per the manufacturer's instructions, prior to the polymersomes treatment. After treatment, the cells were washed and stained with the DNA-staining reagent, Hoechst 33342 (Molecular probes®), for 15 minutes. Cells were rinsed and replaced with the DMEM without phenol red as an imaging medium. Live cell imaging was acquired in the normal growth condition at 37°C supplied with 5% CO_2. Cell imaging study was performed by Zeiss LSM510 Meta confocal microscope (Carl Zeiss Ltd., Germany) equipped with 405 nm, 488 nm

(argon), 561 nm and 640 nm lasers or the laser scanning microscope BD Pathway™ 855 system (BD Biosciences, San Jose, CA, USA).

3.2.17. MTT assay

Tetrazolium salt MTT, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, was used in this study to determine the viability of cells treated with polymersomes. The method has been developed according to the original protocol by Mosmann (Mosmann 1983a). HeLa cells were cultured in 24-well plates at the seeding density of $5x10^4$ cells per well for one day before being treated with polymersomes. After treatment, cells were washed with PBS buffer twice followed by the addition of 0.5 mg/ml of MTT solution (dissolved in PBS buffer). The cells were protected from light and incubated at 37°C with 5% CO_2 for 30 minutes before removal of MTT solution. Then 300 µl acidified isopropanol (0.001 N of HCl in isopropanol) was added to each well to dissolve the blue crystal of insoluble formazan. After this 150 µl of solution was transferred to 96-well plate, the plate was then read on a ELx800 absorbance microplate reader (BioTek, USA) at the wavelength of 570 nm and 630 nm (as a reference). The cells treated with polymersomes were compared with the untreated cell as a control (100% cell viability).

3.2.18. mRNA extraction

HeLa cells were trypsinised and centrifuged after treatment with PBS buffer, polymersomes or Lipofectamine™ 2000. The mRNA extraction was performed by using RNeasy mini kit (cat. no. 74104, Qiagen) according to the procedure provided by the company. The cell pellet was re-suspended and lysed with 350 µl RLT buffer followed by the addition of 350 µl of 70% ethanol to precipitate the nucleic acid, before being transferred to the silica membrane column and centrifuged at 10,000 rpm for 15 seconds. After that the flow-through was discarded and 700 µl of RW1 buffer was added to the column in order to wash the unbound molecules from the column. The column was centrifuged at 10,000 rpm for 15 second and the flow-through was removed before washing with RPE buffer for the removal of traces of salts, and centrifuged to discard the flow-through. This step was performed twice followed by addition of 30 µl of RNase-free water to re-suspend extracted mRNA.

3.2.19. Total RNA concentration

After mRNA extraction step, total RNA concentration was measured for absorbance at the wavelength of 260 nm (nucleic acid content) and 280 nm (protein content) by using NanoDrop8000 UV-Vis spectrophotmeter (Thermo Scientific, UK). The ratio of

nucleic acid/protein of RNA samples was calculated, and samples with a ratio between 1.8-2.0 were used for further cDNA synthesis.

3.2.20. cDNA synthesis

Total mRNA of HeLa cells were reverse-transcribed into cDNA using QuantiTect® Reverse Transcription Kit (cat. no. 205313, Qiagen) according to the protocol provided by the kit manual. Briefly, the genomic DNA elimination step was prepared at 4°C with 1x gDNA wipeout buffer, 1 µg of template RNA and RNase-free water and the reaction was set at 42°C for 2 minutes. After that the reverse transcription reaction was performed by addition of Quantiscript Reverse Transcriptase, Quantiscript RT Buffer and RT primer mix into the previous sample mix and the reaction was carried out at 42°C for 15 minutes before inactivation step at 95°C for 3 minutes. The final cDNA products were kept at -20°C for further study.

3.2.21. Gene expression

The gene expression study was performed by Rotor-Gene® Q real-time PCR cycler (Qiagen, UK) with Rotor-Gene SYBR Green PCR Kit (cat. no. 204072, Qiagen). The PCR reactions were prepared with 1x Rotor-Gene SYBR Green PCR master mix, 1 µM forward primer, 1 µM reverse primer, cDNA template and RNase-free water. The PCR reaction (as shown in *Figure 3.2*) was initially activated at 95°C for 15 minutes, followed by 40-50 cycles of denaturation at 95°C for 15 seconds, annealing at 60°C for 30 seconds, and extension at 72°C for 30 seconds. The melting analysis was also performed at 65-95°C for confirmation of the final PCR product. Fluorescent signal was acquired at the end of extension step in each PCR cycles. The data acquired from the thermal cycler was analysed by Rotor-Gene Q software. Primers used in this work were shown in *Table 3.5.*

HeLa cells were incubated with polymersomes and/or Lipofectamine™ 2000 containing siRNA. In order to check the knockdown efficiency, Silencer Select Negative Control#1 siRNA (Cat no. AM4635, Ambion) has been used as validated negative control, along with the scrambled sequence siRNA as the nucleotide composition control.

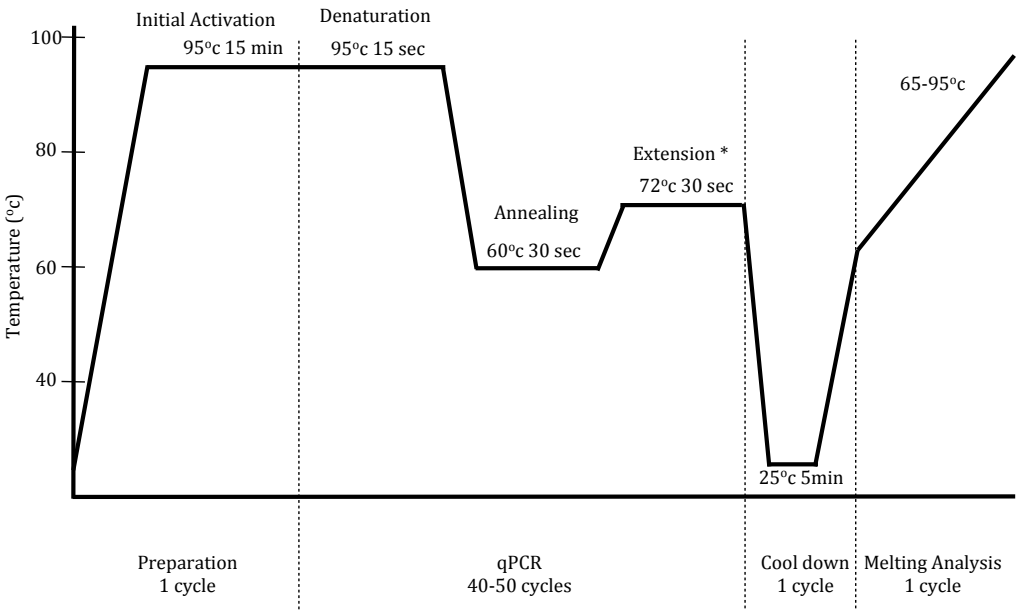


Figure 3.2: Thermal cycling profile for qPCR.

Table 3.5: The details of primers used in this study.

Gene name	Abbreviation	Genbank No.	seq (5'-3')	Tm	Amplicon size	Ref.
Lamin A/C	LMNA	NM_005572.3	FWD: GAG GAC CAG GTG GAG CAG TA	60.26	95	(Tulac, Dosiou, Suchanek, & Giudice, 2004)
			RV: AGG TTG CTG TTC CTC TCA GC	59.60		
18s ribosomal RNA	18s	NM_003286	FWD: GCC ATG CAT GTC TGA GTA CG	60.30	83	(Zhu & Altmann, 2005)
			RV: GAG CGA CCA AAG GAA CCA TA	60.07		
β-Actin	β-Actin	NM_001101.3	FWD: CCA ACC GCG AGA AGA TGA	51.46	97	(Kwon et al., 2009)
			RV: CCA GAG GCG TAC AGG GAT AG	53.24		
Eukaryotic translation elongation factor 1 alpha 1	EEF1A1	NM_001402.5	FW: CTG TAT TGG ATT GCC ACA CG	59.99	124	(Curtis et al., 2010)
			RV: GCA GCA TCA CCA GAC TTC AA	59.99		
Glyceraldehyde-3-phosphate dehydrogenase	GAPDH	NM_002046	FWD: ACA GTC AGC CGC ATC TTC TT	60.02	94	(Mori, Wang, Danenberg, Pinski, & Danenberg, 2008)
			RV: ACG ACC AAA TCC GTT GAC TC	59.97		
Hypoxanthine phosphoribosyltransferase 1	HPRT1	NM_000194.2	FWD: GAC CAG TCA ACA GGG GAC AT	59.82	111	(Fu et al., 2009)
			RV: CTG CAT TGT TTT GCC AGT GT	59.76		
Ribosomal protein L13a	RPL13a	NM_012423.2	FW: AGC TCA TGA GGC TAC GGA AA	59.98	103	(Curtis et al., 2010)
			RV: TAT TGG GCT CAG ACC AGG AG	60.21		

3.2.22. Cell extraction

After treatment, HeLa cells were trypsinised and the cell pellets were mixed and lysed with RIPA buffer (cat. no. R0278, Sigma, UK) containing 10% Protease inhibitor cocktail (cat. no. 11836153001, Roche, UK). Lysed cells were kept at -20^{o}C, or directly measured for total protein concentration by BCA protein assay.

3.2.23. Protein assay

Total protein concentration was measured by bicinchonic acid (BCA) (Smith et al. 1985) using the Pierce® BCA Protein Assay Kit (Thermo Scientific, UK). The assay was performed according to the protocol provided by the company. The bovine serum albumin was used as the standard protein to plot the calibration curve. The protein samples were incubated with the BCA reagent at the ratio of 1 (sample):20 (BCA) (v/v) and incubated at 37^{o}C before colorimetric detection at the absorbance of 570 nm by ELx800 absorbance microplate reader (BioTek, USA).

3.2.24. Protein expression

The protein expression was investigated by using capillary electrophoresis-based automated western blot machine, Simon™ (ProteinSimple, USA). This automated Western blot machine is based on the traditional Western hybridisation technique, but all steps are performed by the machine. Moreover, it provides both qualitative and semi-quantitive analysis for protein expression, whereas conventional Western blot offers only qualitative analysis. For automated Western, protein samples were loaded into capillary columns where size-based separation occurred, followed by fixation and immunoprobing with specific primary antibody. The detection is obtained from an enzyme-labelled secondary antibody that catalyses the chemiluminescent substrates and generates light. All chemiluminescent signals are directly exposed through the detector and presented as an electropherogram, allowing both qualitative and semi-quantitive analysis. To examine the level of Lamin A/C expression, GAPDH was used as internal control as it has molecular weight of 37 kDa, which differs from Lamin A/C (75 kDa). Differences in the molecular weight of the protein of interest and the internal control allows ease of identification and discrimination on the electropherogram obtained from automated Western blot. The location of the peaks on the electropherogram were compared with the marker proteins to identify the presence of the protein of interest according to molecular weights. The areas of each peak (GAPDH and Lamin A/C) were determined and analysed. Lamin A/C protein was normalised with GAPDH as an internal control and And quantified relative to the untreated control sample to obtain the expression value.

The reagent used were Fluorescent standard, Biotinylated ladder, Goat anti mouse secondary antibody HRP conjugated, Streptavidin-HRP, Luminol-S, Peroxide, Separation matrix, Stacking matrix, Wash buffer, Running buffer, DTT. All reagents were prepared according to the manufacturer's recommendation, the antibodies used for lamin A/C (cat. no. ab58529, Abcam, UK) and GAPDH (cat. no. 2118, Cell Signaling, UK) were diluted with antibody diluent provided at 1:100 and 1:25, respectively. All samples was diluted at 2 mg/ml and further mixed with the sample buffer, DTT and fluorescent standard before incubation at 95°C for 5 minutes for protein denaturation. After that, the samples and reagents were loaded into the 384-well plate as per the machine's guidelines. The analysis was performed by Compass software (ProteinSimple, USA).

3.2.25. NF-κB translocation assay

HeLa cells were cultured in the BD Falcon™ 96-well imaging plate with black/clear bottom (BD Biosciences) at a seeding density of $5x10^3$ cells per well. TNF-α was used as a positive control to activate NF-κB translocation in this study. HeLa cells were treated with TNF-α at concentration varied from 0.01-30 ng/ml for 30 minutes. HeLa cells were transfected with empty polymersomes, siRNA/ polymersomes or siRNA/Lipofectamine and incubated for 6 hours. HeLa cells were then washed with PBS buffer and fixed with 3.7% paraformaldehyde for 10 minutes. Washing step with PBS buffer was performed twice followed by permeabilising step with 0.1% Triton™ X-100 for 15 minutes. HeLa cells were washed again with PBS buffer before being incubated with the Rabbit anti-Human IgG NF-κB p65 (1:250 dilution, catalog no. SC-372, Santa Cruz Biotechnology) as a primary antibody for one hour. 0.01% of Tween® 20 was used as an detergent to help remove the remaining antibody before normal washing with PBS buffer. The HeLa cells were incubated with the Goat anti-Rabbit IgG DyLight® 488 (1:400 dilution, Abcam) as secondary antibody together with 1 μg/ml of Hoechst 33342 (Molecular probes®) for one hour in the dark. The cells were rinsed with 0.01% of Tween® 20. The final two washing steps with PBS buffer were carried out to remove all the remaining residual in the well before imaging with BD Pathway™ 855 Bioimager (BD Biosciences, San Jose, CA, USA) with 20x U-Apo 340 objective lens.

The positive cells were scored based on the location of fluorescent signals from DyLight® 488 and normalised by number of cells counted obtained from Hoechst 33342.

3.2.26. Human Interleukine-6 expression

Enzyme-linked immunosorbent assay (ELISA) was used to quantify interleukine-6 expressed in the cells. The Hu IL-6 ELISA kit (catalog number KHC0061) was

purchased from Invitrogen (Camarillo, CA, USA). HeLa cells were grown in 24-well plate with a seeding density of $5x10^4$ cells per well for 24 hours, before being changed to culture media with polymersomes or Lipofectamine™ 2000. After 24 hours of treatment, culture media was collected and used for IL-6 quantification. The assay was performed according to the product information sheet with the standard IL-6 at concentration of 0-500 pg/ml to plot the calibration curve. In brief, 100 µl of samples, standards and controls were added to the anti-IL-6 coated microtitre wells provided from the kit with addition of 50 µl of biotin conjugated anti-IL-6 solution. The plate was incubated for two hours at room temperature before decanting and washing with wash buffer four times. The 100 µl of streptavidin-HRP was added into each well and the plate was incubated for a further 30 minutes at room temperature. The washing step was performed again to remove all remaining streptavidin-HRP residue and 100 µl of stabilised chromogen was added to each well. The plate was kept further in the dark for 30 minutes at room temperature. The reaction was inhibited by addition of 100 µl of stop solution and the absorbance at 450 nm was measured using the ELx800 absorbance microplate reader (BioTek, USA). The concentration of IL-6 of each sample was calculated according to constructed standard curve.

3.2.27. Type I interferon assay

Total RNA from the cells treated with the polymersomes and Lipofectamine™ 2000 was extracted and prepared for realtime-PCR with protocols described in the previous section. RNA concentrations for each sample were measured at the absorbance of 260 nm. cDNA was reverse transcribed with RT^2 First strand kit (catalog no. 330401, Qiagen) according to the product instructions. The genomic DNA elimination step was performed with the mixture of 1 µg of RNA, 2 µl of GE buffer, and addition of RNase-free water to obtain the final volume of 10 µl. The reactions occured at 42°C for 15 minutes before stopping at 4°C for 1 minute. A further step was completed with the addition of 10 µl of reverse-transcription mix (containing 4 µl of 5x buffer BC3, 1 µl of control P2, 2 µl of RE reverse transcription mix and 3 µl of RNase-free water) into the first reaction. The mixture was incubated at 42°C for 15 minutes and immediately placed at 95°C for 5 minutes to stop the reaction. 91 µl of RNase-free water was added into each reaction in preparation for the qPCR experiment. Realtime PCR was prepared with 1150 µl of RT^2 SYBR Green ROX FAST mastermix (catalog no. 330622, Qiagen, UK), 102 µl of cDNA synthesis reaction and 1048 µl of RNase-free water. The mixture was added into the Human Type I interferon response RT^2 Profiler PCR array (catalog no. PAHS-016R, Qiagen) at the volume of 20 µl per well. The array was sealed with the Rotor-Disc heat-sealing film using Rotor-Disc heat sealer. The PCR reaction was performed at 95°C

for 10 minutes to activate the HotStart DNA *Taq* polymerase, followed by 40 cycles of denaturation at 95°C for 15 seconds and extension at 60°C for 30 seconds. The threshold cycles were analysed and determined by the Rotor-Gene software.

RT^2 Profiler PCR arrays (PAHS-016R, Qiagen) which contain 84 genes involved in type I IFN response in human cell was used to study mRNA expression. mRNA expression of samples in all treated conditions were analysed for their cycle threshold (Ct) value. Ct of each samples was normalised with house-keeping genes to obtain the ΔCt. The average of 5 different house keeping genes including β-2-microglobulin (B2M), Hypoxanthine phosphoribosyl-transferase 1 (HPRT1), Ribosomal protein L13a (RPL13A), Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and β-actin was used as reference gene for normalisation. All genes are listed in the *Appendices*.

3.2.28. Statistical analysis

The Student's *t*-test was performed to compare the difference between two groups while an analysis of variance (ANOVA) was used to determine the treatment effect from more than 3 groups which are different samples, times or concentrations, with the addition of a post test using Tukey's test for differences between individual groups and Dunnett's test for comparison with the control group. The statistical significance is represented as *,**,*** for *p* values less than 0.05, 0.01 and 0.01 respectively. The GraphPad Prism v 6.0b (GraphPad Software, Inc., San Diego, CA, USA) was used for analysis in this study.