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Formulation

**Investigating novel cationic polymers
for siRNA delivery for treatment of
allergic disease**

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Abstract

Spleen tyrosine kinase (Syk) is an enzyme which plays a prominent role in IgE-dependent signal transduction in type 1 hypersensitivity reactions. Due to the important role of Syk in the early signalling cascade in mast cells and basophils for inducing allergic reaction, it could be a suitable target for the treatment of allergy. In this project, the efficacy of Syk gene silencing in the inhibition of mast cell degranulation and cytokine induction by small interfering RNA (siRNA) in vitro was evaluated. The evaluation of the inhibition of Syk function was assessed by two novel rat basophil leukaemia (RBL-2H3) derived reporter cell lines which are Neuropeptide Y-Red Fluorescent Protein fusion (NPY-mRFP) and Nuclear factor of activated T-cells-DsRed (NFAT-DsRed) reporter cell lines. In NPY-mRFP reporter cells, red fluorescent protein (RFP) is targeted and stored in the granules and released into the medium during degranulation upon appropriate IgE-dependent stimulation of the cells. In NFAT-DsRed reporter cell, DsRed can be expressed upon appropriate IgE-dependent stimulation of the cells resulting in translocation of NFAT from the cytosol to the nucleus and reporter gene expression. Novel cationic polymers in this project were evaluated for the delivery of siRNA. The first polymer was AGMA29, an amphoteric linear polyamidoamine polymer which has been reported to be useful for gene delivery and with a low toxicity. In addition, two modified poly(glycerol adipate) (PGA) variants were used. PGA is a polyester which can be derivatised with a variety of substituents to make cationic polymers. Derived polymers PGA-50% lysine (PGA-50% Lys) and PGA-20% histidine (PGA-20% His) were evaluated in this work to determine the size, efficacy and toxicity of polyplexes used in the Syk siRNA delivery. Using

optimised polyplex resulted in the knockdown of the Syk function in the release of RFP in NPY-mRFP and the DsRed induction in NFAT-DsRed reporter cell lines, optimal calcium concentration and media used were determined. A new method was established for measuring the release of RFP in the NPY-mRFP cells, which could be used for detecting allergic reactions. The results showed that the polyplexes could be used for transfection when they were prepared in deionised water. 5 RU/Nt of AGMA29/Syk siRNA H polyplex resulted in a 31% decrease in the release of RFP in NPY-mRFP cell lines in comparison to untreated cell or scrambled siRNA. PGA-50% Lys/Syk siRNA H at 10AA/Nt ratio resulted in a 31% decrease in the release of RFP in NPY-mRFP cell lines in comparison to untreated cell and 24% in comparison to scrambled siRNA. The final finding from evaluation of Syk mRNA by RT-qPCR showed that the Syk mRNA knockdown at 5 RU/Nt of AGMA29/siRNA H was low (~26%) after 48 hr of the transfection. Interestingly, almost complete knockdown of Syk mRNA by PGA-50% Lys/Syk siRNA H was achieved at 5, 10, and 20 AA/Nt ratios. This work therefore contributes to the exploration of a novel polyester based polymer for siRNA gene therapy and compares it with a polyamidoamine based polymer. From the results obtained it is concluded that the novel polyester based polymer for siRNA delivery is safe, effective in the transfection of hard to transfect cells and could be used for future siRNA *in vivo* gene silencing applications.

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List of Abbreviations

AA	Amino Acid
AA/Nt	Amino Acid unit to RNA nucleotide
ALN	Alnylam
BCA	bicinchoninic acid
Bp	Base pairs
BSA	Bovine serum albumin
cDNA	Complementary deoxyribonucleic acid
DCM	Dichloromethane
DLinDMA	1,2-dilinoleyloxy-N,N-diethyl-3-aminopropane
DLS	Dynamic light scattering
DNA	Deoxyribonucleic acid
DNP-BSA	2,4-dinitrophenylated bovine serum albumin
DPBS	Dulbecco's phosphate buffered saline
EDTA	Ethylenediaminetetraacetic acid
FcεRI	Fc epsilon IgE receptor I
FBS	heat-inactivated foetal bovine serum
GalNAc	N-acetylgalactosamine
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase

Ggt1	Gamma-glutamyltransferase 1
interferon	IFN
IgE	Immunoglobulin E
IL	Interleukin
ITAM	Immunoreceptor tyrosine-based activation motif
kDa	Kilodalton
MEM	Minimum essential medium
miRNA	Micro-Ribonucleic acid
mRFP	Monomeric red fluorescent protein
NFAT	nuclear factor of activated T-cells
NPY	Neuropeptide Y
Nt	Nucleotide
PAA	Polyamidoamine
PEI	Polyethylenimine
pH	Potential of hydrogen
PLL	Poly-L-lysine
RBL	Rat basophilic leukaemia
RIPA	Radioimmunoprecipitation assay
RISC	RNA Induced Silencing Complex

RNA	Ribonucleic acid
RNAi	Ribonucleic acid interference
RSB	Reducing sample buffer
RT	Reverse transcription
RT-qPCR	Real Time-Quantitative Polymerase Chain Reaction
RTK	Receptor Tyrosine Kinase
RU	Repeating unit
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SH2	SRC homology 2
shRNA	Short hairpin ribonucleic acid
siRNA	Small interfering Ribonucleic acid
STIM-1	Stromal interaction molecule 1
Syk	Spleen tyrosine kinase
RU/Nt	Polymer repeating unit to RNA nucleotide
TAE	Tris – Acetic Acid – EDTA buffer
TFA	Trifluoroacetic acid
t-Boc	tert-Butyloxycarbonyl protecting group

CHAPTER 1: INTRODUCTION

1.1 Allergic diseases

Allergic disease is defined as a hypersensitivity syndrome that is caused by the immune system. Hypersensitivity reactions are classified by Coombs and Gell into four types which are Type I reactions, which are immunoglobulin E (IgE) mediated hypersensitivity reactions, Type II reactions which is immunoglobulin G (IgG) or immunoglobulin M (IgM) mediated hypersensitivity reactions, Type III reactions, which are immune complexation in the blood circulation between antigen and antibody, and Type IV reactions, which are topical immune cell mediated reaction that do not involve antibody in the reaction (Gell and Coombs, 1963). This project will focus on Type I IgE-mediated hypersensitivity reactions.

Type I IgE-mediated hypersensitivity reactions is part of several diseases such as asthma, rhinitis, and dermatitis. An allergic disease can occur at any age in any organ. In Europe in 2011, 25% of children were suffering from allergic disease (Pawankar et al., 2011). By 2025, asthma will represent the most widespread chronic childhood disease. The allergic symptoms that are caused by Type I IgE-mediated hypersensitivity reactions are mainly generated by the mast cells. Mast cells are originally from the hematopoietic stem cells in bone marrow. Mast cells are classified into MC_T which have high levels of tryptase

and MC_{TC} which has both tryptase and chymase (Reber and Frossard, 2014). Mast cells contain granules which contain pharmacological mediators such as histamine that are responsible for allergic reactions. FcεRI receptor (also known as the high affinity IgE receptor) in mast cells induces the allergic reaction. FcεRI consists of α, β, and γ subunits.

IgE protein consists of identical heavy variable chains (V_H), identical light variable chains (V_L), identical constant light chains (C_L), and two identical constant heavy chains. Each heavy chain has four domains (Cε) as shown in Figure 1.1 (Gould and Sutton, 2008). IgE binds to the antigen epitope through the variable chains whereas the IgE binds to to the α-subunit of high-affinity IgE receptor (FcεRI) through the Fc region (Penichet and Jensen-Jarolim, 2010). FcεRI is expressed in both mast cells and basophils (Pawankar, 2001) Human IgE is known to have a high binding affinity to FcεRI with an association constant (K_a) of approximately 10¹⁰ M⁻¹ (Gould et al., 2003). The normal level of IgE in healthy adult serum is approximately 150–250 ng/ml (Criep, 1976). The level of IgE in allergic asthmatic patient serum can be increased (Hansel and Barnes, 2010).

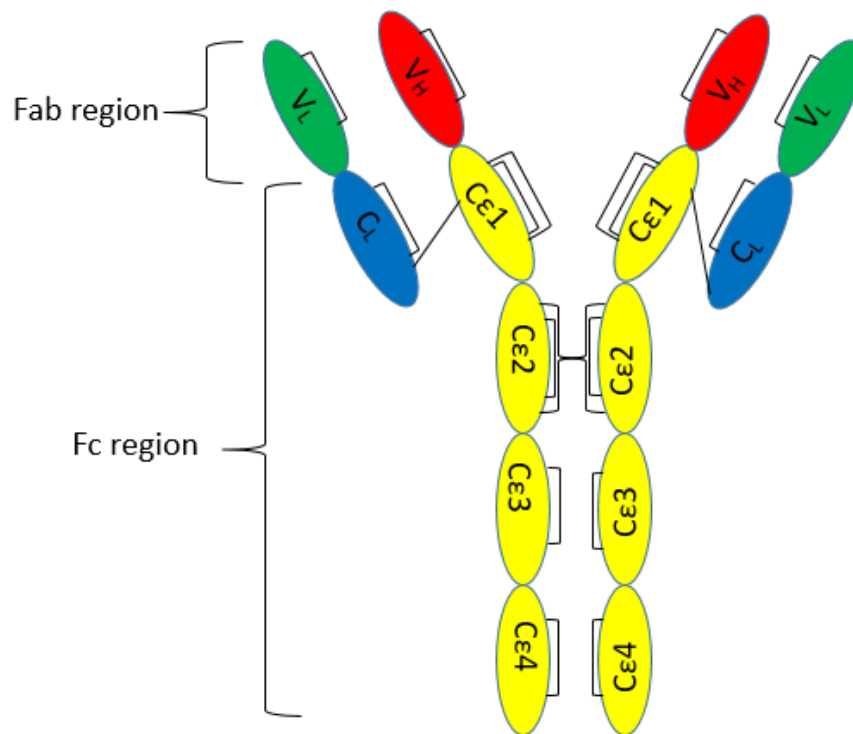


Figure 1.1 Structure of IgE. Yellow colours are constant domains of the heavy chain (C ϵ); blue colours are constant light chains (C λ); red color are variable heavy chains (V_H); green color are variable light chains (V_L); lines within and between chains are intra and inter disulphide bonds.

Immunoglobulin E (IgE) binds to α subunit of Fc ϵ RI. In the allergic reaction, allergen binds to IgEs which will result in the crosslinking of the Fc ϵ RI receptors (Saitō and Okayama, 2005). This will result finally in the degranulation of the mast cell and the release of their allergic mediators such as histamine which can cause bronchoconstriction and vasodilation (Saitō and Okayama, 2005, Razin et al., 1984). In addition, newly synthesised allergic mediators such as

prostaglandins and cytokines such as tumour necrosis factor α (TNF- α) and interleukin 6 (IL-6) will be generated (Siraganian et al., 2010).

1.2 Allergic disease treatment

There are several types of allergic diseases that are caused by several factors. In order to narrow the therapeutic strategies that are used for the treatment of allergic diseases, asthma is selected in this section as an example for the therapeutic approaches for the treatment of an allergy. There are several causes of asthma and the major cause of asthma is the allergic reaction (Ribeiro and Tarlo, 2012). Asthma is selected in this section because it is a prevalent disease. According to world health organisation, 235 million people in the world suffer from asthma (Bergon-Sendin et al., 2014)

One of the possible treatments which is not targeted the allergic reaction is the use of β_2 -adrenoceptor agonists. β_2 -adrenoceptor agonists, such as short acting salbutamol and long acting formoterol, are bronchodilators which cause a rapid inhibition of the symptoms of asthma. The binding of β_2 -adrenoceptor agonists to the receptor cause an increase in the expression of cyclic adenosine 3'5'-monophosphate (cAMP) which will activate the protein kinase A. Protein kinase A facilitates the relaxation of smooth muscle and therefore relieves the symptoms of the bronchoconstriction of asthma. Although β_2 -adrenoceptor agonists cause a bronchodilation which will give relief to the asthmatic patient,

it may increase the severity of the allergic asthma because it did not treat the causes of inflammation such as the release of allergic mediators and masks the effect of inflammation (ANDREW McIVOR et al., 1998).

Another strategy for treatment of allergy is targeting allergic mediators such as histamine. Antihistamine treatment can inhibit the effect of histamine such as bronchoconstriction, vasodilation, and contraction of smooth muscle by antagonising histamine in the histamine receptors (Brown et al., 2008). There are four known histamine receptors which are named H₁, H₂, H₃, and H₄. The receptor mainly targeted to treat allergic reactions is H₁ (Brown et al., 2008). Antagonising H₁ receptors can inhibit the activation of nuclear factor- kappa B (NF- κB) which is one of the transcriptional factors of inflammatory cytokines (Bakker et al., 2001). It was reported that a higher level of activated NF- κB was found in asthmatic patients (Hart et al., 1998). The limitation of using antihistamine therapy is that it targets the histamine receptor which is a late stage of allergic reaction instead of inhibiting histamine from being released. Furthermore, antihistamines will inhibit the effect of histamine only without influencing the effect of other allergic mediators that are released after mast cell degranulation. Also, antihistamine is not effective in the treatment of asthma and other severe allergic reaction.

Leukotriene receptor antagonists such as Montelukast and Zafirlukast are another therapeutic strategy for the treatment of allergy. Montelukast and Zafirlukast mainly antagonise type 1 cysteinyl leukotriene receptor (Hay, 1997). Cysteinyl leukotrienes are highly allergic mediators that can cause

hypersecretion of the mucus and smooth muscle constriction (Lipworth, 1999a). However, the effectiveness of leukotriene receptor antagonists in the treatment of asthma is weak (Barnes, 1999).

Corticosteroids are known as potent anti-inflammatory drugs that are widely used for various inflammatory diseases and as a first line medication for the treatment of asthma (Lipworth, 1999b). They can cause a decrease in cytokine production, decrease in mucus secretion, and airway dilation by increasing the numbers of β 2-adrenoceptors (Barnes and Adcock, 2003). However, corticosteroids can be used for short term treatment since long term treatment is associated with several adverse effects such as increase in the risk of cardiovascular disease, osteoporosis, diabetes mellitus (Sholter and Armstrong, 2000).

Humanised monoclonal anti-IgE antibody such as omalizumab is another medication that can be used for the treatment of allergic reactions. The omalizumab can bind to free IgE in the Fc region and forms a complex and therefore prevent the IgE from binding to the IgE receptor (Rodrigo et al., 2011). The prevention of IgE binding to its receptor causes a blockage of mast cells and basophil from degranulation and the release of allergic mediators (Rodrigo et al., 2011). Omalizumab is known to be a useful therapy for severe allergic asthmatic patients that have a high serum level of IgE (approximately 30-700 IU/mL) and cannot be relieved by long acting β 2-adrenoceptor agonists and corticosteroids even at high dose (National Asthma and Prevention, 2007). The limitations of using monoclonal anti-IgE antibody are that it does not bind to IgE already bound to IgE receptors. In addition, malignancies can occur to a higher extent for asthmatic patient that use Omalizumab than asthmatic patients

using other medications (Soler et al., 2001). The cost of the treatment of allergic patient in the UK by Omalizumab range from £1,665 to £26,640 annually.

Mast cell stabilizers such as cromolyn sodium, ketotifen, and nedocromil sodium can prevent the activation and the degranulation of mast cell via stabilizing the mast cell membrane (Corin, 2000). The efficacy of the cromolyn can be observed after two days of the initiation of the therapy whereas the efficacy of the nedocromil can be observed after 15 min of the initiation of the therapy (Stock and Bielory, 1997, Kjellman et al., 1995). However, cromolyn can influence in the function of other cell type such as granulocyte and B cells (Norris, 1996, Arumugam et al., 2006).

From the above treatments of allergic reactions and the disadvantages of each treatment strategy, this project proposes another treatment strategy targeting the spleen tyrosine kinase protein which is known to have a critical role in the early signalling cascade in mast cells and basophils that occur prior the release of allergic mediators. This pathway will be explained in detail in section 1.3.

1.3 Spleen tyrosine kinase (Syk) Protein

Syk is a tyrosine kinase protein that has a molecular weight of 72 kilodalton (KDa) (Mócsai et al., 2010). Syk consists of a kinase domain and two SRC

homology 2 (SH2) domains, which are called tyrosine domains, and two peptide linkers one of which is between the SH2 domains and the other is between one of the SH2 domains and the kinase domain (Mócsai et al., 2010). Figure 1.2 shows the structure of linear phosphorylated Syk.

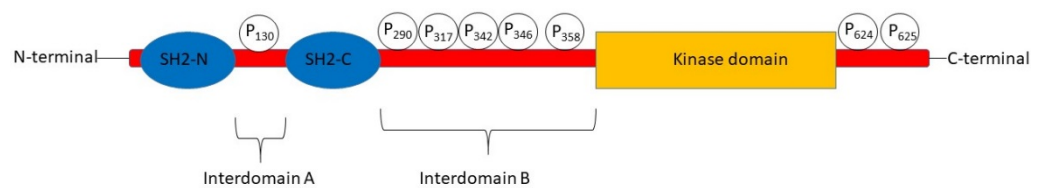


Figure 1.2 Structure of linear phosphorylated Syk. P: phosphate. The number adjacent to each phosphate represent the amino acid number that is phosphorylated. In SykB which is an isoform of Syk, 23 amino acids are missing in interdomain B including P₂₉₀.

Syk has an important signalling role in mast cell and basophil degranulation and the release of allergic mediators. It was reported that the Syk-deficient mast cell type cannot be degranulated when it is sensitised with IgE and stimulated with allergen (Zhang and Siraganian, 1999). In the case of allergic reactions, the Fc region of the IgE binds to the α -subunit of high-affinity IgE receptor (Fc ϵ RI) in the mast cell and the antigen binding site in the Fab region of IgE binds to the antigen (Saitō and Okayama, 2005). This is followed by crosslinking of the Fc ϵ RI receptors via multivalent antigens and aggregation of Fc ϵ RI receptors. When the aggregation of Fc ϵ RI receptors occurs, Lyn which is a tyrosine kinase protein that is associated with the Fc ϵ RI receptor is activated. The activation of

Lyn causes a phosphorylation of the tyrosine-based activation motif (ITAM) which is a part of the β and γ subunits of Fc ϵ RI receptor (Siraganian et al., 2002). SH2 domains of Syk will then bind to the phosphorylated ITAM in the γ subunit of Fc ϵ RI in which the Syk is phosphorylated (Stauffer and Meyer, 1997, Siraganian et al., 2002). Thereafter, a conformational change in the structure of phosphorylated Syk occurs which allows the Syk to bind to its substrate (Siraganian et al., 2002). Thereafter, a signalling cascade is initiated which leads to activation of phospholipase C- γ (PLC- γ) which causes a hydrolysis of phosphatidylinositol-4,5-bisphosphate (PI(4,5)P₂) to inositol-1,4,5-trisphosphate (IP₃) (Gilfillan and Tkaczyk, 2006). IP₃ will bind then to the IP₃ receptor (IP₃R) in the endoplasmic reticulum (ER) which will lead to the release of Ca²⁺ and Stromal interaction molecule 1 (STIM-1) from the ER (Liou et al., 2005). The release of Ca²⁺ and STIM-1 will lead to the influx of calcium from outside the cell via calcium release-activated calcium channel (CRAC) (Reber and Frossard, 2014). This influx of calcium, in turn, leads to the degranulation of the cell and release of allergic mediators such as histamine (Reber and Frossard, 2014). In addition, the influx of Ca²⁺ will cause it to bind to calmodulin (Fric et al., 2012). The Ca²⁺ binding to calmodulin leads to the activation of calcineurin which will dephosphorylate the nuclear factor of activated T-cells (NFAT) transcription factor (Fric et al., 2012). The dephosphorylation of NFAT will unmask a nuclear localisation signal, resulting in NFAT translocation from the cytosol to the nucleus resulting in the induction of chemokines and cytokines (Nakamura et al., 2010). Figure 1.3 shows the mechanism of action of Syk

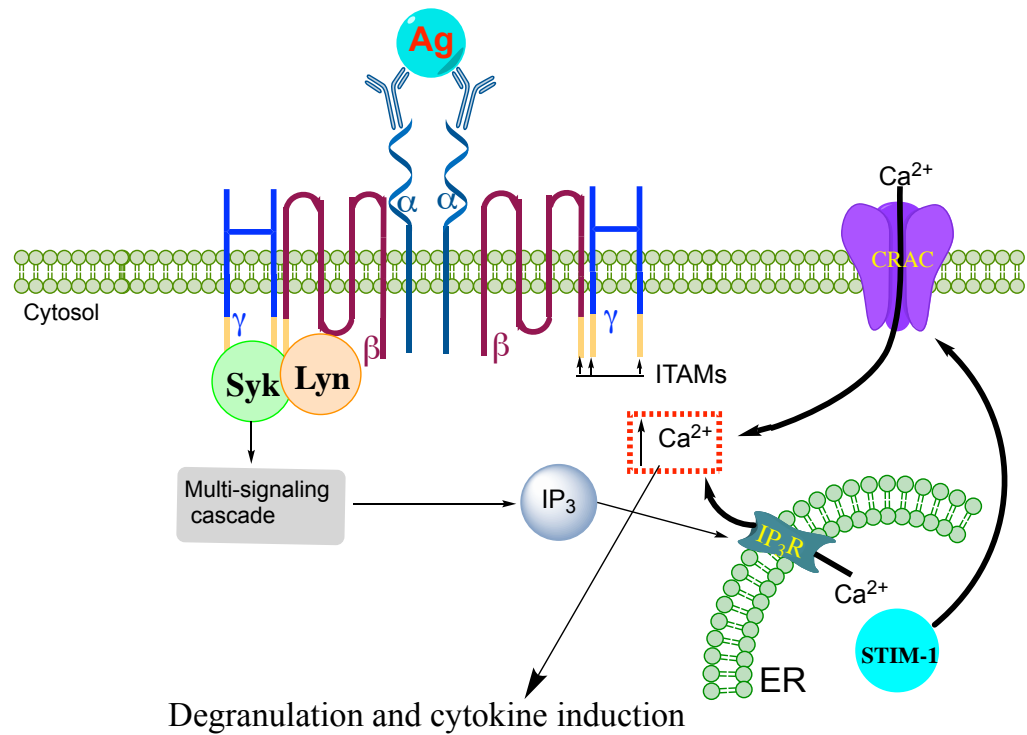


Figure 1. 3 Mechanism of action of Syk after the crosslinking of IgE by allergen. Ag: Antigen/Allergen; CRAC: calcium release-activated calcium channel; ER: endoplasmic reticulum; IP3: inositol-1,4,5-trisphosphate; IP3R: IP3 receptor; STIM-1: Stromal interaction molecule.

NFAT transcriptional factors are classified into five types which are NFAT1, NFAT2, NFAT3, NFAT4, and NFAT5 (Chytil and Verdine, 1996, Graef et al., 2001). Only NFAT5 is not activated by the Ca^{2+} /calcineurin dephosphorylation mechanism described above (Miyakawa et al., 1999). Mast cells have NFAT1 and NFAT2 which are responsible for the IL-13 regulation and the transcription of TNF (Klein et al., 2006). Basophils have NFAT2 which regulates the transcription of IL-4 (Schroeder et al., 2002, Qi et al., 2011).

From the mechanism of action of SYK protein and its role in the signalling cascade of mast cells and basophils prior the release of allergic mediators, knockdown of the expression of SYK by using siRNA nucleic acid therapy could be a potential method for the prevention the release of allergic mediators and treatment of allergic disease.

1.4 Types of nucleic acid therapy

Gene therapy is a method of drug delivery which is was originally devised for the treatment of genetic diseases, but can also be applied to a wide variety of other types of diseases. It is achieved by inserting suitable genetic materials to target specific cells inside the human body in which those cells are the causes of this genetic disease (Anderson, 1998). This genetic materials can treat the disease by fixing the abnormal gene or by adding a new gene (Templeton and Lasic, 2000). Adenosine deaminase (ADA) gene was the first gene therapy that was approved in September 1990 for treatment of ADA deficiency disease by inserting it inside the T cells by using a retroviral vector (Blaese et al., 1995).

There are several types of gene therapy that were used to be delivered to cell such as plasmid DNA (pDNA), antisense oligonucleotide (AON) and siRNA. The pDNA delivery is used in order to either produce a protein or a small hairpin RNA (shRNA). The AON and siRNA are used to inhibit the production of the

target protein by knockdown of the mRNA that is translated to this protein. Therefore, the AON and siRNA have their effect in the cytoplasm where protein synthesis occurs. The siRNA has an advantage over the AON in that the siRNA can bind to RNAi protein complexes which will protect the siRNA from nuclease degradation in addition to transporting the siRNA to its mRNA substrate (Howard, 2013). The main advantage of using pDNA is that DNA is more stable than RNA because the 2' hydroxyl group in the ribose of RNA can be more susceptible for degradation by serum nucleases that break the nucleotides in the phosphodiester backbone region (Bruns et al., 2007, Gary et al., 2007). The pDNA also can bind better to cationic polymer than siRNA by electrostatic interaction because the size of pDNA is several kilo base pair (kbp) of nucleotide whereas the size of siRNA is 21-23 bp of nucleotide so the pDNA form more stable complex with cationic polymer than siRNA (Scholz and Wagner, 2012). Therefore, siRNA may not be completely complexed and form a less well condensed complex with cationic polymer in comparison to pDNA (Gary et al., 2007). The main drawback of delivery of pDNA is that it is required to deliver the pDNA to the nucleus of the cell in order to express its gene of interest.

1.5 RNA Interference

RNA interference (RNAi) is a biological process in which a gene-mediated RNA sequence cause an inhibition of protein production by degrading the mRNA that expressed it (Fire et al., 1998). RNAi was discovered by Craig

Mellow and Andrew Fire in 1998 for which Nobel Prize in medicine or physiology was awarded to them in 2006 (Fire et al., 1998). It is important to know the mechanism of action of RNAi in order to produce an efficient therapy based on the RNAi pathway. RNAi, either natural microRNA (miRNA) or synthetic RNAi such as small interfering RNA (siRNA) or small hairpin RNA (shRNA), has the ability to target a variety of RNA substrates. The first step in the natural RNAi pathway is that most of the miRNA are transcribed as primary RNA (pri-miRNA) which has a hairpin structure (Rodriguez et al., 2004). The pri-miRNA has approximately 80 nucleotides (Lee et al., 2003). The pri-miRNA is polyadenylated in the 3' end and has 7-methyl guanylate (m7G) cap in the 5' end which could indicate that it is transcribed by RNA polymerase II (Cai et al., 2004). The pri-miRNA is then changed to be pre-miRNA by the aid of nucleus enzyme Drosha and its cofactor DGCR8 (Gregory et al., 2004, Denli et al., 2004). The pre-miRNA has approximately 65 nucleotides (Lee et al., 2002). Thereafter, the pre-miRNA migrates from the nucleus to the cytoplasm via the nucleopore complex (NPC) in the nuclear membrane with the help of Exportin-5 (XPO5) protein (Lund et al., 2004). In the cytoplasm, the pre-miRNA binds to the RISC-loading complex (RLC) in which the pre-miRNA is converted to 21-22 nucleotide miRNA by an RNase III enzyme that is called Dicer (Wang et al., 2009, Zamore et al., 2000). The miRNA (and also the natural or synthetic siRNA) binds to pre-RISC in which the unused strand will be removed during the activation of RNA-induced silencing complex (RISC). The RISC consists of four Argonaute proteins (Ago 1-4). The remaining single strand miRNA or siRNA, which is called the guide strand, in the RISC will then bind to the target mRNA and cause its degradation (Howard, 2013). RISC/miRNA guide strand

mainly targets the 3' untranslated region (3'UTR) of mRNA whereas RISC/siRNA guide strand mainly target the open reading frame (ORF) of mRNA (Howard, 2013). From the four Ago proteins, Ago2 is the one that has endonucleolytic activity and it is mainly responsible for endonucleolytic cleavage of mRNA. This access at the nucleotide is complementary to the 9–10th nucleotide position of siRNA guide strand from the 5' end when the siRNA guide strand is fully complementary to the mRNA (Liu et al., 2004, Elbashir et al., 2001b). miRNA needs to be mainly complementary to the mRNA to cause destabilisation and repression of the translation of mRNA (Lam et al., 2015). Figure 1.4 summarises the mechanism of action of RNAi.

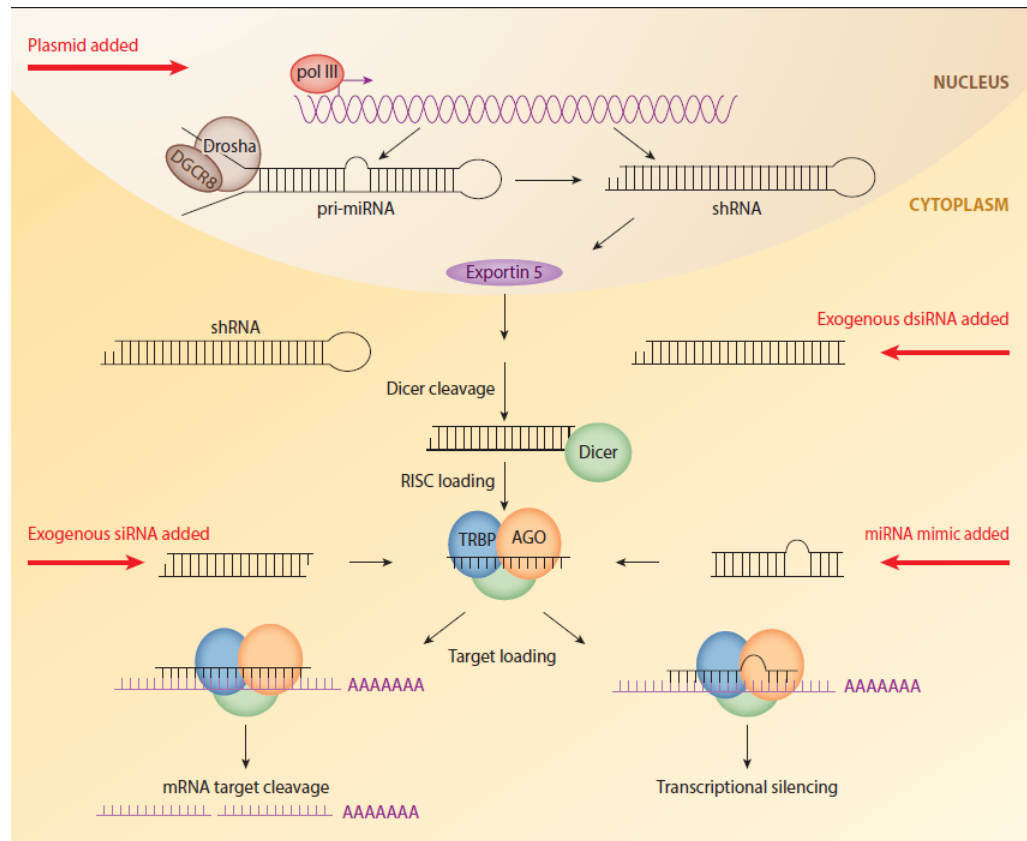


Figure 1.4 Summary of RNAi mechanism of action of RNAi and the pathways of synthetic RNAi therapy entry which are the entry of plasmid DNA that express either pri-miRNA or shRNA to the nucleus, entry of siRNA or miRNA to the cytoplasm (Bobbins and Rossi, 2016)

1.6 Obstacles and solutions of using therapeutic synthetic siRNA

There are several biological barriers that prevent naked siRNA from being used effectively for delivery to target cells. These barriers depend on the targeted organs and the route of administration of siRNA. For example, the number of barriers for the local administration of the synthetic siRNA to the lung,

intranasal, or intraocular areas are lower than the systemic administration of synthetic siRNA (Thomas et al., 2007, Bitko et al., 2005, Fattal and Bochot, 2006). In order to attain an efficient therapeutic siRNA delivery, it is important to know those biological barriers and the methods of crossing them. Figure 1.5 shows the biological barriers of nucleic acid delivery.

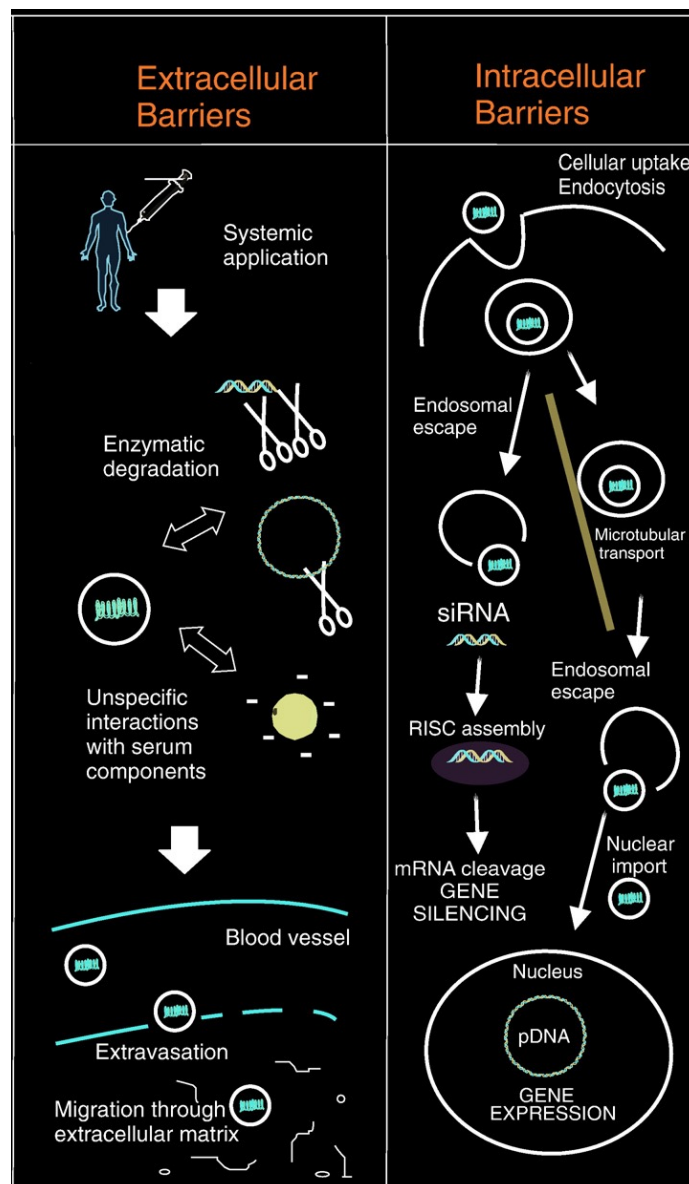


Figure 1.5 Extracellular and intracellular barriers of nucleic acid delivery (Scholz and Wagner, 2012)

Unmodified naked siRNA cannot be successfully delivered in vivo because it can be easily degraded due to the presence of ribonuclease in serum (Soutschek et al., 2004). In addition, significant chemical modification of siRNA can cause an observable reduction in the siRNA potency (Bramsen et al., 2009). However, there are many chemical modifications in the 3' overhang position of the siRNA that enhance its stability by improving its resistance against 3' exonucleases without noticeable decline in the siRNA activity (Bramsen et al., 2009). Additionally, siRNA stability against RNA nuclease can be enhanced by changing the OH group in position 2' of the ribose to OMe in which it can lead also to improve the affinity of the siRNA to the mRNA and reduce its off-target effects (Volkov et al., 2009, Prakash and Bhat, 2007, Bertrand et al., 2002). When siRNA is transfected to the cytoplasm, it has a low half-life in rapidly dividing cells (Bartlett and Davis, 2007).

1.6.1 Cellular uptake and endosomal escape

After the siRNA reaches its target cell, its uptake by the cell membrane is required. The cell membrane has a negatively charged lipid bilayer and proteins that protect the cell contents from the extracellular environment. The cell membrane also has a role in controlling the entry and exit of ions and other substances (Kievit and Zhang, 2011). Because the naked siRNA cannot be administered to the body by injection due to the limitations mentioned above, it is mainly carried by a nanoparticle carrier such as a cationic polymer. The cationic polymer/siRNA polyplex can enter the cell by endocytosis.

Endocytosis is uptake into the cell into vesicles by invagination of the plasma membrane lipid bilayer and the formation of a vesicle called the endosome inside the cell (Doherty and McMahon, 2009, Khalil et al., 2006). There are several types of endocytic uptake pathways. The major endocytosis pathway that is well characterised is clathrin-mediated endocytosis (Takei and Haucke, 2001, Lamaze and Schmid, 1995). In clathrin-mediated endocytosis, the cell membrane surface receptor binds strongly to its ligand such as binding of low-density lipoprotein (LDL) receptor to LDL or binding of transferrin receptor to transferrin (Schmid, 1997, Brodsky et al., 2001). This is followed by invagination of the cell membrane and forming a clathrin-coated endosome (Khalil et al., 2006). The diameter of the formed endosome ranges from 100 to 150 nm (Takei and Haucke, 2001). Another endocytosis type is Caveolae-mediated endocytosis. A caveola is a patch of hydrophobic membrane that consists mainly of cholesterol and glycosphingolipids (Harris et al., 2002). In this form of endocytosis, invagination of the cell membrane occurs and forms a vesicular or tubulovesicular shape that has a diameter between 60-80 nm (Doherty and McMahon, 2009). Two other types of endocytosis that can form larger sized endosome are macropinocytosis and phagocytosis. Macropinocytosis occurs at the ruffled regions of the cell membrane. It mainly occurs when the cell is stimulated with growth factor (Swanson and Watts, 1995). The diameter of macropinosome that is formed from macropinocytosis is between 0.2–5 μm (Kerr and Teasdale, 2009, Schnatwinkel et al., 2004) . Phagocytosis is only found in limited number of cells such as a macrophages to

engulf pathogens and is particularly applicable to larger particle sizes above 500nm (Allen and Aderem, 1996)

Prior the endocytosis of the nanocarrier/siRNA complex, the binding to the cell membrane which is negatively charged can be enhanced by the cationic nanoparticles because electrostatic interaction will occur between the nanoparticles and the cell membrane (Van der Aa et al., 2007). It is important that the nanoparticle escape from the endosome since endosome can transfer the siRNA to the lysosome in which lysosomal degradation will occur (Bonifacino and Traub, 2003). It is hypothesized that endosomal escape can occur because of the proton sponge effect of the nanoparticles that have high buffer capacity in the endosomal stage pH. The pH of early endosomal stage is around 6, whereas it becomes 5 or lower at later stages (Schultz, 1996). Nanoparticles with a high buffer capacity causes a swelling of the endosome and its destruction by increasing the ion concentration inside the endosome (Boussif et al., 1995). Figure 1.6 explains the mechanism of action of polymer endosomal escape by the proton sponge effect.

Even though the proton sponge theory is a commonly recognised mechanism for endosomal escape, it is a highly debated mechanism. It was reported by Benjaminsen et al. (2013) that cell transfection with polyethylenimine (PEI)/DNA polyplexes did not cause an increase in the lysosomal pH. In addition, the PEI/ DNA polyplexes could escape through lysosomal compartment membrane pores which were visualised by electron microscopy.

This could indicate that changes in polymer conformation occurs that cause its escape through the lysosomal membrane holes (Bieber et al., 2002)

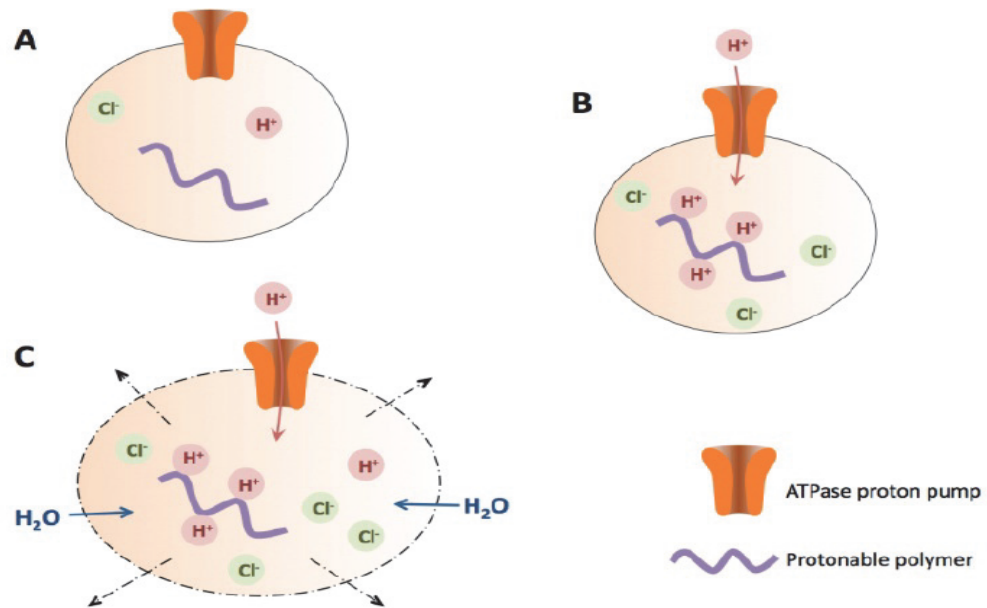


Figure 1.6 The mechanism of polyplex escape from endosome. A) The polyplex enter the endosome. B) The ATPase proton pump enables the hydrogen ions to enter the endosome in which the polymer will be protonated. C) Higher amount of hydrogen ions will enter the endosome which will lead to passive entry of chloride ions and finally water will enter the endosome and endosomal swelling and rupture will occur (Liang and Lam, 2012).

1.6.2 Off-target gene silencing effect

Off-target gene silencing of siRNA is a process in which the synthetic siRNA or miRNA cause unintended downregulation of any gene rather than the targeted gene (Jackson et al., 2003). This can occur if the siRNA is complementary to

any untargeted mRNA (Birmingham et al., 2006). This off-target effect can be reduced firstly by decreasing complementarity to any off-target mRNA since it was recorded that any single mismatch between the synthetic siRNA and the complementary mRNA can lead to a significant change in the siRNA function (Saxena et al., 2003). This can be performed by using sequence alignment tools such as BLAST to detect whether siRNA is complementary to any off-target mRNA or not (Snøve and Holen, 2004). It was suggested that the siRNA should not have more than 15 nucleotide homology to any off-target mRNA (Haley et al., 2010). The off-target effect of siRNA can also be significantly reduced by precise chemical modification (Bramsen et al., 2010). For example, Unlocked Nucleic Acid (UNA), which is an opened ribose ring of the nucleic acid, chemical modification at the 7th nucleotide of the guide strand of siRNA can decrease the off-target effect with only a minor reduction on the efficacy of its mRNA target (Bramsen et al., 2010).

1.6.3 Immunogenicity of siRNA

It was assumed that the siRNA does not cause an immunogenic response to the human cells because it resembles the endogenous siRNA (Elbashir et al., 2001a). However, many innate immunogenic responses were reported from using siRNA which are related to the structure and sequence of siRNA, the type of the transfected cells and the route of the delivery of siRNA (Reynolds et al., 2006). There are many siRNA sequence motifs that can cause an immunogenic response such as UGUGU, GUCCUCAA and UGGC since those motifs can

be identified by Toll-like receptors 7 and 8 (TLR7/8) that are found in the endosome of several immune system cells (Howard, 2013). The length of the siRNA can also influence its immunogenic response since it was reported that the 21mer double strand siRNA does not show any immunogenic response to several types of cells even at high concentration whereas the 25mer double strand siRNA shows some immunogenic response to some cell lines such as HeLa S3, DU145 (Howard, 2013).

1.7 Carriers for gene therapy

In order to overcome the biological barriers that were previously described, the optimised carrier of siRNA should have several criteria such as being non-toxic and capable of forming a stable complex with siRNA (Oh and Park, 2009). This stable complex should be able to protect RNA from the nuclease enzymes and also able to enter the targeted cells and escape from endosome then finally be dissociated from the siRNA to be released to its target mRNA (Oh and Park, 2009). In this section, several vectors will be described with their limitations including both viral and nonviral vectors.

1.7.1 Viral vectors

Viral vectors mainly used for the transfection of plasmid DNA, but can also be used for RNAi therapy by carrying pDNA that express either shRNA or pri-miRNA. In order to synthesise suitable viral vectors for gene therapy, complicated modifications for the vectors are required in order to avoid the virus replication and toxicity.

Viral vectors can be classified into two groups. The first group is enveloped viral vectors which are Retrovirus, Lentivirus, and Herpes simplex virus-1 (HSV-1) (Thomas et al., 2003). The second group is Non-enveloped viral vectors which are adenoassociated virus (AAV) and Adenovirus (Thomas et al., 2003). The first viral vector model that was used for *in vivo* RNAi therapy was by Beverly Davidson's laboratory (Xia et al., 2004, de Fougères et al., 2007). They used recombinant AAV serotype 1 vectors that express shRNA that can knockdown human ataxin-1 gene for the treatment of spinocerebellar ataxia type 1 disease (Xia et al., 2004). Viral vectors can cause an immune response when transfected *in vivo*. For instance, the capsid of viral vectors can induce a cytokine-mediated inflammation and cytotoxic T-lymphocyte (CTL) response that targets the expressed viral gene (Kafri et al., 1998).

1.7.2 Non-viral vectors

Due to safety concerns of using viral vectors for nucleic acid delivery and the complication of producing an efficient viral vector which can be produced in

low scale for siRNA transfection, non-viral vectors recently are the main area of research of siRNA delivery which can offer a safe and easy to be synthesised in large scale alternative for siRNA delivery (Shim and Kwon, 2010, Tomanin and Scarpa, 2004).

1.7.2.1 Lipid based carriers

The first cationic liposomes used for transfection of DNA were discovered by Felgner et al. (1987), (1989). Liposomes are spherical-enclosed membrane vesicles that consist of a phospholipid bilayer (Kraft et al., 2014). Lipid nanoparticles based delivery systems may not have an enclosed phospholipid bilayer (Kraft et al., 2014). Liposomes containing cationic lipids can enhance the binding with pDNA or RNA in which DNA–lipid or RNA–lipid lipoplex is formed. However, liposomes containing cationic lipids forms large lipoplexes with DNA or RNA with high surface charge which narrows their *use in vivo* due to rapid clearance and toxic effects of the cationic lipoplexes (Hirko et al., 2003, Martin et al., 2005). Therefore, the ionisable liposome is the area of research. The ionisable lipid that is used to produce liposomes for gene delivery consists of three parts: the amine head, the hydrophobic tail, and the linker between the amine head and the hydrophobic tail (Kanasty et al., 2013b).

In order to form a low toxic ionisable liposome-RNA lipoplex without affecting the efficacy, the functional groups of the liposome should have pKa that is

unprotonated in the blood circulation but protonated during membrane fusion with the negatively charged cell membrane during endocytosis (Zhang et al., 2011). The effect of the pKa–activity relationship of the ionisable liposome was evaluated by Jayaraman et al. (2012). In their study, 56 ionisable lipids that have different amino lipids head were evaluated for *in vivo* efficiency in knockdown of FVII gene in hepatocyte of female C57BL/6 mice species. The pKas of the amino lipids head in their study ranged from 4.17 to 8.12. They conclude from their study that the optimal pKa for the highest knockdown of FVII gene efficacy was observed at 6.44. It was assumed that at this pKa, the lipid nanoparticles (LNPs) are with low or without surface charge in the blood circulation when injected intravenously, which will diminish the non-specific interactions between the LNPs and negatively charged proteins but being positively charged in the process of endocytosis.

However, the ionisable LNPs can be removed from the circulation through binding to the hepatocyte because of apolipoprotein (Apo) E or A-I. It was reported that the neutral liposome can interact with Apo E and Apo A-I (Mendez et al., 1988, Rensen et al., 1997). The role of Apo E in the delivery of the LNPs was further evaluated *in vivo* by Yan et al. (2005) who used apoE-deficient mice. Their finding showed that the hepatic uptake of neutral liposomes in wild type mice was 20 times higher than that was found in apoE-deficient mice whereas hepatic uptake of negatively charged liposomes was identical. They also found that the blood circulation clearance of neutral liposomes in apoE-deficient mice was slower than that observed in wild type mice, while it was undistinguishable for the negatively charged liposome.

1.7.2.2 Cationic polymers

1.7.2.2.1 Polyelectrolyte theory

The behaviour and the binding of siRNA to the cationic nanoparticles to form a non-viral gene delivery system can be explained by the polyelectrolyte theory. Polyelectrolytes are polymers that contain either positively cationic or negatively anionic charged groups (Dobrynin and Rubinstein, 2005). The polyelectrolyte molecule can dissociate in a polar solution into charged macro-ion which is a charged polymer chain and small counter-ions (Barrat and Joanny, 1997). When the polyanionic molecule such as siRNA is mixed with a polycationic molecule such as a cationic polymer in a polar solution such as an aqueous solution, they form a complex that is called either a polyelectrolyte complex (PEC) or an interpolyelectrolyte complex (IPEC) due to the electrostatic interaction. Moreover, the charged macro cationic-ions bind to the charged macro anionic-ions and their cationic and anionic counter-ions bind together. The ratio of the polycationic to polyanionic molecules in the PEC is called ϕ . When the ϕ value is low, the net charge of the PEC will be negative. When the polycationic polymer increases, there will be two forms of complex. One of them is called stoichiometric complex (S-PEC) that is insoluble in polar solvent and has a neutral charge since the polycationic molecules charges are equal to polyanionic molecules charges ($\phi=1$). The other one is called non-

stoichiometric complex (N-PEC) that is soluble in polar solvent and has a net negative charge ($\phi < 1$). Further addition of polycationic polymers will make all the PECs to become S-PEC in which the complex can be precipitated and aggregated because it is insoluble complex. However, when extra polycationic polymers are added to this insoluble S-PEC, the complex will return to be soluble and the net charge of this complex will be positive (Garnett, 1999). Therefore, it is important to consider this basic aspects of the polyelectrolyte theory in order to form a small size polyplex between the cationic polymer and the anionic siRNA. For example, the ratio of cationic polymer to the siRNA and pKa functional group values of the cationic polymer can influence the properties of the formed polyplex such as solubility and stability.

1.7.2.2.2 Chitosan

Chitosan is a cationic polysaccharide that can be synthesised by deacetylation of the natural polysaccharide chitin (Romøren et al., 2002). The gene delivery properties of chitosan depend on both the degree of acetylation and its molecular weight (Alameh et al., 2012). Chitosan can be used as a carrier for *in vivo* gene application since it is a biocompatible polysaccharide (Hirano et al., 1990, Bersch et al., 1995). It was reported that a therapeutic effect can be found for treatment of type 2 diabetes mellitus in animals from using pDNA that express glucagon-like peptide-1 in which the chitosan was used to form the chitosan/pDNA polyplex (Jean et al., 2011). The transfection efficiency and toxicity of chitosan/siRNA polyplex for a variety of *in vitro cell* lines was

evaluated by Malmo et al. (2012). Their finding shows that the complete de-N-acetylated form of chitosan is more efficiently in delivery of siRNA than the partially de-N-acetylated form and the toxicity of chitosan/siRNA polyplex was low.

However, it was mentioned that chitosan has a low endosomal escape which can decrease the transfection efficacy (Ishii et al., 2001). In addition, chitosan has a low solubility which can negatively influence *in vitro* and *in vivo* application of chitosan (Chang et al., 2010).

1.7.2.2.3 Polyethylenimine (PEI)

PEI was firstly used for gene delivery by Boussif *et al.* (1995) who found that PEI/DNA polyplex can cross the central nervous system barrier to the brain of a new born mouse. There are two types of PEI: linear PEI (lPEI) and branched PEI (bPEI). lPEI contains only secondary amines in its backbone and the primary amine is on the terminal of the polymer chain (Brissault et al., 2003). bPEI contains primary, secondary, and tertiary amines (Zhuk et al., 1965). Due to the presence of variety of amino groups of bPEI, it has a better complexation and buffer capacity than lPEI (Kircheis et al., 2001).

The PEI polymer has high positive charge density which can condense the pDNA and siRNA effectively to form a positively charged PEI/ pDNA and PEI/ siRNA polyplexes (Werth et al., 2006, Intra and Salem, 2008). In addition, the high positive charge density character of PEI enable it to strongly protect pDNA and siRNA from nuclease degradation. PEI has a variety of amine groups and therefore it has a high buffer capacity since amine groups can be protonated (Pandey and Sawant, 2016). The primary amine in PEI is responsible for the binding and the condensation of the gene, whereas the secondary and tertiary amines in PEI are important for the endosomal escape by proton sponge effect (Thomas and Klibanov, 2002). There are other advantages of PEI such as the availability of different molecular weights and its high water solubility (Pandey and Sawant, 2016).

However, PEI can be toxic when used at high concentration (Boussif et al., 1995). The toxicity of PEI is correlated with the molecular weight in which the lower molecular weight of PEI is less toxic than the higher molecular weight (Fischer et al., 1999). However, the higher molecular weight of PEI is more effective in transfection than the lower molecular weight (Parhiz et al., 2013).

1.7.2.2.4 Linear Polyamidoamine (PAA)

Linear PAA is a polymer that resembles a peptide chain in which each repeating unit has an amide group and a tertiary amine group (Richardson *et al.*, 2001).

The synthesis of the PAA can be performed by mixing primary or secondary amines with acrylamide derivatives as shown in Figure 1.7.

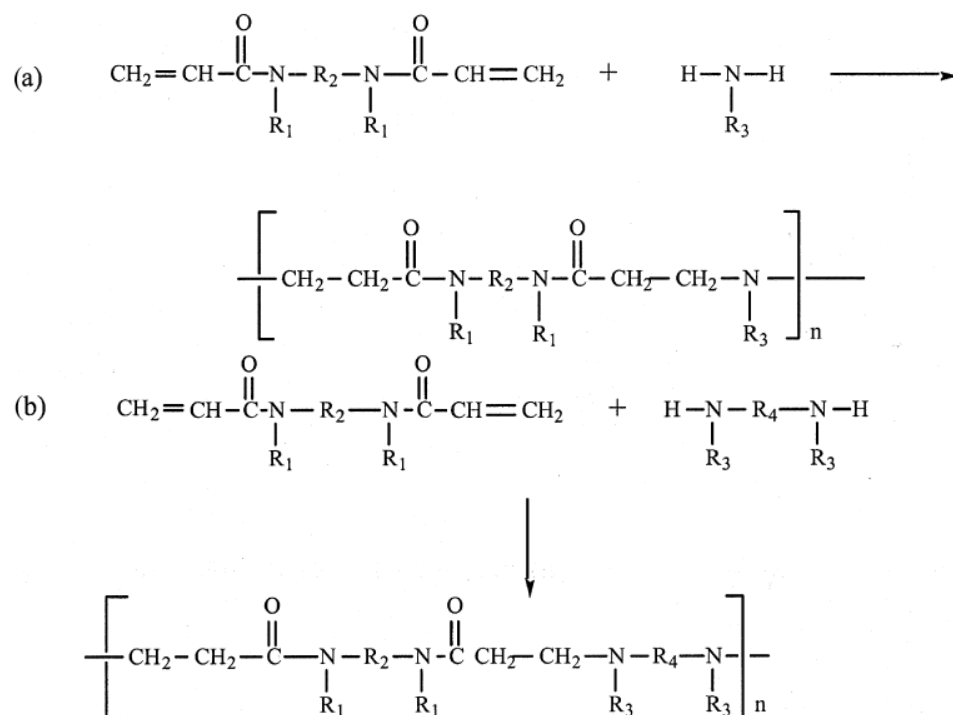


Figure 1.7 Preparation of linear PAA. a) Preparation of linear PAA from primary amine. B) Preparation of linear PAA from secondary amine (Ferruti *et al.*, 2002)

One of the main advantages of PAA is that many polymer variants can be formed by using monomers that have different functional groups. Furthermore, chemical functional groups can be added to the repeating unit such as sulphide or carboxylic group by the choice of appropriate monomers (Ferruti *et al.*, 2002). Therefore, several formulations of PAA with different characteristics can be produced. For instance, PAA can be synthesised to be highly cationic in order to enhance the condensation and the cellular uptake of the gene but it will be

more toxic. In addition, PAA can be prepared to have disulphide bonds in order to enhance the stability and intracellular delivery since disulphide bond can be easily cleaved in the reducible environment in the cytosol which can lead therefore to the release of the gene in the cell (Piest *et al.*, 2013). Poly (ethylene glycol) (PEG) can be introduced to the PAA in order to produce PAA copolymer with terminal PEG (Garnett *et al.*, 2009). This terminal PEG can decrease the phagocytosis of the PAA polyplex when it is administrated parentally since it can form a hydrophilic layer that decrease the adsorption between the serum contents and the polyplex (Rackstraw *et al.*, 2002).

Amphoteric PAA is PAA that contains acidic functional group such as carboxylic acid (Ferruti, 2013). The basic amphoteric PAA is commonly more biocompatible than the PAA that has the same net average positive charge but without an acid functional group (Ferruti, 2013).

1.7.2.4 Polyester

Polyester polymer is a polymer that contains ester backbone in the monomer structure. Polyester polymer known to be highly biodegradable polymer because ester bonds can be hydrolysed (Bilensoy, 2010, Sunshine et al., 2012). One of the example of polyester polymer is a Poly(β -amino esters) (PbAEs). PbAEs can be synthesised by adding N,N'-dimethylethylenediamine, piperazine, and 4,4'-trimethylenedipiperidine to 1,4-butanediol diacrylate (Lynn and Langer, 2000).

It was reported that gene transfection for hard to transfect cells such as human macrovascular cells and lymphoma/leukemia cells was successfully accomplished by PbAEs (Shmueli *et al.*, 2012, Zhao *et al.*, 2012). PbAEs efficiency in gene transfection of retinal pigment epithelial (ARPE-19) cells was evaluated and the result shown that the transfection efficiency was significantly more than Lipofectamine 2000 and X-tremeGENE HP commercial reagents (Sunshine *et al.*, 2012).

1.8 Clinical trials of RNAi

There have been many clinical trials that were performed in phase I, II, and III as shown from table 1.1. For example, cyclodextrin polymer was used as an siRNA delivery system for clinical trial of cancer research. Adamantane-PEG (AD-PEG) and adamantane-PEG-transferrin (AD-PEG-Tf) were added to a cyclodextrin polymer in order to form complexes through binding of the adamantane to the cyclodextrin to enhance its *in vivo* properties (Kanasty *et al.*, 2013a). The siRNA was targeted to oncogenic EWS-FLI1 gene in order to treat Ewing's sarcoma in animal model (Kanasty *et al.*, 2013a). This cyclodextrin/siRNA complex was administered intravenously and shows an antiproliferative effect (Kanasty *et al.*, 2013a). In addition, many liposome/siRNA formulations have been used in clinical trials for treatment of cancer, hypercholesterolemia and transthyretin-mediated amyloidosis (Kanasty *et al.*, 2013a). Stabilized nucleic acid-lipid particles (SNALP) were used for clinical trials for siRNA delivery mainly by companies such as Alnylam

Pharmaceutical and Tekmira Pharmaceutical (Scherman et al., 2017). SNALP consists of 1,2-dilinoleyloxy-N,N-diethyl-3-aminopropane (DLinDMA) cationic lipid and 1,2-distearyl-sn-glycero-3-phosphocholine (DSPC) (Scherman et al., 2017). A PEGylated lipid was added to the SNALP in order to enhance its stability in the blood circulation (Scherman et al., 2017).

It can be seen from table 1.1 and examples above that many of those methods are complicated and expensive. In addition, some of those methods used only naked chemically modified siRNA which cannot be administered systemically since degradation of the siRNA can occur by nuclease.

Table 1.1 Example of RNAi clinical trials (Bobbin and Rossi, 2016)

Disease	Therapeutic Name	Target	Delivery	Clinical status	Company	NCT number
Hypercholesterolemia	ALN-PCSSC	PCSK9	SC injection of LNP	Phase I	Alnylam	NCT02314442
Paroxysmal nocturnal hemoglobinuria	ALN-CC5	C5	SC injection of ESC-GalNAc	Phase I/II	Alnylam	NCT02352493
HCC; neural endocrine cancer	TKMPLK1/ TKM-080301	PLK1	IV infusion of LNP	Phase I/II	Arbutus	NCT01262235 NCT02191878
Advanced/metastatic cancer	pbi-shRNA STMN	STMN	Intratumoral injection of LNP	Phase I	Gradalis	NCT01505153
HCC; solid tumors; myeloma; lymphoma	DCR-MYC	MYC	IV infusion of LNP	Phase I Phase I/II	Dicerna	NCT02110563 NCT02314052
Hepatitis C virus	TT-034	Three conserved regions of HCV	IV infusion of AAV	Phase I/II	Benitec	NCT01899092 NCT02315638

Dry eye	SYL1001	TRPV1	Ocular-topical of naked siRNA	Phase I/II	Sylentis	NCT01776658
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AAV: adeno-associated virus; ESC:enhanced stabilization chemistry; GalNAc: *N*-acetylgalactosamine; LNP; lipid nanoparticl

1.9 Project Aims and Objectives

To evaluate new cationic polymers which may form the basis for siRNA delivery systems which may be appropriate for anti-allergic therapies. The systems will be evaluated after validation of suitable siRNA reporter systems based on the RBL-2H3 reporter cell lines which are NPY-mRFP and NFAT-DsRed reporter cell lines.

One of the linear PAAs that is used in this project is AGMA1. AGMA1 is an amphoteric PAA which has a carboxylic group that can be protonated at low pH and amine group that can be protonated at high pH (Ferruti *et al.*, 2007). It can be produced by adding (4-aminobutyl) guanidine (agmatine) to BAC as shown in Figure 1.8. It was reported that AGMA1 with a 20 kDa molecular weight (AGMA20) has a low cytotoxicity for *in vivo* applications since it did not show cytotoxicity for a dose up to 7 mg/ml (Cavalli *et al.*, 2010). AGMA1 also mimics the tripeptide Arg-Gly-Asp (RGD) motif that can bind to different integrin receptor family (Parkhouse *et al.*, 2008). Therefore, AGMA1 can bind to integrin receptors which can enhance the endocytosis of AGMA1 to the cell.

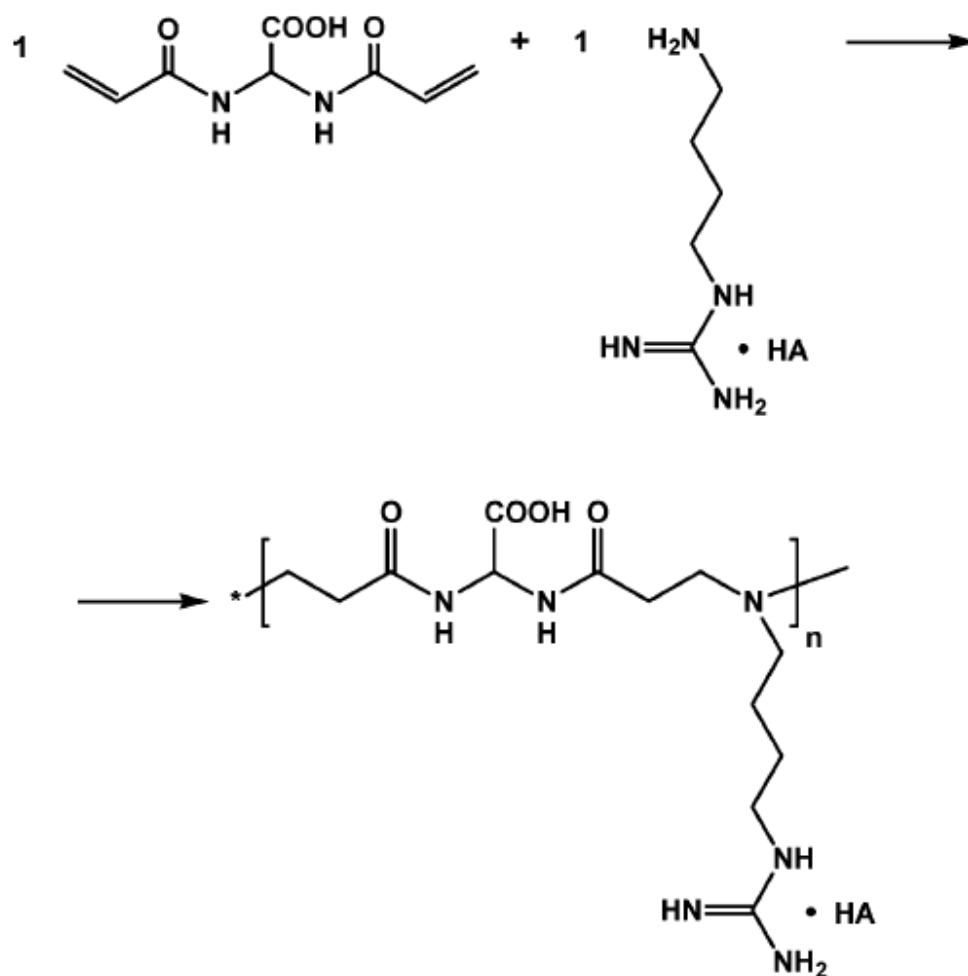


Figure 1. 8 Synthesis of AGMA1 (Ferruti et al., 2007)

Poly (glycerol adipate) (PGA) polymer will also be evaluated which is a polyester cationic polymer carrier for siRNA delivery. PGA can be synthesised by dissolving divinyl adipate (DVA) with glycerol in anhydrous Tetrahydrofuran (THF) in the presence of Novozym 435 as shown in Figure 1.9 (Taresco et al., 2016). The presence of the hydroxyl group in the PGA monomer has an advantage that it can be covalently modified by reaction with the carboxyl group of any of several positively charged amino acids such as lysine, arginine, and histidine. The presence of the positively charged amino acids will provide

an additional character to the biodegradability of PGA to be a cationic polyester. Therefore, two novel synthesised PGA derived polymers will be evaluated which are PGA-50% lysine (PGA-50% Lys), in which 50% of the pendant hydroxyl groups of the polymer are covalently bound to a lysine moiety. The second polymer was PGA-20% histidine (PGA-20% His), in which 20% of the pendant hydroxyl groups of the polymer were covalently bound to histidine. The synthesis requires the reactive amine groups of the amino acids to be protected during synthesis and the protective groups removed before using the polymers. The repeating unit structure of PGA-50% Lys and PGA-20% His is shown in Figure 1.10.

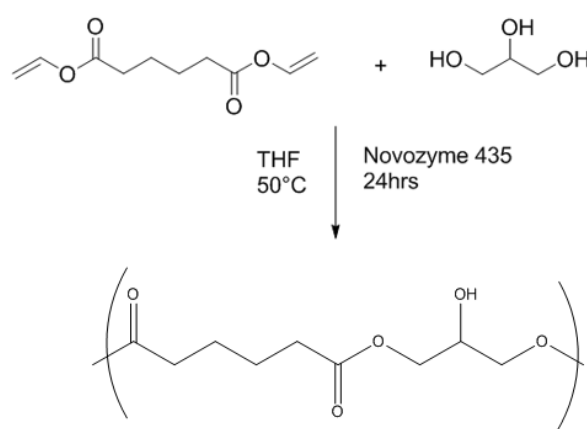
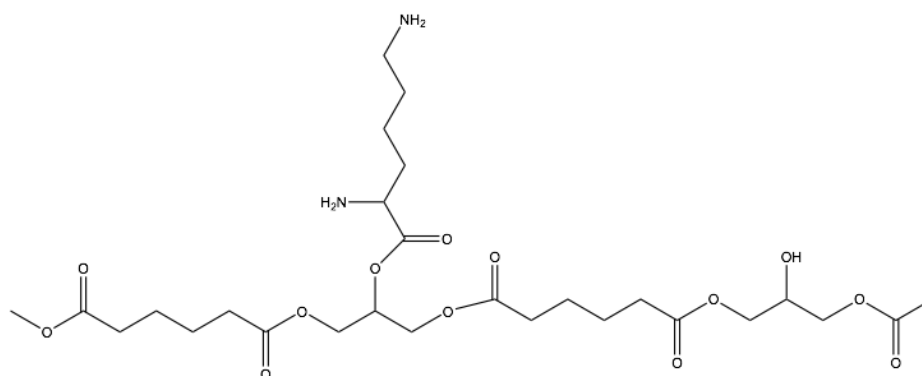


Figure 1. 9 Synthesis of PGA (Taresco *et al.*, 2016)

A)



B)

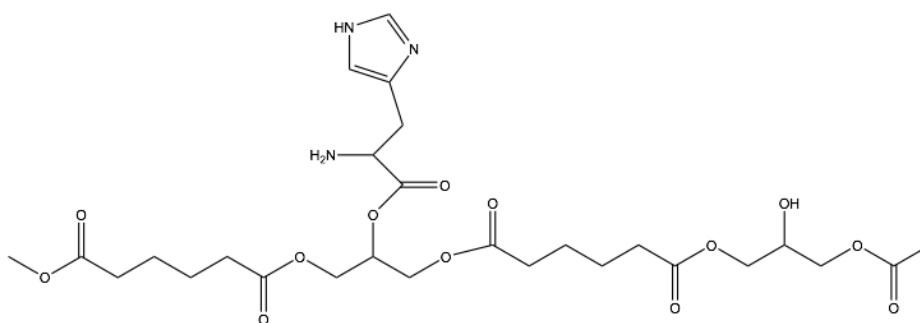


Figure 1. 10 Structure of A) PGA-50% Lys B) PGA-20% His

AGMA29 and PGA derived polymers have advantages that they can be produced easily and it is cheap to produce them. Also, it was reported that AGMA29/DNA polyplex can induce gene expression in liver when it is administered intravenously (Cavalli *et al.*, 2010).

The objectives of this project are the following:

1- Formulation a complex of polymer–siRNA polyplex of AGMA1 and PGA derived polymers in an optimal condition will be determined. The size of the polyplex needs to be optimised in order to determine the most stable form of polyplex that can be used to transfect the targeted cell.

2- The conditions of NPY–mRFP and NFAT-DsRed reporter cell lines will be optimised in terms of the highest release of RFP in NPY–mRFP reporter and the highest expression of DsRed in the NFAT-DsRed reporter.

3- The transfection efficiency of polymer–Syk siRNA polyplex in the knockdown of Syk mRNA and Syk protein will be evaluated. In addition, the optimised condition of NPY–mRFP and NFAT-DsRed reporter cell assays as a quantitative method in terms of evaluating the change of release of RFP in NPY–mRFP reporter cell and change of expression of DsRed in the NFAT-DsRed after the knockdown of Syk by polymer–Syk siRNA polyplex will be assessed.

CHAPTER 2: MATERIALS AND METHODS

2.1 Materials

Most of the chemicals and solutions used were purchased from Sigma unless specified in Table 2.1. The AGMA1 polymer which has been designated AGMA29 for our studies were designed by our collaborators Prof Paolo Ferruti and Elisabetta Ranucci and synthesised by Dr Fabio Fenili of the Department of Organic and Industrial Chemistry, University of Milan, Italy (Ferruti et al., 2007). The AGMA1 that is used is in lyophilised powder form and has a weight average molecular weight (M_{wt}) of 28700 Da and a number average molecular weight (M_n) of 20800.

PGA and PGA derived polymers which are PGA-50% lysine (PGA-50% Lys) and PGA-20% histidine (PGA-20% His) were synthesised by Dr. Vincenzo Taresco in our department (Taresco et al., 2016).

Table 2. 1 list of materials and corresponding supplier

Product	Company
DNP-BSA	Life Technology, USA
iBind TM Flex 100X additive	Thermo-scientific, USA

iBind™ Flex 5X buffer	Thermo-scientific, USA
iBind™ Flex card	Thermo-scientific, USA
iScript Advanced Reaction Mix	Bio-Rad, USA
iScript Advanced Reverse Transcriptase	Bio-Rad, USA
Mini-PROTEAN®	Bio-Rad, USA
nuclease-free water	Bio-Rad, USA
Pierce's BCA™ Protein Assay Kit	Thermo-scientific, USA
Pierce ECL Western Blotting Substrate	Thermo-scientific, USA
Pierce GAPDH primary antibody	Thermo-scientific, USA
Purified anti-Syk Antibody (SYK-01 clone)	BioLegend, UK
Re-blot Plus stripping solution	Millipore, USA
RIPA lysis buffer	VWR Life Science AMRESCO
SsoAdvanced Universal SYBR Green Supermix	Bio-Rad, USA
SingleShot SYBR Green Syk primers	Bio-Rad, USA
SingleShot SYBR Green Ggt1 primers	VWR Life Science AMRESCO
Trifluoroacetic acid ≥99% Technical (TFA)	Bio-Rad, USA
TGX™ precast polyacrylamide gel	Bio-Rad, USA
Trans-Blot® Turbo™ Transfer Pack	

The amplicon length of Syk primer is 81 bp, the targeted splice variant is ENSRNOT00000016942.6 and the primer efficiency is 97 %. The amplicon length of Ggt1 primer is 116 bp, the targeted splice variant is ENSRNOT00000074533 and the primer efficiency is 97 %. GGT1 is the gene that encodes type I gamma-glutamyltransferase which catalyses the transfer of the glutamyl moiety of glutathione. It was selected for RT-qPCR instead of GAPDH primer because there are 11 transcripts (splice variants) that produce GAPDH so GAPDH gene is not suitable to be used as a reference gene. Also the Ggt1 primer was validated previously in Franco Falcone Lab as a reference gene.

Four Syk 21mer siRNA sequences were selected according to the study of Sanderson et al. (2010) together with a set of scrambled sequences as tabulated below (Table 2.2). They were purchased from Sigma-Aldrich in powder form and re-suspended in RNase-free water to a concentration of $1 \mu\text{g} \cdot \mu\text{l}^{-1}$.

Table 2.2 siRNA sequences and their targeted locations in rat Syk mRNA (RefSeq_dna: NM_012758)

siRNA	Sequence	bp location in rat Syk mRNA	MWt (g/mol)
G	CCUCAUCAGGGAAUAUGUGdTdT	697-715	13304
H	GCCUGCUGCACGAAGGGAAAdTdT	936-954	13349
I	GUCAUGCAGCAGCUGGACAdTdT	1570-1588	13334
J	GUGCUGAUGAAAACUACUAdTdT	1862-1880	13274
Scrambled siRNA			
G	GUCAGGCUAUCGUAUGACAdTdT		13304

H	AGACGGAUUCGCCGAACGGdTdT	13349
I	GCGCAUGCAAUCGGUAGACdTdT	13334
J	GAGUCCGGUAUAACAAUAdTdT	13274

2.2 Methods

2.2.1 Physicochemical characterization

2.2.1.1 Deprotection of PGA polymers from t-BOC

PGA-50% Lys or PGA-20% His (200 mg) was dissolved in DCM (18 ml), and then TFA (2 ml) was added. The solution was stirred at room temperature using a magnetic follower for 5 h. The solvent was then evaporated in a rotary evaporator. The resultant powder was dissolved in tetrahydrofuran (THF) (2 ml). The solution was added gradually to cold petroleum ether (50 ml) and the mixture was centrifuged at 4000 g for 15 min at 5°C. The supernatant was discarded, and the pellet was stored at 5°C.

2.2.1.2 Determination of the molecular weight of PGA

The molecular weight of deprotected PGA was determined using the PL-GPC 50 Plus Polymer Laboratories system (Varian, USA) in which two mixed bed (D) columns at 50°C were used. PGA (5 mg) was dissolved in DMF containing LiBr (0.1%, 1 ml), which was used as the mobile phase, and the flow rate was 1 mL min⁻¹. Polymethylmethacrylate calibration kits (Agilent Technologies, USA) were used, in which the Mn range was from 505 to 1810000 g mol⁻¹.

2.2.1.3 ¹H-NMR analysis of PGA

Deprotected PGA-50% Lys or PGA-20% His (5 mg) was dissolved in acetone-d₆ (600 µl) (Sigma-Aldrich, USA), and ¹H NMR was analysed using a Bruker 400 MHz spectrometer, and the chemical shift values are expressed in ppm.

2.2.1.4 Titration of PGA-50% Lys

Deprotected PGA-50% Lys (18 mg) was dissolved in 0.1 M sodium chloride solution (18 ml) then the pH was adjusted by HCl (0.1 M) to be 2.33. Thereafter, the solution was pH-metrically titrated forward by NaOH (0.1 M, 20 µL) and the pH was recorded after each addition of NaOH until the pH reached 11.3.

2.2.1.5 Gel retardation assay

2.2.1.5.1 Measurement of polyplex formation in phosphate buffer

Tris-acetate-EDTA buffer (TAE) (1X) was prepared by diluting TAE (20 ml, 50X) in deionised water (980 ml). Agarose powder (1.2% w/v, 1.2 g) was dissolved in TAE (1X, 100 ml). The resultant solution was heated in a microwave to dissolve the agarose powder. The molten gel was mixed with ethidium bromide (1 µg·ml⁻¹). Monosodium phosphate (26.5 mg) was mixed

with disodium phosphate (82.5 mg) and dissolved in water (100 ml) to obtain phosphate buffer (5 mM) that has a pH of 7. Glycerol (30% v/v) was mixed with bromophenol blue (0.1% w/v) in deionised water to obtain 6X loading buffer dye. Polyplexes (20 µl) containing 1, 2, 3, 4, 5 or 8 repeating units (RU) of polymer per nucleotide of siRNA were prepared by adding different amounts of polymers to a fixed amount of siRNA (1 µg) in the prepared phosphate buffer solution (pH 7). The amount of polymer required to prepare the 1-, 2-, 3-, 4-, 5- and 8-RU/Nt polyplexes was calculated using equation 2.1. After 30 min incubation, the samples (20 µl) were mixed with 6X loading buffer (2.5 µl), and the mixture (22.5 µl) was loaded onto a prepared agarose gel (1.2% w/v, 100 ml) containing ethidium bromide (1 µg/ml). This gel was then run at 80V for 45–60 min in TAE buffer. The gel was then viewed using a UV transilluminator GeneGenius (Syngene) and analysed using the SynGene software. The gel was then stained with Coomassie Blue staining solution (0.1 g/L) for 1 h and destained overnight in destaining solution. The staining solution was prepared by mixing Brilliant blue R250 (0.1% w/v) with methanol (50% v/v) and glacial acetic acid (10% v/v) in deionised water. The destaining solution was prepared by mixing methanol (10% v/v) with glacial acetic acid (10% v/v) in deionised water. An image of the gel was taken under white light with the same imaging software.

$$\text{Amount of polymer} = \frac{\text{Ratio of RU:Nt} \times \text{Amount of siRNA} \times \text{MW of RU of polymer}}{\text{Average MW of siRNA nucleotide}}$$

Equation 2.1. Formula for calculating the amount of polymer used, where Mw = molecular weight and RU = repeating units.

2.2.1.5.2 Measurement of polyplex formation at different pH values

Polyplexes (20 μ l) containing 1, 2, 3, 4, 5 or 8 RU of polymer per nucleotide of siRNA were prepared by adding different amounts of polymer to a fixed amount of siRNA (1 μ g) in RNase-free water. This experiment was performed at different pH values (pH 8, 7, 6, 5 and 4), and the pH was adjusted using NaOH (1 M) and HCl (1 M). Thereafter, the gel retardation assay and staining and destaining of the gel were performed as described in section 2.2.1.5.1

2.2.1.5.3 Measurement of polyplex formation at different salt concentrations

Polyplexes were prepared by adding a selected amount of polymer to a fixed amount of siRNA dissolved at different NaCl concentrations (0, 10, 20, 30, 50, 80, 110 and 150 mM). Thereafter, the gel retardation assay and staining and destaining of the gel were performed as described in section 2.2.1.5.1

2.2.1.6 Dynamic light scattering (DLS) analysis

Polyplex solutions (80 μ l) were prepared as described in sections 2.2.1.5.1, 2.2.1.5.2, or 2.2.1.5.3. The polyplex sizes were then determined by light scattering using a Viskotek DLS 802 at 20°C with the scattering angle was fixed at 90°. The laser power was adjusted to provide an intensity of 300–1000 kcps. The size was determined from an average of 8 measurements.

2.2.2 Biological characterisation

2.2.2.1 Genetically modified cell lines

The cell line RBL-2H3 is stably transfected with a neuropeptide Y- monomeric red fluorescent protein (NPY-mRFP) fusion construct that targets the granules (Falcone et al., 2017).

The cell line RBL-2H3 is stably transfected with the human FcεRI receptor α chain and NFAT-DsRed genes (Wang et al., 2014a). The reporter cell lines were a kind gift from Franco Falcone Lab.

NPY is a peptide that consists of 36 amino acid which was discovered by Tatemoto et al. (1982) which is localised in secretory granules. NPY-mRFP protein can be localised and being stable in the granules of RBL-2H3 cell line (Azouz et al., 2012). Therefore, after the degranulation of NPY-mRFP reporter cell line after the sensitisation of the cells by IgE followed by stimulation of the cell with anti-IgE, the exocytosis of NPY-mRFP occurs which can be measured.

NFAT-DsRed reporter cell line was designed by transfection of the NFAT-DsRed-Express2 plasmid which contains NFAT promoter sequence with a DsRed reporter gene to RBL-703/21 cell line (Wang et al., 2014a). The RBL-703/21 cell line is RBL-2H3 that is transfected with the gene encoding the human FcεRI α (Danish, 2014). Therefore, this NFAT-DsRed reporter cell line can be sensitised with either human or mouse IgE. Also, the NFAT-DsRed

protein can be synthesised after the sensitisation of the NFAT-DsRed reporter cell line by IgE followed by stimulation of the cell with anti-IgE. Figure 2.1 illustrates the mechanism of action of NPY-mRFP and NFAT-DsRed reporter cell lines.

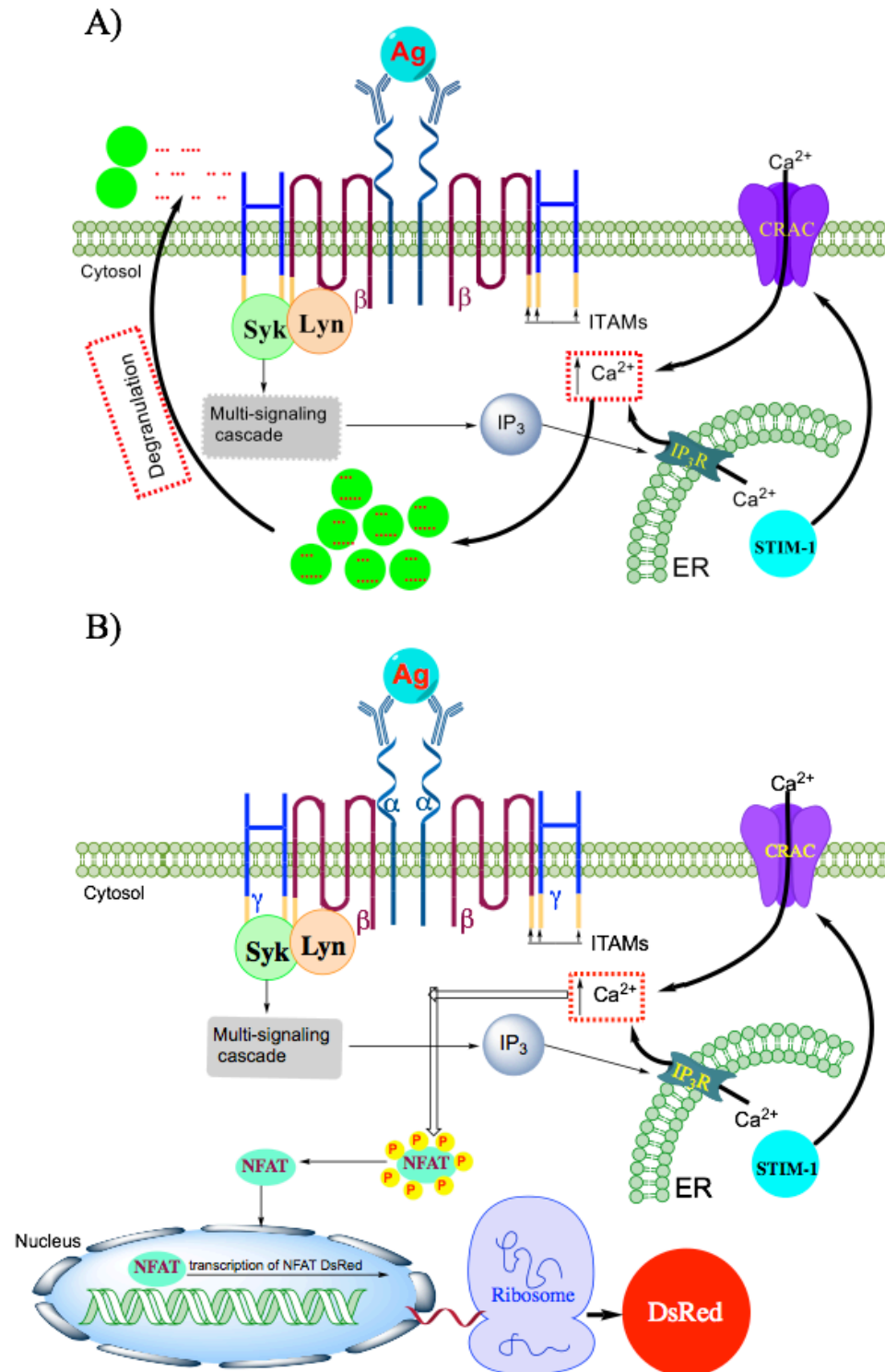


Figure 2.1 The mechanism of action of reporter cell lines after the crosslinking of IgE by allergen. A) NPY-mRFP reporter cell line; B) NFAT-DsRed reporter cell line. Ag: Antigen/Allergen; CRAC: calcium release-activated calcium channel; ER: endoplasmic reticulum; IP₃: inositol-1,4,5-trisphosphate; IP₃R:

IP3 receptor; STIM-1: Stromal interaction molecule 1. For NPY-mRFP cell line, green circles are granules and red color dots are red fluorescent protein.

2.2.2.2 Cell passaging of the NPY-mRFP and NFAT-DsRed reporter cell lines

The cell culture medium was prepared by adding heat-inactivated foetal bovine serum (FBS) (10% v/v), L-glutamine (2 mM), penicillin (100 U·ml⁻¹) and streptomycin (100 µg·ml⁻¹) to Eagle's minimum essential medium (MEM).

Cells from both cell lines were grown in a flask with a filter cap that allows for gas exchange. The passaging was performed when the cells reached confluence. First, the supernatant was removed and the flask was washed twice with Dulbecco's phosphate-buffered saline (DPBS) (10 ml) without calcium or magnesium. Then, Trypsin-EDTA solution (1X, 2 ml) was added to the flask to induce detachment of the cells, and the flask was incubated at 37°C in a 5% CO₂ humidified atmosphere for 10 min. Thereafter, MEM (8 ml) was mixed with the Trypsin-EDTA solution in the flask, and 9 ml of the mixture was discarded and 1 ml was retained in the flask. Finally, MEM (13 ml) was added to the flask and the flask was incubated at 37°C in a 5% CO₂ humidified atmosphere. For the NPY-mRFP reporter cell line, G418 sulphate (Fisher, UK) (1 µg/ml) was added to the media to maintain the transfected NPY-mRFP gene. For the NFAT-DsRed reporter cell line, G418 sulphate (1 µg/ml) and blasticidin S (Invitrogen, USA) (20 µg/mL) were added to the media to maintain the human FcεRI receptor α chain and NFAT-DsRed genes, respectively.

2.2.2.3 Effect of calcium on the degranulation of NPY-mRFP and Red fluorescent protein induction in NFAT-DsRed.

2.2.2.3.1 Effect of calcium on degranulation of the cells

NPY-mRFP reporter cells were seeded in transparent 96-well plates (4.5×10^4 cells per well) and incubated overnight at 37°C in a 5% CO₂ incubator. The medium was replaced with monoclonal anti-dinitrophenyl (anti-DNP) mouse IgE diluted in MEM ($1 \mu\text{g}\cdot\text{ml}^{-1}$), which was incubated at 37°C in a humidified 5% CO₂ incubator for 12–18 h. The cells were then washed with DPBS, and the cells were stimulated with DNP-BSA diluted in MEM with FBS, MEM without FBS, or DPBS ($1 \mu\text{g}\cdot\text{ml}^{-1}$ final concentration of DNP-BSA for each medium). Different amounts of calcium were mixed into the medium and it was incubated for 60–90 min at 37°C in a 5% CO₂ incubator. Thereafter, the supernatant was transferred to a black 96-well plate, and the RFU was measured with a SPARK 10M Tecan plate reader (Tecan Austria GmbH, Austria) at an excitation wavelength of 530 nm and an emission wavelength of 590 nm. For the negative control, different amounts of calcium diluted in MEM with FBS, MEM without FBS, or DPBS were used without mixing with DNP-BSA.

2.2.2.3.2 Effect of calcium on the production of red fluorescence protein in the crosslinking of human IgE receptors

Triton X-100 (0.5 ml) was dissolved in Dulbecco's phosphate-buffered saline (DPBS) (49.5 ml) to obtain a 1% (v/v) Triton solution. NFAT-DsRed reporter cells were seeded in a transparent 96-well plate (4.5×10^4 cells per well) and incubated overnight at 37°C in a humidified 5% CO₂ incubator. The medium was then replaced with human serum in MEM (2% v/v), for which the serum samples were obtained from patients who were allergic to grass, and incubated at 37°C in a humidified 5% CO₂ incubator for 12–18 h. The medium was replaced with goat anti-human IgE (ε chain-specific) antibody (Sigma) that was diluted in MEM with FBS ($1 \mu\text{g} \cdot \text{ml}^{-1}$), mixed with different amounts of calcium and incubated at 37°C in a 5% CO₂ incubator for 24 h. Thereafter, the medium was replaced with Triton lysis solution (1% v/v). The lysed cells were transferred to black 96-well plates, and the RFU was measured with a SPARK 10M Tecan plate reader at an excitation wavelength of 530 nm and an emission wavelength of 590 nm.

2.2.2.3.3 Effect of calcium on the production of red fluorescent protein in the crosslinking of rat IgE receptors

This experiment is similar to the experiment in section 2.2.2.3.2, except that anti-DNP mouse IgE diluted in MEM ($1 \mu\text{g} \cdot \text{ml}^{-1}$) was used instead of human serum, and DNP-BSA diluted in MEM with FBS ($1 \mu\text{g} \cdot \text{ml}^{-1}$) was used instead of the anti-human IgE (ε chain-specific) antibody.

2.2.2.4 Inhibition of the degranulation of NPY-mRFP

Cells of the NPY-mRFP reporter cell line were seeded in a transparent 96-well plate (1.5×10^4 cells per well, Costar) and incubated overnight at 37°C in a 5% CO₂ incubator. The cell medium was replaced with polymer/siRNA complexes of different concentrations and ratios that were preincubated in deionised water for 30 min and then mixed with serum-free MEM. The cells were then incubated at 37°C in a 5% CO₂ incubator for 4 h and washed with PBS. Next, MEM was added and the cells were incubated at 37°C in a humidified 5% CO₂ incubator overnight. The medium was replaced with mouse IgE diluted in MEM ($1 \text{ mg} \cdot \text{ml}^{-1}$) and incubated at 37°C in a 5% CO₂ incubator for 12–18 h. The cells were washed with DPBS then stimulated with medium containing DNP-BSA (21 mole DNP/mole BSA) (Life Technology, USA) that was dissolved in MEM ($1 \text{ } \mu\text{g} \cdot \text{ml}^{-1}$) for 60–90 min at 37°C in a 5% CO₂ incubator. Thereafter, the supernatant was transferred to a black 96-well plate, and the relative amount of fluorescence protein (RFU) was measured using a SPARK 10M Tecan plate reader (Tecan Austria GmbH, Austria) at an excitation wavelength of 530 nm and an emission wavelength of 590 nm.

2.2.2.5 Inhibition of Red fluorescent protein induction in NFAT-DsRed

The NFAT-DsRed reporter cell line was seeded, incubated and transfected with polymer/siRNA complexes at different ratios, as shown in section 2.2.2.4. The medium was then replaced with mouse IgE diluted in MEM ($1 \text{ mg} \cdot \text{ml}^{-1}$) and incubated at 37°C in a 5% CO₂ incubator for 12–18 h. The cells were then washed with DPBS then stimulated with MEM containing DNP-BSA ($1 \text{ } \mu\text{g} \cdot \text{ml}^{-1}$)

¹) for 24 h. Thereafter, the medium was replaced with Triton lysis solution (1% v/v), and the lysed cells were transferred to a black 96-well plate, where the RFU was measured with a SPARK 10M Tecan plate reader at an excitation wavelength of 530 nm and an emission wavelength of 590 nm.

2.2.2.6 Resazurin viability assay

Resazurin solution was prepared by dissolving Resazurin sodium salt (65 mg) in phenol-free Hanks' balanced salt solution (50 ml). The solution was then filtered through a Minisart high-flow 0.2-µm filter (Sartorius, Germany) and stored at 5°C.

For the NPY-mRFP reporter cell, the resazurin assay was performed on the same cells after the degranulation assay in section 2.2.2.4. After the degranulation assay, resazurin solution that was diluted in MEM (10% v/v) and incubated for 60 min at 37°C in a 5% CO₂ incubator. Thereafter, the supernatant was transferred to a black 96-well plate, and the RFU was measured with a SPARK 10M Tecan plate reader at 540 nm (excitation wavelength) and 590 nm (emission wavelength).

The NFAT-DsRed reporter cell, the resazurin assay was performed in separate wells to those used for red fluorescent protein induction assay but with the same treatment in section 2.2.2.5 (the reasons for using separate wells will be explained in section 4.3.3). After the red fluorescent protein induction assay without the lysis step, resazurin solution was diluted in MEM (10% v/v) and incubated for 60 min at 37°C in a 5% CO₂ incubator. Thereafter, the supernatant

was transferred to a black 96-well plate, and the RFU was measured with a SPARK 10M Tecan plate reader at 540 nm (excitation wavelength) and 590 nm (emission wavelength).

2.2.2.7 Analysis of Syk protein by Western blotting

2.2.2.7.1 Preparation of cell lysate

The NPY-mRFP reporter cell line was seeded in a transparent 96-well plate, incubated, transfected with polymer/siRNA complexes at different ratios as shown in section 2.2.2.4. Next, the cells were incubated for 2 or 3 days at 37°C in a 5% CO₂ incubator. Thereafter, the medium was removed and the cells were washed twice with cold DPBS (100 µl/well). Then, cold RIPA lysis buffer with protease inhibitor cocktails (1X final concentration) was added (20 µl/well), and the cells were incubated on ice for 15 min, after which the lysate was transferred to a cold 1.5 ml microcentrifuge tube. The lysate was centrifuged at 14,000 g for 15 min at 4°C. The supernatant was transferred to a new 1.5 ml microcentrifuge tube and the pellet was discarded. The lysate was stored at -20°C.

2.2.2.7.2 Quantification of the lysed protein

The concentration of protein in the cell lysate was measured using the BCA assay. BCA working reagent was prepared by mixing BCA Reagent A (19.6 ml) with BCA Reagent B (0.4 ml). Serial dilutions of BSA were prepared, with the

concentrations ranging from $2 \text{ mg}\cdot\text{ml}^{-1}$ to $0 \text{ mg}\cdot\text{ml}^{-1}$, and transferred with the cell lysate to a transparent 96-well plate (Nunc™, USA) (25 μl from each dilution). Thereafter, the prepared BCA working reagent was added to each well (200 μl), and the plate was gently shaken and then incubated for 30 min at 37°C . The absorbance at 562 nm was measured with the SPARK 10M Tecan plate reader.

2.2.2.7.3 SDS-PAGE

Tris-Cl (100 mM) was dissolved in deionised water with glycerol (20% v/v), sodium dodecyl sulfate (SDS) (4% w/v), bromophenol blue (0.2% w/v), and dithiothreitol (100 mM) to obtain a 2X reducing sample buffer (RSB). SDS (10 g) was dissolved in deionised water (10 L), and then glycine (144 g) and Tris (30 g) were added and dissolved to obtain a 1X SDS buffer. The cell lysate was mixed with an equal volume of 2X RSB and heated at 100°C for 5 min. The sample was then loaded in a precast Tris-Glycine 4–20% TGX mini gel and run in a Mini-PROTEAN™ Tetra Cell at 180V. Thereafter, the gels were blotted against a 0.2- μm nitrocellulose membrane using a Trans-Blot® Turbo™ Blotting System (Bio-Rad, USA) that was run at 25V for 3 min.

2.2.2.7.4 Western blotting

Western blotting onto a nitrocellulose membrane was performed with the Western blot iBind™ Flex system (Invitrogen, USA). The Western blot iBind™ Flex system consists of the iBind™ Flex Western Device, iBind™ Flex Mini

Insert, iBind™ Flex card and the prepared iBind™ Flex solution. The Western blot analysis was performed according to the manufacturer's protocol. The blotted nitrocellulose membrane was incubated for 10 min with the prepared iBind™ Flex solution (10 ml). iBind™ Flex 5X buffer (10 ml) was mixed with iBind™ Flex 100X additive (500 µl) and water (39.5 ml) to obtain a 1X iBind™ Flex solution. The iBind™ Flex card was loaded onto the stage of the iBind™ Flex Western Device, and the membrane of the card was wetted with the prepared iBind™ Flex solution (5 ml). The blotted nitrocellulose membrane was then loaded above the card and wetted with the prepared iBind™ Flex solution (1 ml), after which the lid of the device was closed. There are four well inserts in the iBind™ Flex Mini Insert. The primary anti-Syk antibody SYK-01 (Biolegend, USA) was diluted in 2 ml of iBind™ Flex solution ($2\text{ }\mu\text{g}\cdot\text{ml}^{-1}$) and loaded in the first well insert. iBind™ Flex solution was loaded in the second well insert (2 ml). Anti-mouse IgG antibody (Sigma-Aldrich, USA) was diluted in 2 ml of iBind™ Flex solution (1:1000) and loaded in the third well insert. The fourth well insert was filled with iBind™ Flex solution (6 ml). The blotted nitrocellulose membrane was incubated for 3 h, and then an equal volume of detection reagent 1 (peroxide solution) and detection reagent 2 (luminol enhancer) (0.7 ml each) (ThermoFisher Scientific, USA) was mixed and added to the membrane. Thereafter, chemiluminescent images of the membrane in the high binning mode were taken with a FUJI-LAS 4000 charge-coupled device camera (Fujifilm, Japan). Following this, stripping of the nitrocellulose membrane was performed by adding diluted Re-blot Plus stripping solution (Millipore, USA) (15 ml,) to a plastic tray in which the membrane was submerged and incubated at room temperature for 15 min in a shaking incubator.

For probing of the GAPDH reference protein, the same blotting method was used except that diluted primary GAPDH loading control antibody GA1R (ThermoFisher Scientific, Canada) in the iBind™ solution (1:1000, 2 ml) was loaded in the first well insert instead of the primary anti-Syk antibody SYK-01. Thereafter, the band intensity of Syk and GAPDH were analysed and the % of Syk was calculated from the following equation.

$$\% \text{ of Syk} = \frac{\left(\frac{\text{Band intensity of Syk siRNA H}}{\text{Band intensity of GAPDH siRNA H}} \right)}{\left(\frac{\text{Band intensity of Syk Scrambled siRNA H}}{\text{Band intensity of GAPDH Scrambled siRNA H}} \right)} \times 100$$

Equation 2.2 The percentage of Syk protein was determined by calculating the percentage of the relative band intensity of cells transfected with the AGMA29–Syk siRNA H polyplex to the relative band intensity of cells transfected with the AGMA29–Scrambled siRNA H polyplex at the same AGMA29 concentration and for the same period of transfection.

2.2.2.9 Analysis of Syk mRNA by RT-qPCR assay

2.2.2.9.1 Extraction of the mRNA

Cells of the NPY-mRFP reporter cell line were seeded in a transparent 96-well plate, incubated, transfected with polymer/siRNA complexes at different ratios, and then incubated again as shown in section 2.2.2.4. Next, the cells were incubated for 1 or 2 days at 37°C in a 5% CO₂ incubator. SingleShot cell lysis buffer) (960 µl) was mixed with proteinase K solution (20 µl) and DNase

solution (20 µl) to obtain the SingleShot cell lysis master mix. Thereafter, the medium was removed and the cells were washed with DPBS (125 µl/well), after which the cells were lysed with the prepared SingleShot cell lysis master mix (20 µl/well) and incubated at room temperature for 10 min. The lysate was transferred to a PCR tube, incubated at 37°C for 5 min and again incubated at 75°C for 5 min. The lysate can be stored at -20°C for up to 2 months or at -80°C for up to 12 months.

2.2.2.9.2 Reverse transcription of the mRNA

iScript Advanced Reaction Mix (40 µl) was mixed with iScript Advanced Reverse Transcriptase (10 µl) and nuclease-free water (60 µl) to obtain the SingleShot reverse transcription master mix. The cell lysate (9 µl) was mixed with the prepared SingleShot reverse transcription master mix solution (11 µl) in a PCR tube and incubated at 42°C for 30 min, which was followed by incubation at 85°C for 5 min. The resultant cDNA can be stored at -20°C for up to 12 months.

2.2.2.9.3 RT-qPCR reaction

SsoAdvanced Universal SYBR Green Supermix (148.5 µl) was mixed with SingleShot SYBR Green qPCR Control Assay primer (14.9 µl), which was Syk for the target gene and Ggt1 for the reference gene, and nuclease-free water (79.7 µl). The cDNA (6 µl) was added to the prepared SingleShot qPCR master mix (27 µl) in a PCR tube, and an aliquot of 10 µl was transferred to Strip Tube

(Qiagen, Germany). The sample was then run in a Rotor-Gene Q (Qiagen, Australia). The running conditions were as follows: step 1: 95°C for 2 min, step 2: 40 cycles with each cycle set at 95°C for 2 s and then 60°C for 30 s, step 3: 65°C for 5 s, and step 4: 95°C for 5 s. The ratio of the amount of Syk mRNA in the treated cells to that in the untreated cells was calculated using the Pfaffl equation.

$$Ratio = \frac{E_{target}^{\Delta C_{ttarget}}}{E_{ref}^{\Delta C_{tref}}}$$

Equation 2.3. The Pfaffl equation. E_{target} = efficiency of the primers for the cDNA of interest, which is Syk in this case, E_{ref} = efficiency of the reference primer, which is Ggt1 in this case, $\Delta C_{ttarget}$ and ΔC_{tref} = threshold difference between treated and untreated RBL cells for the target or reference reaction

CHAPTER 3: PHYSICOCHEMICAL CHARACTERISATION OF AGMA29-Syk siRNA POLYPLEXES

3.1 Introduction

The physicochemical characterisation of polyelectrolyte complex formation between a cationic polymer and siRNA is essential for preparing polyplexes that can be used for in vitro and in vivo transfection. There are many limitations to polyelectrolyte complexation between a cationic polymer and siRNA or the transfection of the polyplex to its target site, such as the low capacity of polymers to bind to siRNA and the low stability of the formulated polyplex. Both these factors can result in poor protection of the RNA and poor uptake of the polyplex by cells or inhibition of its release from the endosome. Moreover, siRNA is known to be more challenging than pDNA in terms of stable complex formation with cationic polymers which was claimed to be because it acts as a rigid rod due to its small size that cannot be condensed (Spagnou et al., 2004). However, 21 bp small size DNA which has approximately the same length of siRNA was evaluated by Danish (2014) and compared with siRNA in terms of forming a small polyplex with different types of PAA. The finding of this evaluation was that the PAA can form a smaller polyplex with 21 bp DNA than with siRNA. This could indicate that the additional hydroxyl group of siRNA is the cause of less binding of siRNA to cationic polymer in comparison to DNA.

Therefore, siRNA may not be completely encapsulated by the cationic polymers, or they may form large aggregated polyplexes on account of disturbances in electrostatic interaction (Spagnou et al., 2004).

The stability of polyplexes formed with siRNA depends on several factors. For example, optimisation of the ratio of the number of repeating unit (RU) of cationic polymers to the number of nucleotide (Nt) of siRNA can play a critical role in formulating a stable polyplex. A low RU/Nt ratio can result in incomplete complexation of the polyplex or the formation of large aggregated polyplexes, whereas a high RU/Nt ratio may result in the formation of a polyplex with high cytotoxicity (Fischer et al., 2003). The molecular weight of the cationic polymer is another factor that can influence the stability of the polymer/siRNA polyplex. It was reported by Godbey et al. (1999) that the transfection efficiency of PEI that has 70 kDa molecular weight in human endothelial cell derived cell line was higher than PEI that has 10 kDa molecular weight suggesting that high molecular weight PEI can have more transfection efficiency than low molecular weight PEI. However, the cytotoxicity of the high molecular weight PEI can be higher than low molecular weight PEI (Kunath et al., 2003). The functional groups of the cationic polymer can play an important role in the stability, transfection efficiency and biocompatibility of polymer/siRNA polyplex. For example, the primary amines have high ability to bind to nucleic acid because it is highly cationic and the higher ability of cationic polymer to bind to nucleic acid to form a stable complex can be correlated to the number of primary amines present (Wolfert et al., 1999, Reschel et al., 2002). The tertiary amino groups can be highly protonated at lower pH which is required for endosomal escape

that will cause an enhancement in the transfection efficiency (Piest and Engbersen, 2010). In addition, the predominately basic amphoteric PAA that contains acidic functional group such as carboxylic acid can be less cytotoxic than PAA that has similar net average charge at physiological pH (Danish, 2014, Ranucci et al., 2009).

In this chapter, the physicochemical characteristics of AGMA1 binding to siRNA will be evaluated. The AGMA1 molecule used here has an average weight molecular weight of 28700 Da, and it was named AGMA29. The physicochemical characterisation of AGMA1/pDNA polyplexes and their in vitro and in vivo transfection efficacy has been evaluated by Cavalli et al. (2010). They evaluated three samples of AGMA1 that were composed of the same monomer and were named AGMA5, AGMA10, and AGMA20 on account of their molecular weight, which was 4500, 10100, and 20500 Da, respectively. In their study, stable AGMA10/pDNA and AGMA20/pDNA polyplexes with diameter ≤ 161 nm were formulated at a 15:1 or 30:1 w/w ratio at pH 7.4. Moreover, when the pH was changed to 5, they were able to formulate more stable AGMA10/pDNA and AGMA20/pDNA polyplexes with diameter ≤ 95 nm. Thereafter, they assessed the transfection efficiency of AGMA10/pDNA and AGMA20/pDNA polyplexes that carried the GFP protein in HeLa cells, and the result showed that the transfection efficiency was good even at a 5:1 w/w ratio. Based on these results, it would be interesting to evaluate whether AGMA29 forms a stable polyplex with siRNA, as polyplex formation is more challenging with siRNA than with pDNA.

3.2 Aims and objectives

The aim of this chapter is to identify the optimal conditions for formulating an AGMA29/siRNA polyplex. To this end, the optimal polymer/siRNA polyplex ratio and pH that results in the release of minimum free siRNA and polymers and the formation of complexes of the optimal size need to be determined. In addition, the smallest polyplex size that could be optimal to be used for transfection in the target RBL-2H3 cells also needs to be determined. Further, the effect of different NaCl concentrations on the binding of the polymer/siRNA complex will also be examined in order to determine the optimal NaCl concentration.

3.3 Results and discussion

3.3.1 Complexation characteristics and Size of AGMA29-siRNA polyplex

The complexation behaviour of the AGMA29 polymer in the presence of the siRNA was examined by the gel retardation assay (Figure 3.1). The gel retardation assay is a useful technique that can be used for analysing the binding

of cationic polymers to DNA or RNA. In the gel retardation assay, free siRNA migrate toward the anode after gel electrophoresis because of the presence of a negatively charged phosphate backbone that does not bind to the cationic polymer. The siRNA that is completely electrostatically bound to the cationic polymer is neutralised and therefore does not migrate toward the anode or cathode and can be detected in the loading well. The excess cationic polymer that does not bind to siRNA migrates toward the cathode. The siRNA can then be detected under a UV transilluminator because the ethidium bromide (EtBr) that is present in the agarose gel intercalates with the nucleic acids of the siRNA and emits high fluorescence under the UV transilluminator. The EtBr can bind to siRNA by intercalating between the nucleotide base pairs or by electrostatic interaction between the positive charges of EtBr and the negative charges of the phosphate backbone of siRNA (Nordmeier, 1992). The cationic polymer used in this experiment is AGMA29, which has an amide group that can be detected in agarose gel when the gel is stained with Coomassie brilliant blue R250.

In this experiment, the pH of the AGMA29 polymer was measured after it was dissolved in phosphate buffer, and it was adjusted to 7. It can be seen from Figure 3.1 that the mobility of siRNA did not change after the addition of AGMA29 at RU:Nt ratios of 1:1, 2:1, and 3:1; this shows that AGMA29 was only partially bound to the siRNA. It can also be seen from Figure 3.1 that only very low levels of EtBr fluorescence were seen in the wells for 1:1, 2:1, and 3:1 RU:Nt ratios suggesting that although only part of the siRNA was complexed, the part that was complexed was tightly bound to polymer.

At RU:Nt ratios of 4:1 and 5:1, the free siRNA percentage was 19.59% and 6.27%, respectively, in comparison to the free siRNA in the first lane; this means that the polymer-siRNA binding was enhanced at these ratios. At the RU:Nt ratio of 8:1, it was estimated that the siRNA was completely bound to the AGMA29 polymer. However, there were some free polymers that did not bind to the siRNA at the 8:1 RU:Nt ratio that were detected by brilliant blue dye staining.

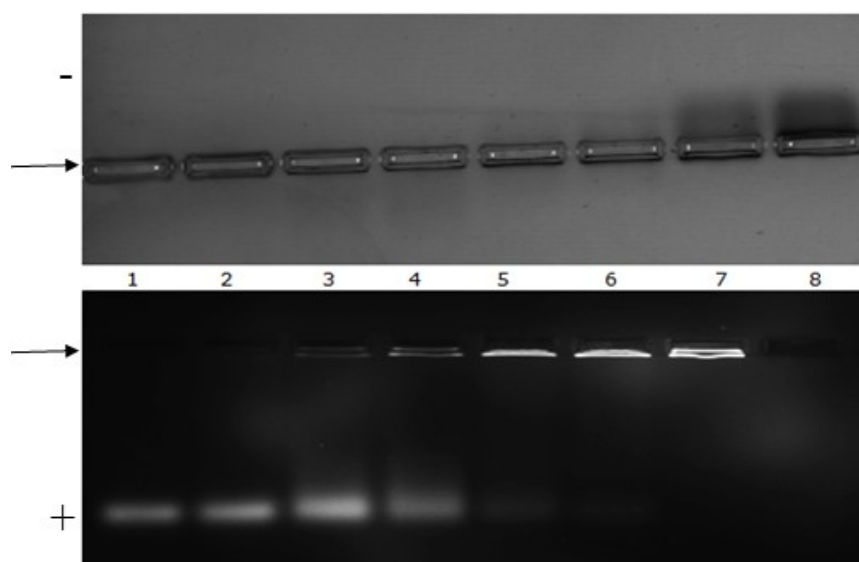


Figure 3.1 Binding of AGMA29 to siRNA at different RU:Nt ratios. Lane 1 contains free siRNA. Lanes 2–7 contain polyplexes with RU:Nt ratios of 1:1, 2:1, 3:1, 4:1, 5:1 and 8:1, respectively. Lane 8 contains free polymer. The loading wells are marked with arrows. The anode site and cathode site are marked with + and – respectively. The upper image shows the migration of polymer components stained with brilliant blue dye, while the lower image shows the migration of the siRNA component by EtBr staining and UV visualization. Both the upper and lower images represent the same gel.

The polyplex diameters corresponding to the RU:Nt ratios were determined using Viskotek dynamic light scattering (DLS) 802 (Figure 3.2). It can be seen from Figure 3.2 that the mean diameter of the polyplex increases with increase in the RU/Nt ratio: the diameter was 36, 746, 560, 1120 and 1260 nm for the 1:1, 2:1, 3:1, 4:1 and 5:1 RU/Nt ratios, respectively. However, at the RU/Nt ratio of 8:1, the mean polyplex diameter shows a slight decrease and reaches 1238 nm. The increase in the size of the polyplex in correspondence with the RU:Nt ratio can be explained by polyelectrolyte theory, according to which the proportion of stoichiometric complexes (S-PECs) that is insoluble in a polar solvent increases with increase in the RU:Nt ratio. This means that increase in the RU/Nt ratio leads to aggregation of the polyplex to form larger complexes until it reaches its biggest diameter at the 5:1 RU/Nt ratio; most of the polyplexes are expected to be S-PECs at this ratio. Because the 8:1 RU/Nt ratio results in full complexation and the release of extra free cationic polymers (Figure 3.1), the proportion of S-PECs might have decreased at this ratio and a small proportion of nonstoichiometric complexes (N-PECs) that were soluble in polar solution were probably formed. However, this decrease was not significant also the size of the AGMA/siRNA polyplex is high at this ratio. Attempts to solve this problem will be described in section 3.3.2.

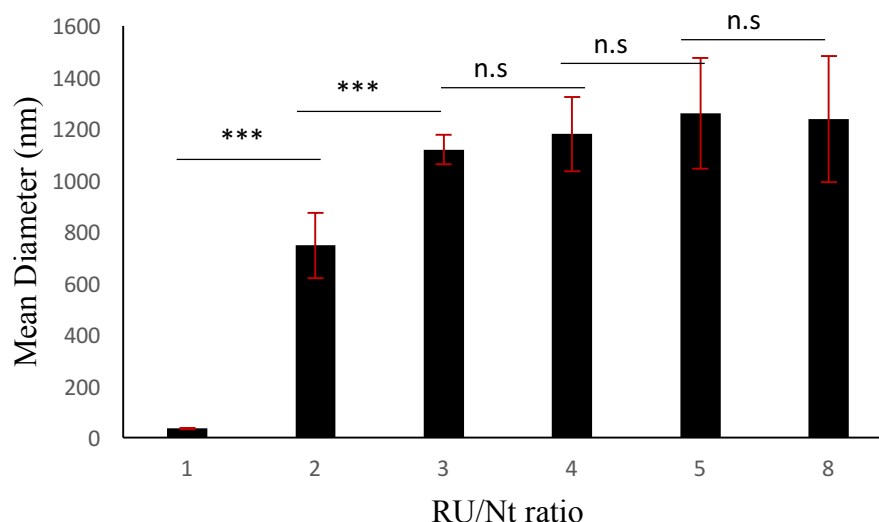


Figure 3. 2 The mean diameter ($\pm$ SD) of the AGMA29/siRNA polyplex at different RU:Nt ratios. A two-tailed *t*-test with different variables was used to compare each ratio with the adjacent ratio. P value < 0.001 (***), n.s. = not significant ($p > 0.05$) ($n = 8$).

3.3.2 Effect of pH on AGMA29 polymer complexation

This experiment was performed using the gel retardation assay, in which the AGMA29/siRNA complexes were prepared at different RU:Nt ratios (1:1, 2:1, 3:1, 5:1, and 8:1) and different pH levels (pH 4, 5, 6, 7, and 8), as shown in Figure 3.3. The results show that AGMA29 can bind to siRNA in a pH range of 4 to 8. This could be attributed to the amphoteric nature of AGMA29, which is positively charged at all the physiological pH levels (Cavalli et al., 2010). AGMA29 has three functional groups—the carboxyl, ter-amino, and guanidine groups—which had pK_A values of 2.25, 7.45, and ≥ 12.1 , respectively, when

reverse titration were performed (Franchini et al., 2006). The anionic carboxylic group of AGMA29 becomes neutral at low pH ranges which is perhaps lower than 2.25 (Ferruti et al., 2007). Since the pH in the present experiment was in the range of 4-8, it can be assumed that the carboxylic group was in the anionic state at all pH conditions that were used in this experiment.

Figure 3.3 shows the results of the gel retardation assay, which demonstrate the complexation behaviour of AGMA29/siRNA at pH 4, 5, 6, 7, and 8 (Figure 3.3). The lower images in Figure 3.3 show the EtBr fluorescence emitted after the binding of AGMA29 to siRNA at a particular pH value. The fluorescence emitted was indicator of the amount of bound siRNA, free siRNA and migrated siRNA. The upper images in Figure 3.3 show brilliant blue dye staining of the free excess AGMA29 polymers at the same pH as the upper image. Full complexation occurred at an RU:Nt ratio of 3:1 at pH 4, whereas a small percentage of free siRNA was observed at the anode at the same ratio at pH 5, 6, and 7 (1.78%, 7.95%, and 4.13% siRNA, respectively) (Figure 3.3 and Table 3.1). The binding ability of AGMA29 to the siRNA at pH 8 was lower, and full complexation was not observed even at an RU:Nt ratio of 8:1. This could be attributed to a decrease in the net positive charge of the polymer at this pH, at which the medium-strength ter-amino group of AGMA29 has a PKa value of 7.4 and cannot be protonated, and participates in the electrostatic interaction with siRNA at pH 8. Therefore, it is expected that only the guanidine group will be protonated at pH 8. The free excess AGMA29 polymer can thus be detected at pH 4, 5, 6, and 7, at RU:Nt ratios of 5:1 and above. At pH 8, no excess AGMA29 polymer was detected; this was probably because full complexation did not occur even at an RU:Nt ratio of 8:1 at this pH. The amount of estimated

free siRNA that did not bind to the AGMA29 polymer is summarised in Table 3.1. It can be assumed from Table 3.1 and Figure 3.3 that the RU/Nt ratio of 3:1 is the optimal ratio at all pH levels, except for pH 8, since the findings from Figure 3.3 do not show the presence of any free polymers at this ratio for the evaluated pH range

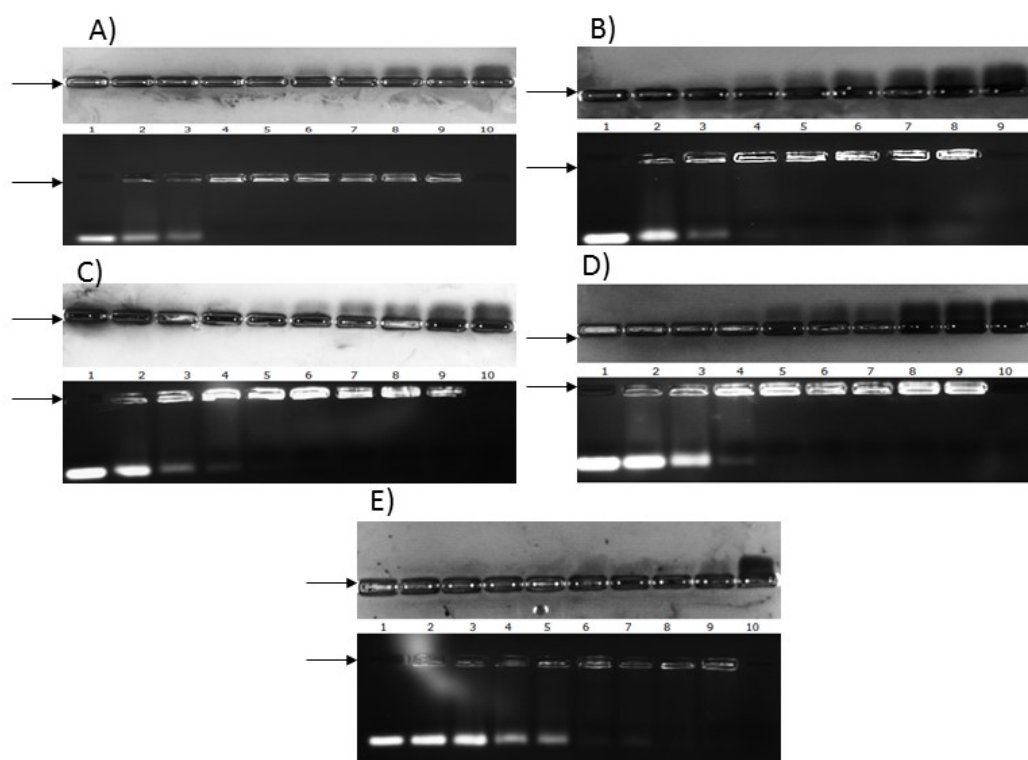


Figure 3.3. Binding of AGMA to siRNA at (A) pH 4, (B) pH 5, (C) pH 6, (D) pH 7, and (E) pH 8. The loading wells are marked with arrows. Lane 1 contains free siRNA. Lanes 2–9 contain polyplexes with RU:Nt ratios of 1:1, 2:1, 3:1, 4:1, 5:1, 6:1, 7:1 and 8:1 respectively*. Lane 10 contains free polymer. The upper image shows the migration of polymer components after staining with Coomassie brilliant blue dye, while the lower image shows the migration of siRNA components by EtBr staining and UV visualization. In pH 5, lane 9 is the free polymer and 8:1 RU:Nt ratio was not measured.

Table 3.1. The estimated percentage of free siRNA at different pH values and different polymer/siRNA ratios

AGMA29/siRNA ratio (RU:Nt ratio)	% of free siRNA				
	pH 4.0	pH 5.0	pH 6.0	pH 7.0	pH 8.0
1:1	58.87	65.42	70.19	82.21	99
2:1	30.04	21.92	24.04	42.47	89.6
3:1	0	1.78	7.95	4.13	47.8
4:1	0	0	1.65	0	42.74
5:1	0	0	0	0	4.32
6:1	0	0	0	0	5.44
7:1	0	0	0	0	≤ 1
8:1	0	0	0	0	≤ 1

Cavalli et al. (2010) showed that AGMA20 can form complexes of different sizes with DNA at pH 5.0 and pH 7.4. They used AGMA/DNA polyplex ratios of 5:1, 15:1, and 30:1 w/w. Their results showed that the AGMA20/DNA polyplexes (of all ratios) prepared at pH 5 were smaller than those prepared at pH 7.4. Therefore, the sizes the RU/Nt ratio of 3:1 which is probably the lowest

optimal ratio for full binding of the AGMA29/siRNA polyplexes at pH 4, 5, 6, and 7 (Figure 3.4) will be evaluated. As shown in Figure 3.4, the size of the polyplex is smallest at pH 7 at a diameter of 526 nm. This diameter was smaller than that obtained when the phosphate buffer was used in section 3.3.1. However, the size of the AGMA29/siRNA polyplex at pH 7 is still high which could be less effective for transfection. Therefore, the effect of NaCl on the size and binding of the AGMA29/siRNA polyplex under the optimal pH of 7 and optimal Ru/Nt ratio of 3.1 will be evaluated in section 3.3.3.

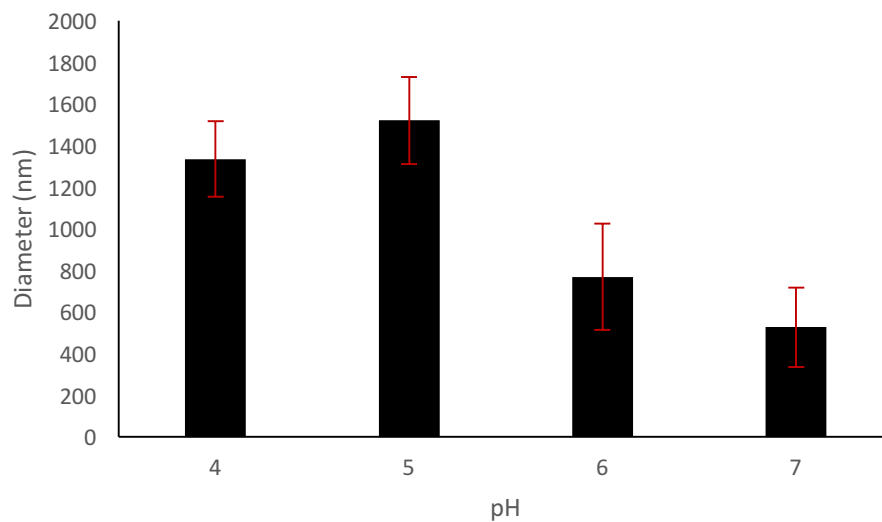


Figure 3. 4. The diameter ($\pm$ SD) of the AGMA/siRNA polyplex at different pHs at the RU/Nt ratio of 3:1 (n = 8)

3.3.3 Effect of NaCl on binding and size of the AGMA29-siRNA polyplex

In this experiment, the influence of NaCl on the complexation behaviour of the AGMA29/siRNA polyplex was determined by a gel retardation assay in which the 3:1 RU/Nt ratio was used at pH 7.0 (which were determined to be the optimal ratio and pH in section 3.3.2). The AGMA29/siRNA polyplex was prepared in water containing NaCl in a concentration range of 0 to 150 mM. The complexation of AGMA29/siRNA was enhanced when a low concentration of NaCl was used, and the maximum binding was observed at NaCl concentration of 20 mM (as this was the concentration at which the lowest amount of free siRNA was detected in the anode). Even though the percentage of free siRNA formed at NaCl concentrations of 10 and 20 mM was less than that in the deionised water condition, the EtBr fluorescence observed in the wells at 10 and 20 mM NaCl was higher. This could mean that the polyplex that is formed in the deionised water is more stable, since it may cause displacement of EtBr to a higher extent than the 10 and 20 mM NaCl conditions. Addition of NaCl at a concentration higher than 20 mM can negatively influence the binding of the polymer to siRNA. The estimated percentage of free siRNA increased from 0.5% to 81.78% when the NaCl concentration was increased from 20 mM to 150 mM. This is probably because the anions (Cl^-) resulting from the dissociation of NaCl neutralized the positively charged AGMA29 and led to a decrease in the binding of AGMA29 to the siRNA (Takahashi et al., 2000). Figure 3.6 shows

the results of the analysis in which the percentage of free siRNA that did not bind to AGMA29 are shown.

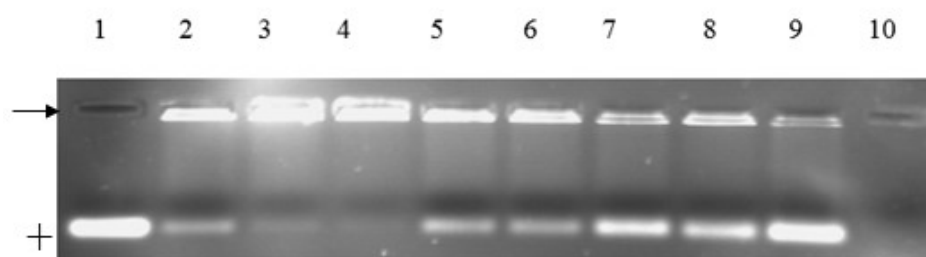


Figure 3. 5. Binding of AGMA29 to siRNA at different NaCl concentrations at an RU/Nt ratio of 3:1 and pH of 7.0. The loading wells are marked with arrows. The anode site is marked with +. Lane 1 contains free siRNA. The NaCl salt concentrations in lanes 2–9 are 0, 10, 20, 30, 50, 80, 110 and 150 mM, respectively. Lane 10 contains free polymer.

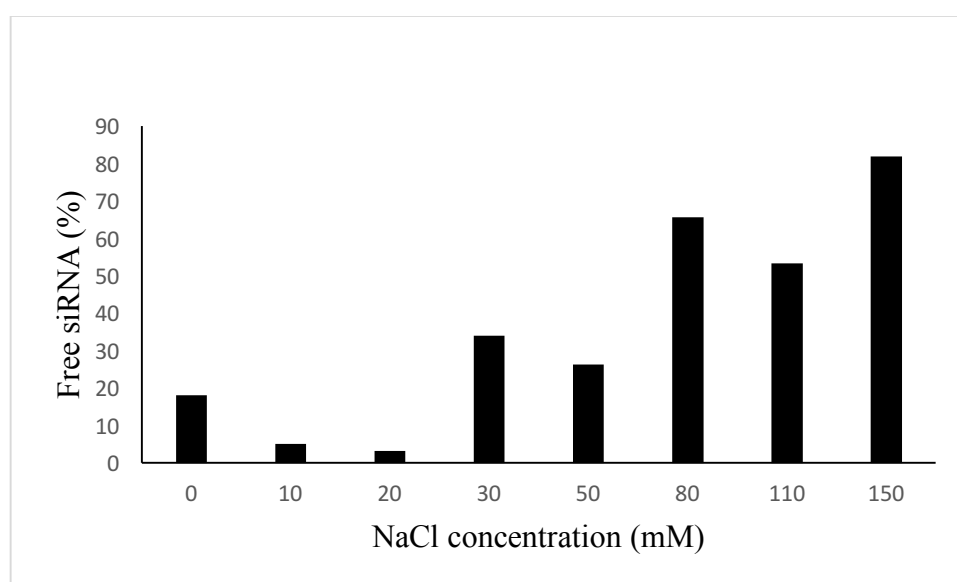


Figure 3.6. Agarose gel analysis of the percentage of free siRNA that did not bind to AGMA29 at an RU:Nt ratio of 3:1 at different concentrations of NaCl.

Since it is expected that the AGMA29 can form complex with siRNA in pure water that is more stable than complex in 20 mM NaCl, another experiment was performed in order to determine whether the AGMA29–siRNA polyplex is more stable when prepared in pure water or in 150 mM NaCl solution. To this end, the size of the polyplex was measured by Dynamic light scattering (DLS), since a smaller polyplex means that the siRNA has condensed and bound to AGMA29 more efficiently. It is important for the AGMA29–siRNA polyplex to be small so that it can be easily transfected into the cell. It was reported that the siRNA–polyethylenimine polyplex can be transfected into cells only when its diameter is less than 150 nm (Grayson et al., 2006). Full complexation is possible with a high AGMA29/siRNA polyplex ratio, with or without NaCl, as confirmed by agarose gel electrophoresis (Figure 3.7). As shown in Figure 3.7, all the complexes formed were in the loading wells in the presence of both deionised water and 150 mM NaCl and there was no free siRNA (no EtBr bands were observed toward the anode side). The size of the polyplex was measured by DLS as shown in Figure 3.8. The size of the AGMA29–siRNA polyplex was significantly greater when it was prepared in 150 mM NaCl solution than when it was prepared in deionized water. The diameter of the AGMA29–siRNA polyplexes when they were prepared in deionised water was 90 ± 39 nm and 107.8 ± 43.6 nm at an RU/Nt ratio of 10:1 and 30:1 respectively. According to Perez et al. (2009), addition of NaCl can result in an increase in the size of the cationic polymer–siRNA complex. In their study, they used ethylenediamine-core polyamidoamine dendrimers for the complexation of siRNA as these dendrimers selectively inhibit enhanced green fluorescence protein (Perez et al.,

2009). Their results showed that the diameter of the complex in the absence of NaCl was 30–130 nm, whereas the diameter of the complex when it was prepared using media containing 150 mM NaCl was more than 800 nm (Perez et al., 2009). Therefore, the AGMA29–siRNA polyplex is more stable when it is prepared in deionised water. Moreover, there was no difference in the effect of the 30:1 and 10:1 RU/Nt ratio on the size, and therefore, the stability of the complex.

From the above results, it seems that an RU/Nt ratio of 10:1 or lower is optimal for the preparation of the AGMA29–siRNA polyplex in deionised water.

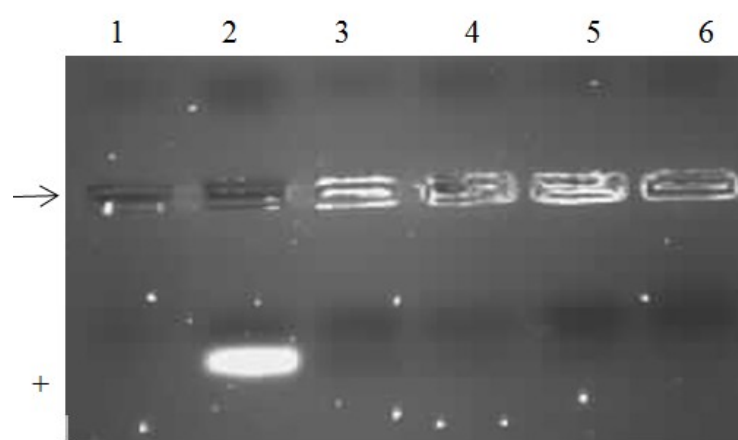


Figure 3.7. Binding of AGMA29 to siRNA in the presence of 150 mM NaCl and deionised water. Lane 1: free polymer; Lane 2: free siRNA; Lane 3: 10:1 RU/Nt AGMA-siRNA prepared in deionised water; Lane 4: 10:1 RU/Nt AGMA-siRNA prepared in 150 mM NaCl solution; Lane 5: 30:1 RU/Nt AGMA-siRNA prepared in deionised water; Lane 6: 30:1 RU/Nt AGMA-siRNA prepared in

150 mM NaCl solution. The anodic end is marked with +. The loading wells are marked with arrows.

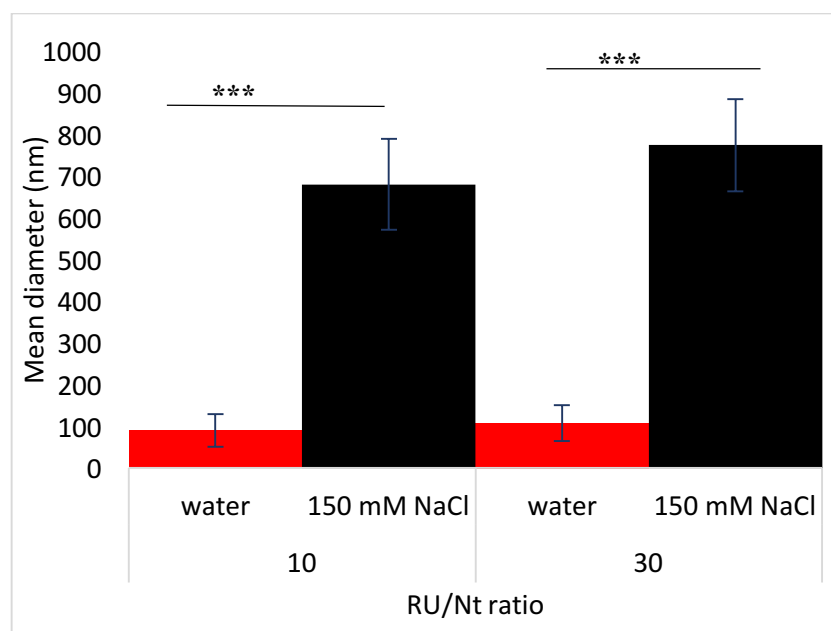


Figure 3.8. The mean diameter ($\pm$ SD) of the AGMA29 polymer-siRNA complex at pH 7. A two-tailed *t*-test with different variables was used to compare the complexes prepared in deionised water to the complexes prepared in 150 mM NaCl solution. P value < 0.001 (***) (n = 8)

3.4 Conclusion

The aim of this chapter was to study the physicochemical properties of AGMA29 binding to the Syk siRNA. The results indicated that AGMA29 binding to the siRNA at different pHs in the range of pH 4 to pH 8 was achieved

successfully. This is probably attributable to the amphoteric properties of AGMA29, since it has a carboxylic group and basic amine groups. In addition, the optimal ratio for the binding of AGMA29 to the siRNA that shows a full complexation was successfully determined. Thereafter, the sizes of the polyplexes were measured at this ratio under different pH conditions, and the ideal pH was determined that shows the smallest size which was at pH 7. Therefore, effect of NaCl on the binding of AGMA29 to the siRNA and the size of the complex formed was examined at optimised pH and polyplex ratio. The results indicated NaCl can in general negatively influence the size of the polyplex. Therefore, the optimised conditions for preparing a small polyplex that can be used for transfection of RBL-2H3 derived cell lines were successfully established.

CHAPTER 4: OPTIMISATION OF SYK KNOCKDOWN ASSAYS USING AGMA29

4.1 Introduction

Syk plays a pivotal role in early signalling in allergic reactions, which involve mast cells and basophils. High-affinity IgE receptors that are expressed in mast cells and basophils have immunoreceptor tyrosine-based activation motifs (ITAMs). These motifs induce the phosphorylation of Syk when the crosslinking of IgE with the allergen occurs, and this eventually leads to the degranulation of cells and cytokine induction, the mechanism of action of which is explained in detail in section 1.3. Based on these mechanisms, it is expected that the knockdown of Syk mRNA will inhibit both degranulation of the cells and cytokine induction.

In order to study the effect of the treatment in prevention of allergic mediator from being released (such as evaluation of knockdown of syk), it is important to find a suitable cell line as model for the evaluation of mast cell allergic mediator release. One main reason for this is that mast cells are located in tissues and are therefore difficult to obtain in large amounts, high purity and without activation during the purification steps. The parental RBL cell line was created by Leonard et al. (1971) by injecting rats with 2-(α -chlor- β -isopropylamine ethylnaphthalene to generate basophilic leukaemia cell lines. However, the

cytological characterisation of the cells produced are different from the normal mastocytes (Eccleston et al., 1973).

Kulczycki Jr et al. (1974) successfully adapted rat basophilic leukaemia to a cell line which was named RBL-1. Even though RBL-1 has IgE receptors that can bind to rat IgE successfully and at high affinity, it was shown that neither IgE + antigen sensitisation nor A-23187 Ca^{2+} ionophore cause a release of histamine and therefore it cannot be used for the evaluation of degranulation (Kulczycki Jr et al., 1974, Siraganian et al., 1975). RBL-2H3 cell line is one of the sublines that was produced from RBL-1 cloning that has advantage that can be induced to degranulate and release histamine after IgE + sensitisation and subsequent antigenic stimulation (Barsumian et al., 1981).

In order to evaluate the level of inhibition of degranulation and cytokine induction in mast cells and basophils due to knockdown of Syk mRNA, it is important to find a suitable assay. The β -hexosaminidase assay is used to evaluate degranulation of RBL-2H3 cells (Silverman et al., 2006). β -hexosaminidase is an enzyme that is stored in mast cell and basophil granules (Schwartz et al., 1979). When the degranulation of RBL-2H3 occurs, the relative level of secreted β -hexosaminidase can be measured by using a 4-nitrophenyl *N*-acetyl- β -D-glucosaminide substrate (Tanifuji et al., 2010). The substrate is cleaved by β -hexosaminidase enzyme to produce *p*-nitrophenol and its absorbance can be measured after the cleavage reaction is inhibited with glycine solution. Decrease in the release of β -hexosaminidase due to knockdown of the Syk mRNA by siRNA was evaluated by Sanderson et al. (2010). They found

that RBL-2H3 cells that are treated with Syk siRNA then sensitised with anti-DNP IgE antibody followed by stimulation with DNP-BSA can release less β -hexosaminidase in comparison to the RBL-2H3 cells that are treated with GAPDH siRNA which could indicate that Syk siRNA can inhibit the degranulation of RBL-2H3 cells.

In this chapter, a novel RBL-2H3 cell line that was stably transfected with the neuropeptide Y-monomeric red fluorescent protein (NPY-mRFP) fusion construct that is targeted to granules will be used to evaluate cellular degranulation. The red fluorescent protein (RFP) is present in the granules of the cells and is released on degranulation of the cell. This assay is more convenient than the β -hexosaminidase assay, since the 4-nitrophenyl *N*-acetyl- β -D-glucosaminide substrate and glycine solution are not required for evaluating cellular degranulation. This can decrease variability as no substrate is needed which will remove the experimental sources of error and variability. The knockdown of Syk mRNA is expected to cause inhibition of Syk protein expression. This will reduce degranulation of the NPY-mRFP reporter cells, and subsequently cause a decrease in the release of RFP.

NPY is a brain peptide, which was discovered by Tatemoto et al. (1982), that acts a neurotransmitter. It was reported by Lang et al. (1997) that the c-terminal of the human pro-neuropeptide Y (hpNPY) can be fused with green fluorescent

protein (GFP) to target secretory granules of the PC12 neuronal cell line. The RBL-2H3 cell line transiently transfected with NPY-mRFP cDNA was evaluated by Azouz et al. (2012), and it was reported that NPY-mRFP is pH insensitive and therefore it can be detected in the acidic secretory granules. They found that the amount of NPY-mRFP released is similar to the amount of endogenous β -hexosaminidase secreted when degranulation of cells is triggered by Ca^{2+} ionophores.

For the evaluation of cytokine induction, the luciferase assay was used to analyse genetically modified RBL-2H3, which is called RS-ATL8 (Nakamura et al., 2010). RS-ATL8 is RBL-SX38 that is transfected with the nuclear factor-activated T cell-regulated luciferase (NFAT-regulated luciferase) reporter plasmid (Nakamura et al., 2010, Wiegand et al., 1996). RBL-SX38 is RBL-2H3 that is transfected with a human Fc ϵ RI-expressing gene (Wiegand et al., 1996). NFAT is localised from the cytosol to the nucleus after it is dephosphorylated during allergic reactions. Therefore, NFAT-regulated luciferase is expressed during cellular activation via the IgE receptor and can be detected by the luciferase substrate, and the level of expressed luciferase can be measured by chemiluminescence. Another way of measuring cytokine induction is by using the NFAT-DsRed reporter cell line. NFAT-DsRed cells are RBL-2H3 cells that are stably transfected with the human Fc ϵ RI receptor α chain and the NFAT-DsRed gene (Wang et al., 2014a). The NFAT-DsRed gene consists of the NFAT promoter sequence and a DsRed reporter gene. After crosslinking of the IgE by the allergen and nuclear translocation of NFAT, this cell line produces DsRed

fluorescent protein in the cytosol, which can be visualised by fluorescence microscopy or measured in a spectrofluorometer.

Calcium influx into the cell cytosol is important for cellular degranulation (Reber and Frossard, 2014). After crosslinking of the IgE with the allergen in allergic reactions, the signalling cascade that is activated results in the activation of inositol triphosphate, which binds to the inositol triphosphate receptor in the endoplasmic reticulum and causes the release of Ca^{2+} and stromal interaction molecule 1 (STIM-1) from the endoplasmic reticulum (Gilfillan and Tkaczyk, 2006, Liou et al., 2005). The release of Ca^{2+} and STIM-1 leads to the influx of calcium from outside of the cell into the cell via calcium release-activated calcium channels and causes degranulation of mast cells or basophils (Liou et al., 2005). In addition, when calcium influx into mast cells and basophils occurs, calcineurin interacts with NFAT (Hogan et al., 2003). This interaction leads to dephosphorylation of NFAT, which is translocated from the cytosol to the nucleus (Pandey et al., 2004a). The main advantage of the NFAT-DsRed reporter cell line is that expression of NFAT-DsRed can be induced by NFAT when IgE crosslinking occurs, and the resulting fluorescence can be measured without the need for a substrate, such as in the luciferase assay for the RS-ATL8 cell line (Nakamura et al., 2010). The increase in calcium influx into cells leads to an increase in the production of DsRed in NFAT-DsRed reporter cells via dephosphorylation of NFAT, which results in the translocation of NFAT into the nucleus of the cell and increases transcription of the NFAT-DsRed gene.

4.2 Aims and objectives

The aim of this chapter was to evaluate the transfection efficiency of the AGMA29–Syk siRNA polyplex in two genetically modified rat basophilic leukaemia (RBL-2H3) reporter cell lines—NPY–mRFP and NFAT-DsRed—and to evaluate the suitability of these cell lines for the detection of Syk knockdown. This involved, firstly the determination of the optimal calcium concentration that results in the release of the highest amount of RFP in the NPY–mRFP reporter cell line and the highest expression of NFAT-DsRed in the NFAT-DsRed reporter cell line. Then the inhibition of RFP release in the NPY–mRFP reporter cell line and NFAT-DsRed expression in the NFAT-DsRed reporter cell line by the AGMA29–Syk siRNA polyplex was evaluated. Finally, knockdown of Syk by the AGMA29–Syk siRNA polyplex was evaluated by Western blot analysis and then by real-time quantitative polymerase chain reaction (RT-qPCR).

4.3 Results and discussion

4.3.1 Effect of calcium on the release of RFP and the expression of NFAT-DsRed

This experiment was performed in order to determine the optimal conditions for the release of RFP from the granules of the NPY–mRFP reporter cells. The

optimisation of the release of RFP from the granules can be evaluated by determining the calcium concentration that produces the highest signal-to-noise ratio. Calcium (at varying concentrations) and DNP-BSA (the allergen) were dissolved in Dulbecco's phosphate-buffered saline (DPBS), Eagles minimum essential medium (MEM) with foetal bovine serum (FBS) or MEM without FBS. The main advantage of the NPY-mRFP reporter cell line is that the degranulation of cells can be easily detected by fluorescence, since RFP is released from the cells on degranulation and there is no need for the biochemical assay of a substrate. Figure 4.1 shows the dose-dependent effect of calcium on the release of RFP from NPY-mRFP cells. It can be seen that the release of RFP increased with increasing calcium concentrations in DPBS and MEM without FBS media in the presence of DNP-BSA in which the increase was significant when 5 or 6 mM of calcium added to MEM without FBS media and above 5 mM of calcium added to DPBS. However, the same effect was noted even in the absence of DNP-BSA. This could mean that the cells responded to the increasing calcium concentration and released RFP, or that the increase in the calcium concentration negatively influenced the viability of the cells, which led to their death and release of their contents, including RFP. With regard to MEM containing FBS, the increase in the calcium concentration influenced the release of RFP in the presence or absence of DNP-BSA when 6 mM of calcium were added or above that concentration.

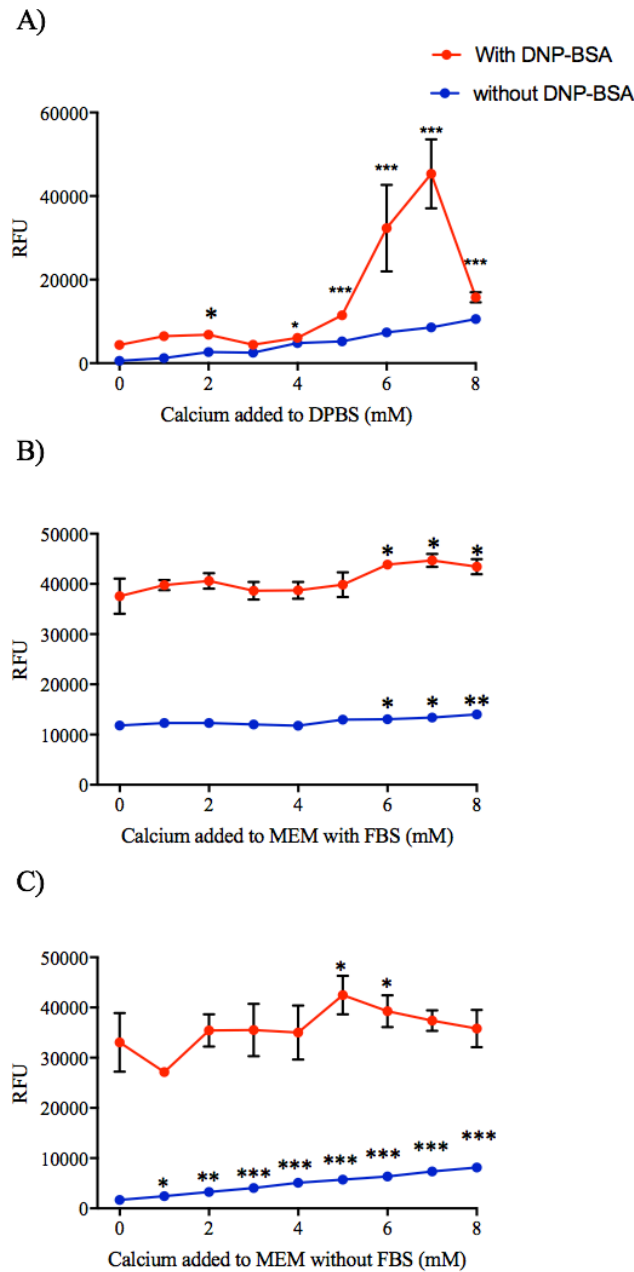


Figure 4.1 Effect of calcium on degranulation and release of red fluorescence protein in NPY-mRFP reporter cells after sensitisation with mouse IgE anti-DNP and stimulation with the DNP-BSA allergen dissolved in (A) DPBS, (B) MEM with FBS and (C) MEM without FBS. The results are presented as RFU $\pm$ SD values. A one-tailed t -test with different variables was used to compare cells that were not treated with calcium with cells that were treated with calcium. P value < 0.001 (***), P value < 0.01 (**), P value < 0.05 (*) (n = 4).

The signal-to-noise ratio of Figure 4.1 was analysed by dividing the Relative fluorescence units (RFU) reading of calcium mixed with DNP-BSA to the concomitant concentration of calcium in the same medium in the absence of DNP-BSA, as shown in Figure 4.2. From Figure 4.2, it can be observed that the highest signal-to-noise ratio was obtained when calcium was not added in the case of all three media, and the optimal result was obtained with MEM without FBS, for which the signal-to-noise ratio was 20.

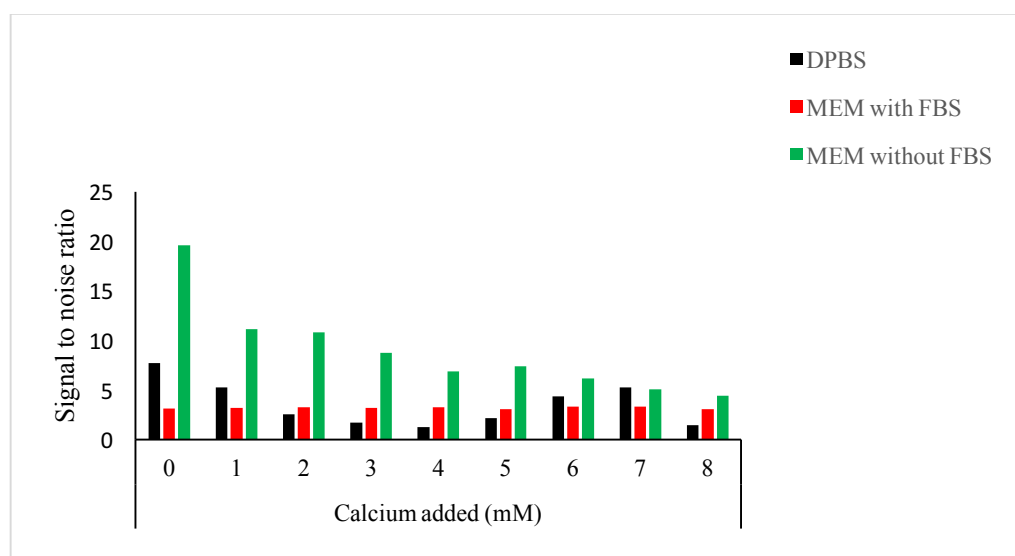


Figure 4.2. Signal-to-noise ratio obtained with different calcium concentrations in the different media.

The second determination was the effect of calcium on the production of DsRed in NFAT-DsRed reporter cells. To this end, calcium at varying concentrations

and anti-human IgE were dissolved in MEM with FBS. From the results in Figure 4.3, it can be seen that calcium caused an increase in DsRed induction only when higher concentrations of calcium (that is, 10 and 20 mM) were used. However, high signal-to-noise ratios were not obtained, even after the addition of calcium. This indicates either low expression of the NFAT-DsRed gene, which led to low production of DsRed, or low expression of the human FcεRI receptor α chain. Thus, further investigation is required from the viewpoint of enhancing the expression of the NFAT-DsRed gene.

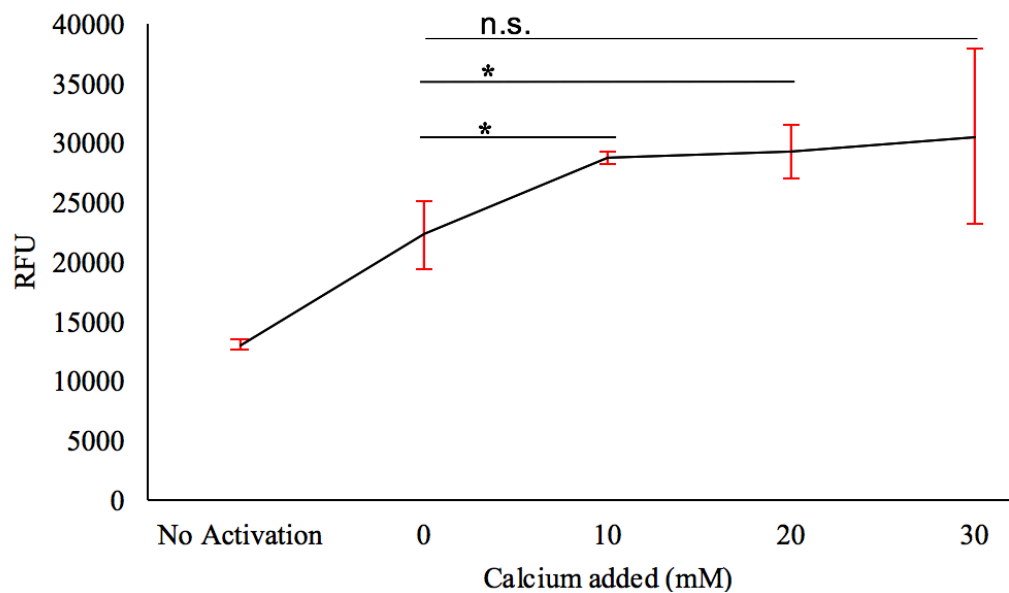


Figure 4.3. Effect of calcium on the expression of DsRed in NFAT-DsRed cells. The negative control was cells that were neither sensitised with human serum nor stimulated with anti-human IgE. A one-tailed *t*-test with different variables was used to compare cells that were not treated with calcium with cells that were treated with calcium. The results are expressed as the RFU $\pm$ SD values. * $p < 0.05$. n.s. = not significant ($p > 0.05$) ($n = 8$).

Due to the low signal-to-noise ratio observed when the NFAT-DsRed reporter cells were sensitised with human serum and stimulated with anti-human IgE, the NFAT-DsRed reporter cells were sensitised with anti-DNP mouse IgE and stimulated with DNP-BSA. From Figure 4.4, it can be seen that, in general, the signal-to-noise ratio was higher when the NFAT-DsRed reporter cells were sensitised with anti-DNP mouse IgE and stimulated with DNP-BSA than when the cells were sensitised with human serum and stimulated with anti-human IgE. Interestingly, when the NFAT-DsRed reporter cell line was sensitised with anti-DNP mouse IgE without DNP-BSA stimulation, the RFU was higher than that in the non-sensitised cells. It was reported by Pandey et al. (2004b) that stimulation of RBL-2H3 by SPE-7 monomeric IgE alone without an allergen can stimulate the influx of calcium and translocation of NFAT from the cytosol to the nucleus. In their study, they transfected RBL-2H3 cells with the EGFP-NFAT gene, and translocation of the expressed EGFP-NFAT from the cytosol to the nucleus was observed after sensitisation with IgE. It is well known that the SPE-7 IgE is highly cytokinergic (Bax et al., 2012). However, in the present experiment, the RFU was higher after the sensitisation of the cells with anti-DNP mouse IgE and stimulation with DNP-BSA than after sensitisation with only anti-DNP mouse IgE. Further, the addition of 10 mM calcium to DNP-BSA resulted in an increase in the RFU compared with the addition of 5 mM of calcium ($p = 0.006$). However, any further increase in the calcium concentration did not result in an increase in the RFU of the cells after they were sensitised with anti-DNP mouse IgE and stimulated with DNP-BSA. Therefore, it could be concluded from this result that the optimal condition for the highest level of

DsRed induction is the addition of 10 mM of calcium in a medium containing DNP-BSA (the final concentration of the calcium in the media $\approx$ 12 mM).

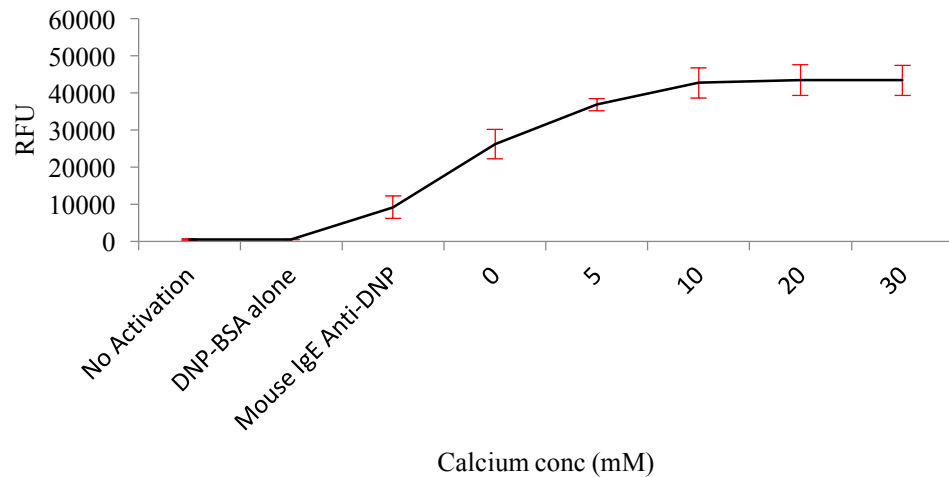


Figure 4.4. Effect of calcium on the expression of DsRed in NFAT-DsRed reporter cells after sensitisation with mouse IgE anti-DNP and stimulation with DNP-BSA (RFU $\pm$ SD values are presented, n = 8).

4.3.2 Efficacy of the AGMA29–Syk siRNA polyplex in inhibiting the degranulation of NPY-mRFP

This section discusses the efficacy of the AGMA29/Syk siRNA polyplex in inhibition of the production of Syk protein and, therefore, inhibition of its function. The aim of this experiment is to inhibit expression of the Syk gene with the AGMA29–Syk siRNA complex. Inhibition of the Syk protein is expected to lead to blockage of the signalling pathway regulating degranulation, which will therefore lead to a decrease in the release of preformed RFP from the

NPY-mRFP reporter cells. At the beginning of the experiment, AGMA29-siRNA J polyplexes were prepared at an AGMA29 concentration of 10 RU/Nt, as this was found to be the optimal concentration in chapter 3. It was reported that Syk siRNA J can inhibit Syk expression by 32% in comparison to untreated cells, when it is transfected in the RBL-2H3 cell line by electroporation (Sanderson et al., 2010). From the results in Figure 4.5, it seems that siRNA J did not result in a decrease in RFU when the amount of siRNA used was less than 500 ng per well. Although the mean RFU when cells were transfected with 500 ng/well of siRNA J was lower than that of the scrambled siRNA J at the same concentration, this reduction in RFU did not confirm knockdown of the Syk protein since the p value was not significant (>0.05). This is probably because the Syk siRNA J was not effective in knocking down the Syk protein. Therefore, the experiment was repeated for different types of Syk siRNAs.

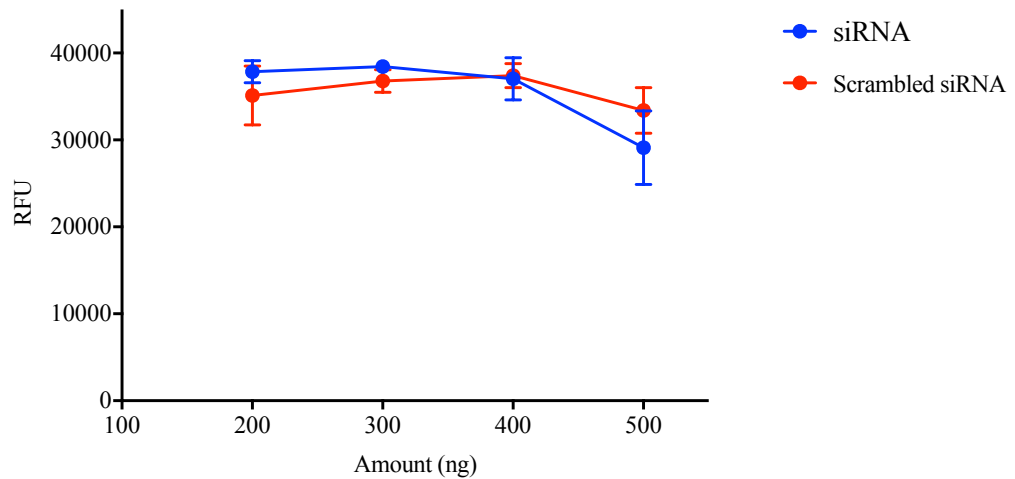


Figure 4. 5 Mean RFU $\pm$ SD values of NPY-mRFP cells after 2 days of treatment with the 10 RU/Nt AGMA29–siRNA J polyplex. This experiment has been performed in triplicate.

Another experiment was performed in order to evaluate the efficiency of AGMA29 in the delivery of the Syk siRNAs G, H, I and J and the efficiency of these siRNAs in the knockdown of the Syk gene, which can lead to inhibition of the degranulation of the cells. Figure 4.6 shows that when 400 ng of the siRNA was added to each well, no decrease in the fluorescence was observed with any of the siRNAs. This could mean that the Syk gene was not efficiently knocked out when the siRNAs were added at this concentration. However, when the Syk siRNAs H and J were added at 500 ng/well, a decrease in the fluorescence to 33% and 26%, respectively was observed. Their toxicity was low compared to their high silencing efficacy, as the cell viability was 87% in the case of both siRNAs. It is important for the accuracy of the measurement silencing efficacy that the viability percentage is high because the lower number of viable cells

will cause lower level of RFP being released that is not relevant to the silencing efficacy of the polyplex.

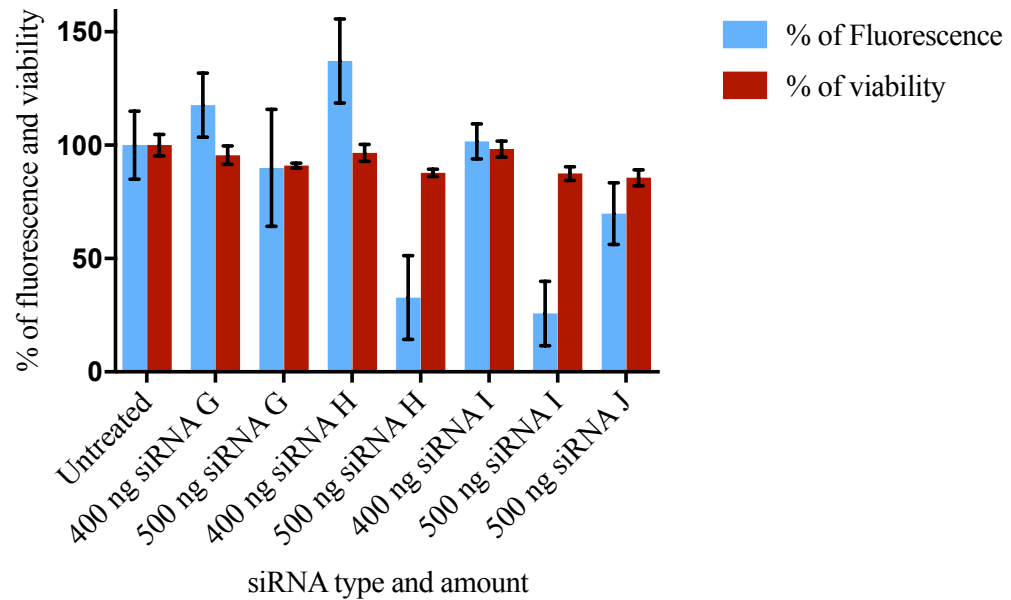


Figure 4. 6 Percentage of fluorescence and viability ($\pm$ SD) of NPY-mRFP cells after 2 days of treatment with 10 RU/Nt AGMA29–siRNA polyplexes. The amount of siRNA per well is 400 ng or 500 ng. This experiment has been performed in triplicate.

Based on the preliminary results, further evaluation of 500 ng of Syk siRNA H was performed in order to determine the reproducibility of the experiment and to determine whether lower or higher concentrations of AGMA29 can result in a higher rate of knockdown of the Syk gene in comparison to the 10 RU/Nt concentration of AGMA29 in the AGMA29/Syk siRNA H polyplex. In addition, scrambled siRNA H was used as a negative control in order to evaluate whether the decrease in percentage fluorescence was caused by knockdown of the Syk

gene by the AGMA29/Syk siRNA H polyplex or by other unspecific causes such as off-target effects. Figure 4.7 shows the percentage fluorescence and viability determined from three independent experiments after 2 days of transfection of the NPY-mRFP reporter cell line with 5, 10, and 15 RU/Nt AGMA29–Syk siRNA H and AGMA29–scrambled siRNA H polyplexes. The Lipofectamine RNAimax transfection reagent was used as a positive control. The viability percentage for each independent result was higher than 85%, except when 15 RU/Nt AGMA29–Syk siRNA H was used in experiment 3, in which case the viability percentage was 63%. It can be seen from the results that the variability between experiments was quite high, and taking an average of the 3 results, the only significant differences could be seen in the 5 RU/Nt of AGMA29/Syk siRNA H polyplex and the lipofectamine positive control. In order to analyse the data and determine whether there were significant differences at any ratio, the average of the mean $\pm$ SD values of this three independent experiments was calculated as shown in Figure 4.8. The figure shows that only the 5 RU/Nt AGMA29–Syk siRNA H polyplex and Lipofectamine RNAimax–Syk siRNA H lipoplex exhibited a significant reduction in fluorescence percentage compared to the AGMA29–scrambled siRNA H polyplexes. This could indicate that the higher the concentration of AGMA29 in the AGMA29–Syk siRNA H polyplexes, the lower the efficiency of transfection of the siRNA H in the NPY-mRFP reporter cell line. This is probably because AGMA29–Syk siRNA H polyplexes containing a higher concentration of AGMA29 are more stable: that is, the cationic AGMA29 may have a strong electrostatic bond with the anionic siRNA, which could inhibit the release of siRNA in the cytosol. It is important that the siRNA dissociated from the cationic polymer efficiently to interact with

the mRNA and cause its knockdown (Wang et al., 2014b). Further investigations are required in order to determine whether the inhibition of RFP release was caused by reduction of the degranulation of NPY-mRFP reporter cells as a result of Syk mRNA knockdown (and subsequent inhibition of Syk signalling), or whether there were unknown factors involved.

Another experiment was performed in order to observe the knockdown of Syk after 72 hrs of transfection of the NPY-mRFP reporter cell line with AGMA29–Syk siRNA H polyplexes. However, a high standard deviation of the percentage fluorescence was observed, which was probably caused by overgrowth of the cells, making the results unreliable (Data not shown).

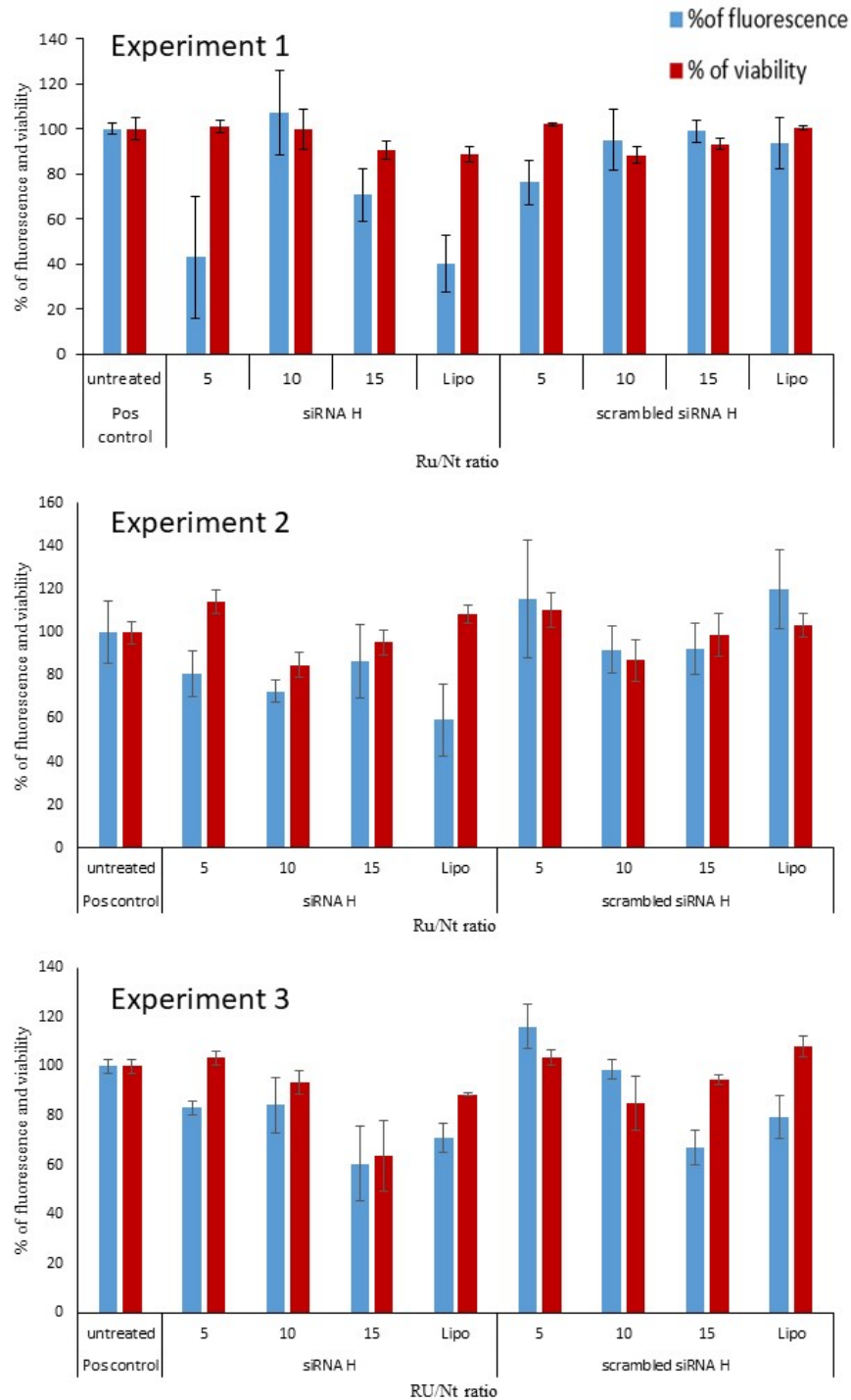


Figure 4.7 Percentage of fluorescence and viability ($\pm$ SD) of the NPY-mRFP cells in three independent experiments after 2 days of treatment of the cells with 5, 10 and 15 RU/Nt AGMA29-siRNA polyplexes and Lipofectamine RNAimax. The amount of siRNA H or scrambled siRNA H per well is 500 ng. The cells were sensitised with mouse anti-DNP IgE (SPE-7) and then stimulated

with DNP-BSA. The non-transfected cells represent 100% fluorescence and viability. Lipo: Lipofectamine RNAiMax (n = 3)

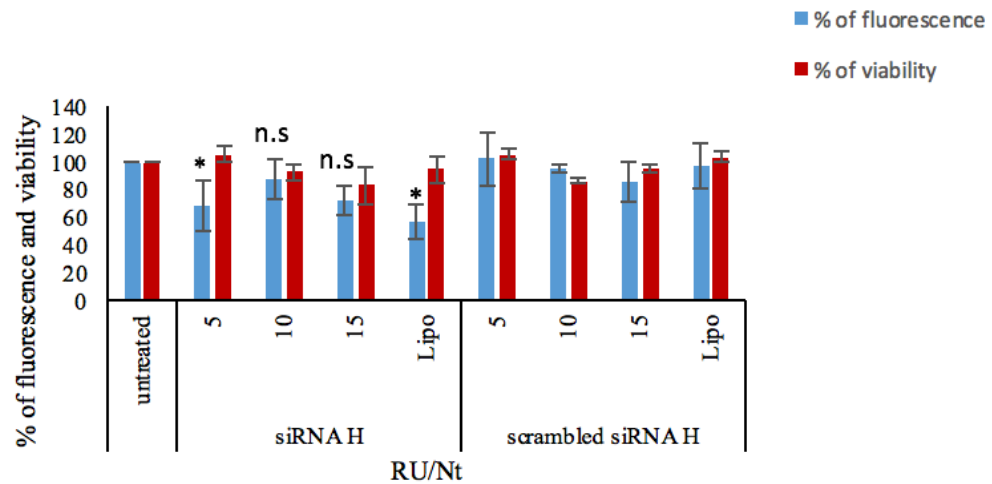


Figure 4.8 The average of the mean $\pm$ SD values from the three experiments in Figure 4.7. A one-paired tailed *t*-test with different variables was used to compare between siRNA H and scrambled siRNA H for AGMA29/siRNA polyplexes of the same AGMA29 concentration. * $p < 0.05$, n.s. = not significant ($p > 0.05$). Lipo: Lipofectamine RNAiMax (n = 3)

4.3.3 Efficacy of the AGMA29-Syk siRNA polyplex in inhibition of the expression of DsRed in the NFAT-DsRed reporter cell line

Inhibition of the Syk protein by the AGMA29/siRNA H polyplex was evaluated in the NFAT-DsRed reporter cell line. Inhibition of Syk is expected to result in inhibition of the NFAT signal transduction cascade and subsequent inhibition of

NFAT translocation from the cytosol to the nucleus. The presence of NFAT in the nucleus leads to expression of the NFAT-DsRed reporter gene, so the knockdown of Syk could inhibit NFAT translocation from the cytosol to the nucleus, which in turn could lead to inhibition of the expression of the NFAT-DsRed reporter gene and therefore inhibition of DsRed production. In this experiment, the cells need to be lysed before DsRed can be measured. However, before the cells are lysed, their viability needs to be assessed based on resazurin staining. Reduction of the blue dye resazurin to the highly red fluorescent resorufin in cellular mitochondria can be detected at an excitation wavelength of 540 nm and an emission wavelength of 590 nm (as shown in Figure 4.9), which is very close to the detection wavelength of DsRed (excitation wavelength, 530 nm; emission wavelength, 590 nm). Therefore, the first experiment in this section was performed in order to determine whether the viability assay can influence the results of DsRed measurement.

The first experiment was performed as described in 2.2.2.5, but without knockdown of the NFAT-DsRed reporter cell line with the AGMA29-Syk siRNA H polyplex. However, before lysis was induced with the Triton lysis solution, the resazurin solution was added to the cells and they were incubated for 1 h at 37°C in a 5% CO₂ incubator and then washed twice with DPBS. As shown in Figure 4.10, even though the cells treated with resazurin were washed twice with DPBS to remove the highly red fluorescent resorufin before lysis was induced, the RFU was approximately 20-fold higher than that in the cells that were not treated with the resazurin solution. This could indicate that some part

of the resorufin molecules were still inside the cells and resulted in the increase in the RFU reading. Therefore, one of the limitations of this experiment is that the the analyses resazurin viability assay will be performed in the in separate wells.

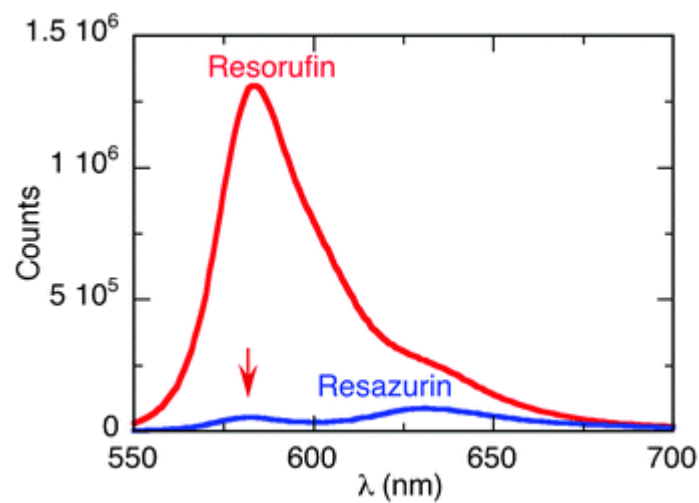


Figure 4.9. The emission wavelengths of resazurin and resorufin on excitation at 532 nm (Alejo et al., 2011)

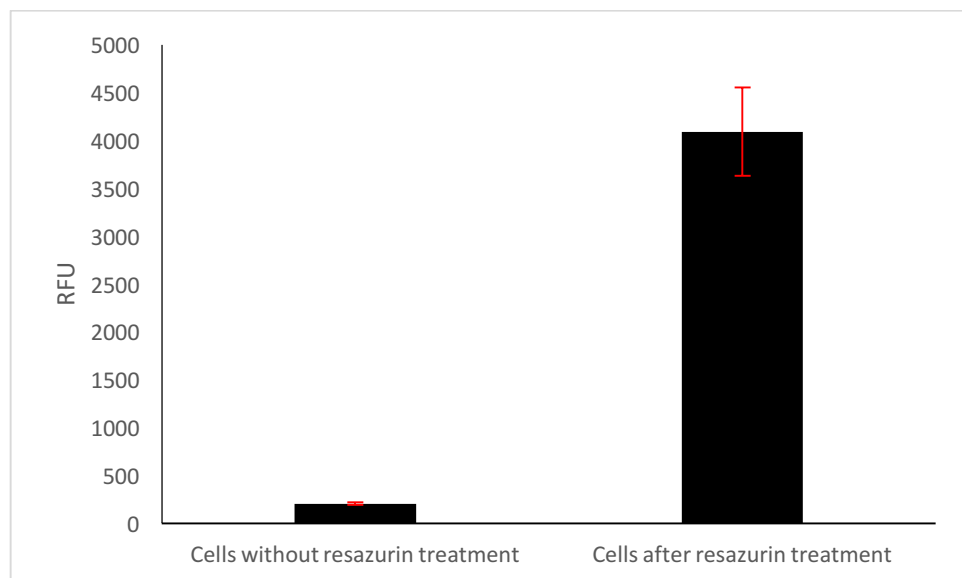


Figure 4.10. Resazurin interference in the RFU of the NFAT-DsRed reporter cell lysate sensitised with mouse IgE and stimulated with DNP-BSA. The mean $\pm$ SD values are shown (n = 4).

In another experiment, the viability assay was performed in different wells from those used for the DsRed production assay; however, the same type of transparent 96-well plate and the same conditions were used. Syk siRNA H was used, as the previous experiment showed that it gave the best results for Syk knockdown. It can be seen from Figure 4.11 that both the AGMA29–Syk siRNA H and the AGMA29–scrambled siRNA H polyplexes are not cytotoxic and the lowest viability was observed with the 15 RU/Nt AGMA29–scrambled siRNA H polyplex. However, the fluorescence percentage was highly variable between the different AGMA29–siRNA treatments. The results were also in contrast to the expectations, as the scrambled siRNA H resulted in a lower fluorescence percentage than the Syk siRNA H at all AGMA29–siRNA polyplex concentrations.

The limitations of this experiment is that viability cannot be assessed in the same cells used for the DsRed production assay. This can be solved by finding another viability assay that does not interfere with the RFU value of the NFAT-DsRed reporter cell lysate sensitised with mouse IgE and stimulated with DNP-BSA. One of the viability assay is lactate dehydrogenase (LDH) assay. LDH assay is a colorimetric assay used to evaluate the integrity of the cell membrane by quantifying the released LDH to the media due to the damage of the cell membrane. Therefore, the released LDH in the supernatant can be evaluated in

another well plate prior the lysis of the NFAT-DsRed reporter cell which will prevent the interfering of the viability result to the RFU of the NFAT-DsRed reporter cell. However, the LDH viability assay reagents are quite expensive. In addition, if the cell death occurred at any steps prior the stimulation of the cells with DNP-BSA, LDH assay can give a false viability result. The other viability assay which could be used is trypan blue exclusion assay. In this assay, the trypan blue stains the non-viable cell so the viability can be evaluated by counting the numbers of viable cells and non-viable cells in hemacytometer then finding the percentage of viable cells. However, assessing the viability in this experiment by this method is complicated because the adherent cells in each well need to be detached by trypsinisation prior the counting in the hemacytometer. One of the most convenient method for assessing viability of NFAT-DsRed reporter cell is using cell culture black well plates instead of using transparent well plates because the lysis of the cells will not be required and therefore the resazurin viability assay can be assessed in the same cells.

Based on these results, the next sections will focus on the use of the NPY-mRFP reporter cell line, rather than the NFAT-DsRed reporter cell line, for evaluating the knockdown of Syk.

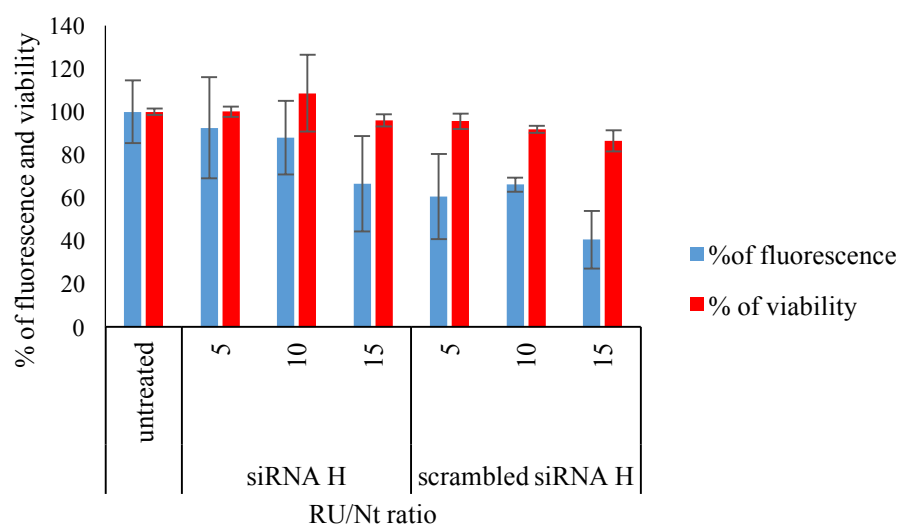


Figure 4.11. Percentage of fluorescence and viability ($\pm$ SD) after 2 to 3 days of treatment of the NFAT-DsRed reporter cell line with 5, 10 and 15 RU/Nt AGMA29–Syk siRNA polyplexes. The amount of siRNA H or scrambled siRNA H per well is 500 ng.

4.3.4 Validation of Syk protein knockdown with Western blot analysis

In this section, the level of Syk protein in the NPY-mRFP reporter cell line was evaluated 48 and 72 h after transfection of the cells with AGMA29–Syk siRNA polyplexes. The concentration of the protein in each treatment was equilibrated after the protein level was measured with the BCA assay described in section 2.3.8.2. The GAPDH protein level was used as the control for each treatment, to evaluate the relative percentage of Syk protein. If the AGMA29–Syk siRNA H polyplex is successfully transfected into the cells, the band intensity of the cells

transfected with the AGMA29–Syk siRNA H polyplex will be lower than that of the cells transfected with the AGMA29–Scrambled siRNA H polyplex at the same AGMA29 concentration. Figure 4.12 and Figure 4.13 show the western blot images obtained after 2 and 3 days of transfection, respectively. The images show that the bands representing the Syk protein are weak in intensity compared to those of the GAPDH protein; this made it difficult to determine whether knockdown of the Syk protein had occurred.

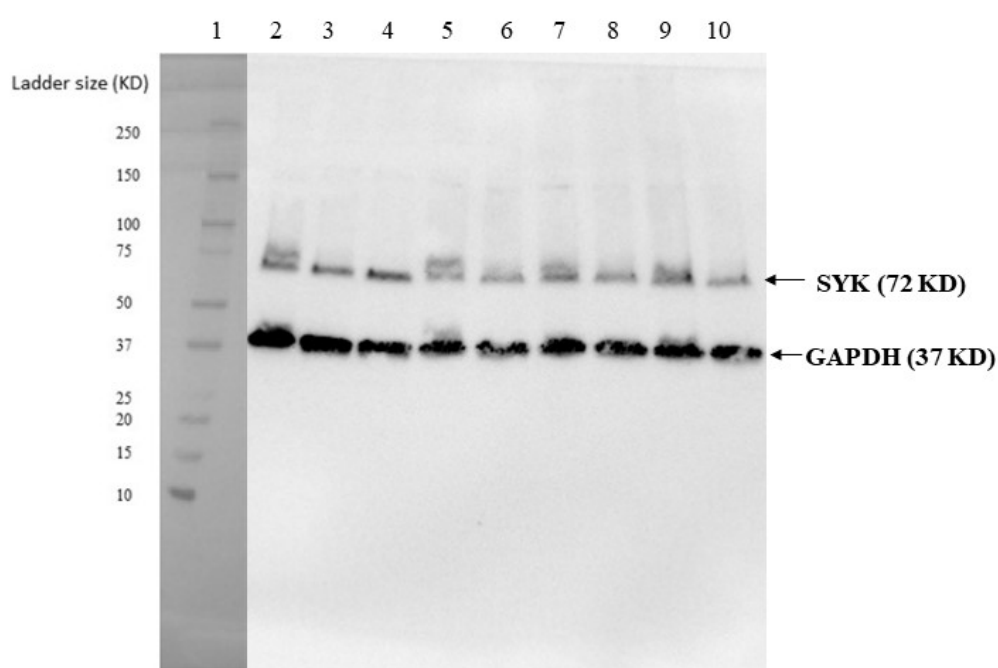


Figure 4.12. Chemiluminescence image analysis of Western blots of the Syk and GAPDH proteins in cells treated with the AGMA29–siRNA polyplex for 48 h before lysis was induced. Lane 1 represents the kaleidoscope ladder; lanes 2, 3 and 4 represent the cells treated with 5, 10 and 15 RU/Nt AGMA29–siRNA polyplexes, respectively; lane 5 represents the cells treated with the Lipofectamine–siRNA polyplex; lanes 6, 7 and 8 represent cells treated with 5, 10 and 15 RU/Nt AGMA29–Scrambled siRNA polyplexes, respectively; lane 9

represents cells treated with the Lipofectamine–Scrambled siRNA polyplex; and lane 10 represents the untreated cells.

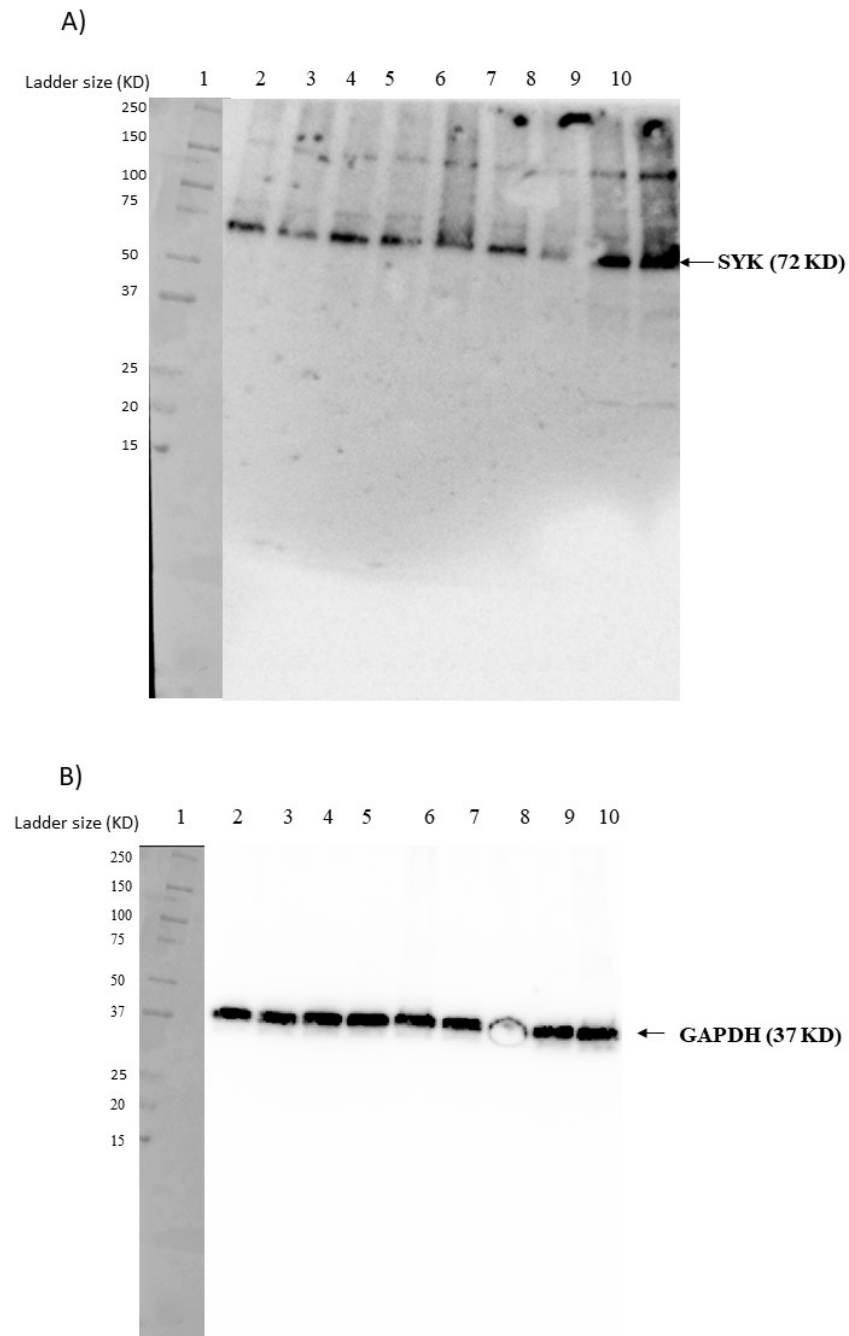


Figure 4.13. Chemiluminescence image analysis of Western blot membranes of the (A) Syk and (B) GAPDH proteins for cells treated with AGMA29–siRNA

polyplexes for 72 h before lysis was induced. Lane 1 represents the kaleidoscope ladder; lanes 2, 3 and 4 represent the cells treated with 5, 10 and 15 RU/Nt AGMA29–siRNA polyplexes, respectively; lane 5 represents the cells treated with the Lipofectamine–siRNA polyplex; lanes 6, 7 and 8 represent the cells treated with 5, 10 and 15 RU/Nt AGMA29–Scrambled siRNA polyplexes, respectively; lane 9 represents the cells treated with the Lipofectamine–Scrambled siRNA polyplex; lane 10 represents the untreated cells.

Due to the low band intensity of the Syk protein in the Western blot membrane, cells transfected with the AGMA29–Syk siRNA H polyplex were compared to cells transfected with the AGMA29–Scrambled siRNA H polyplex at the same concentration with the ImageJ software by using equation 2.2.

Figure 4.14 shows the results of the calculation of the percentage of Syk protein. The results do not show the percentage of Syk protein for 3 days of transfection with the 15 RU/Nt AGMA29–Syk siRNA H polyplex because the GAPDH band after 3 days of transfection of the cells with the 15 RU/Nt AGMA29–scrambled siRNA H polyplex could not be clearly observed in the well (lane 8, Figure 4.13). This could be due to the presence of air bubbles during the transfer of the protein from the Tris-glycine 4–20% TGX mini gels to the nitrocellulose membrane. The results show that after 2 days of transfection with 5 and 10 RU/Nt AGMA29–Syk siRNA H polyplexes, the percentage of Syk protein was

54% and 37%, respectively. 3 days after transfection, the values were 64% and 57%, respectively. No decrease in this percentage was observed with the 15 RU/Nt AGMA29–Syk siRNA H polyplex after 2 days of transfection, which confirms the previous assumption in section 4.3.2., that higher AGMA29 concentrations in the AGMA29–Syk siRNA H polyplexes result in stronger electrostatic bonding between AGMA29 and the siRNA, which hinders dissociation of the siRNA from AGMA29 and its efficient interaction with the Syk mRNA.

However, it is not clear whether the decrease in percentage of the Syk protein is due to the knockdown of Syk mRNA or not. This can be validated by using RT-qPCR, as shown in section 4.3.5.

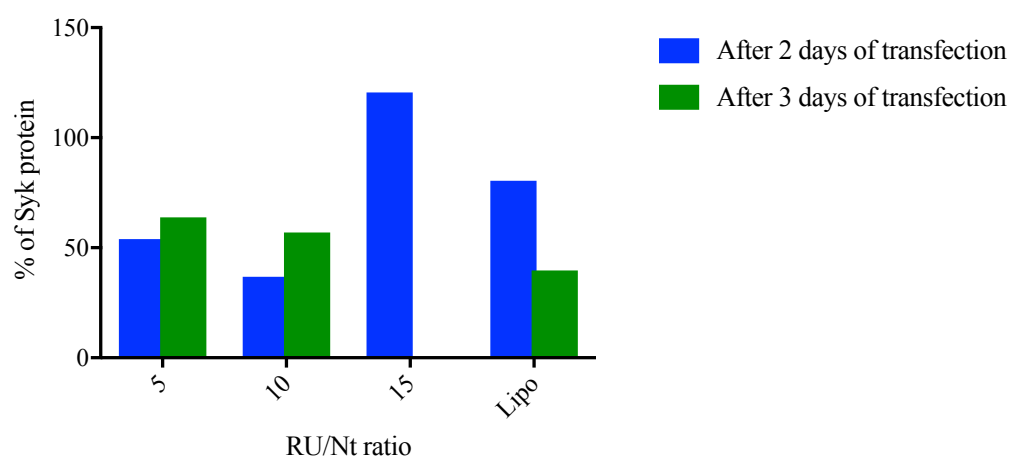


Figure 4.14. Percentage of Syk protein after 2 and 3 days of transfection of the cells with the AGMA29–Syk siRNA H polyplex compared to cells transfected with the AGMA29–Scrambled siRNA H polyplex at the same AGMA29 concentration and the same period of transfection. Lipo: Lipofectamine RNAimax. The 15 RU/Nt ratio was not measured after 3 days of transfection of

the cells because the GAPDH band with the 15 RU/Nt AGMA29–scrambled siRNA H polyplex could not be clearly observed in the well (lane 8, Figure 4.13).

4.3.5 Validation of Syk mRNA knockdown with RT-qPCR

In this section, the level of Syk mRNA was evaluated by RT-qPCR. This experiment is important for directly demonstrating the efficacy of the AGMA29–Syk siRNA H polyplex in the knockdown of Syk mRNA, because the experiments in the previous sections indirectly determined Syk mRNA knockdown by analysing Syk protein activity in RBL-2H3 cells after degranulation and the level of Syk protein by Western blot analysis. The efficacy of Syk siRNA H in the knockdown of Syk mRNA was evaluated by Sanderson et al. (2010) by cell electroporation of the RBL-2H3 cell line. In their study, they found that Syk siRNA H can result in a 59% knockdown of Syk mRNA after 2 days of transfection of the cells.

It is important to first determine whether the primers of Syk cDNA and the reference Ggt1 cDNA amplify only their target cDNA without amplifying other cDNAs. This can be determined by measuring the melting point of the amplified Syk cDNA and Ggt1 cDNA fragments. If any non-target genes are amplified, the cycle threshold (Ct) reading for the green fluorescent SyBr signal will be less than expected, which will cause an error in the calculations and final results.

Figures 4.15 and 4.18 show a single melting point for the amplified Syk CDNA and ggt1 CDNA fragments after 1 and 2 days of transfection of the cells with the 5, 10 and 15 RU/Nt AGMA29–Syk siRNA H or AGMA29–Scrambled siRNA H polyplex; thus, the amplification process was reliable. In addition, Ct for each Syk cDNA and ggt1 cDNA fragment has sigmoidal shape which indicates that the amplification process without unexpected errors occurred as shown from Figures 4.16 and 4.19.

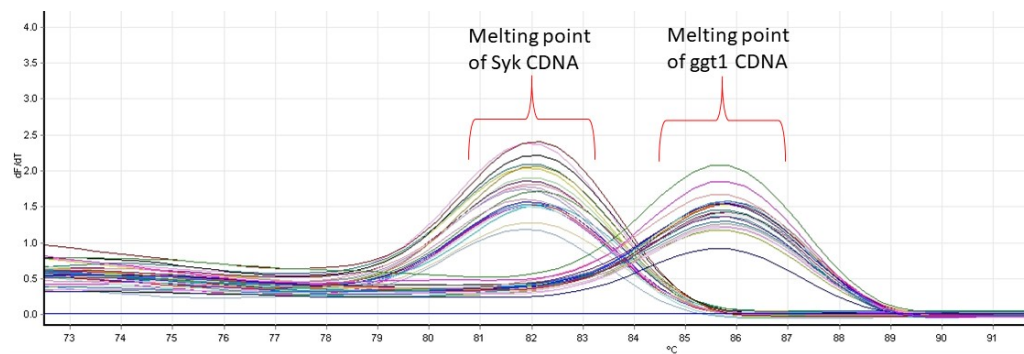


Figure 4.15. Melting points of the amplified Syk cDNA and ggt1 cDNA after 1 day of transfection with the AGMA29–Syk siRNA H or AGMA29–Scrambled siRNA H polyplex.

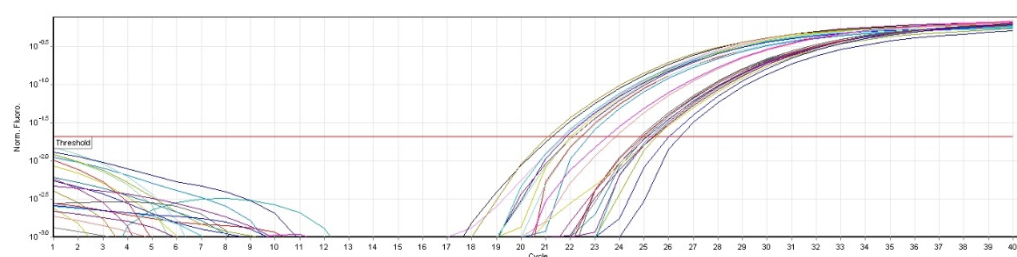


Figure 4. 16 The Ct of the amplified Syk cDNA and ggt1 cDNA after 1 day of transfection with the AGMA29–Syk siRNA H or AGMA29–Scrambled siRNA H polyplex.

The level of Syk mRNA was evaluated after 1 or 2 days of transfection of the cells, because the drop in the level of Syk mRNA was expected to appear before the decline in the level of Syk protein. There was no decline in the level of the Syk mRNA 1 day after transfection of the cells (Figure 4.17 and Table 4.1). Therefore, another experiment was performed to evaluate the level of Syk mRNA after 2 days of transfection.

The percentage of Syk mRNA (which was calculated according to equation 2.3) after transfection with the 5 and 15 RU/Nt polyplexes AGMA29/Syk siRNA H comparing to AGMA29/Scrambled siRNA H was 74% and 87%, respectively (Table 4.2). Although the percentage of Syk mRNA expression at the 10 RU/Nt concentration was 81% comparing to AGMA29/Scrambled siRNA H at the same ratio, it was not different compared with the untreated cells. Therefore, it could not be concluded that the AGMA29–Syk siRNA H polyplex can cause knockdown of Syk mRNA at an AGMA29 concentration of 10 RU/Nt.

It can be concluded from the RT-qPCR results that the lower the AGMA29 concentration, the higher is the efficiency of mRNA knockdown, since the 5 RU/Nt concentration was optimal. This result is in agreement with the findings in section 4.3.2 and section 4.3.4. However, the percentage of Syk mRNA knockdown at 5 RU/Nt of AGMA29/siRNA H is still low at 26%.

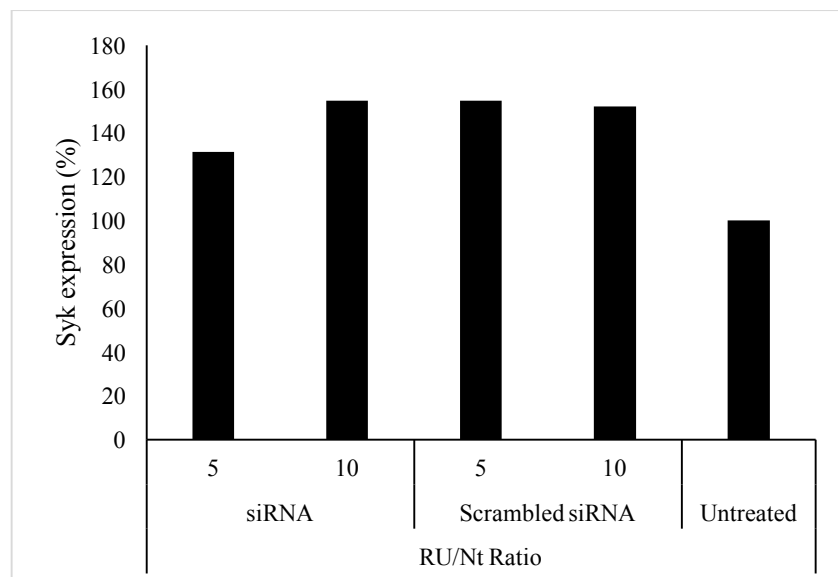


Figure 4.17. Percentage of Syk expression in the NPY-mRFP reporter cell line after 1 day of transfection with the AGMA29–Syk siRNA H or AGMA29–Scrambled siRNA H polyplex. The untreated cells represent 100% expression.

Table 4.1 Percentage of Syk expression in the NPY-mRFP reporter cell line after 1 day of transfection with the AGMA29–Syk siRNA H polyplex

RU/Nt ratio	% of Syk mRNA compared to scrambled siRNA H	% of Syk mRNA compared to untreated cells
5	85	131
10	102	154

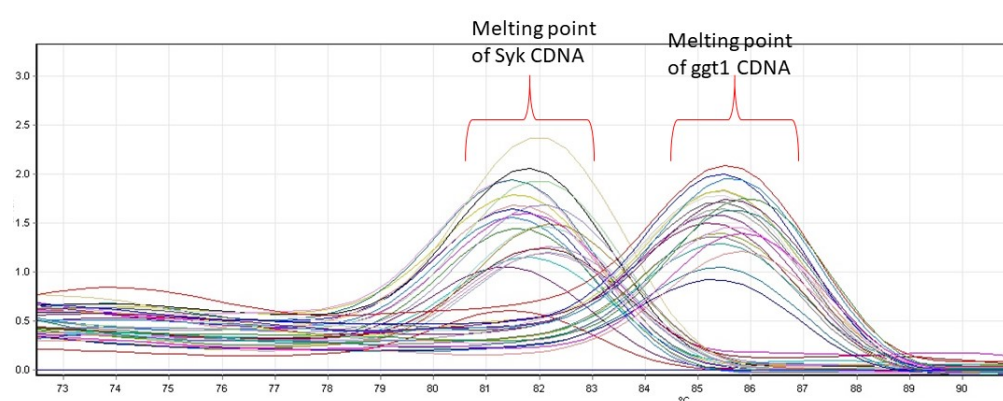


Figure 4.18. Melting point of the amplified Syk cDNA and ggt1 cDNA after 2 days of transfection with the AGMA29–Syk siRNA H or AGMA29–Scrambled siRNA H polyplex.

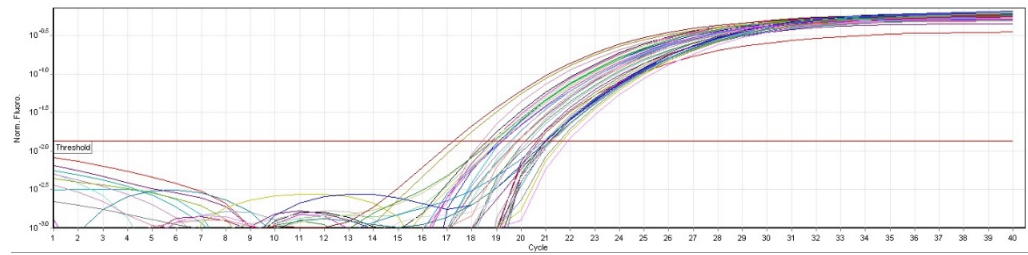


Figure 4. 19 Ct of the amplified Syk cDNA and ggt1 cDNA after 2 days of transfection with the AGMA29–Syk siRNA H or AGMA29–Scrambled siRNA H polyplex.

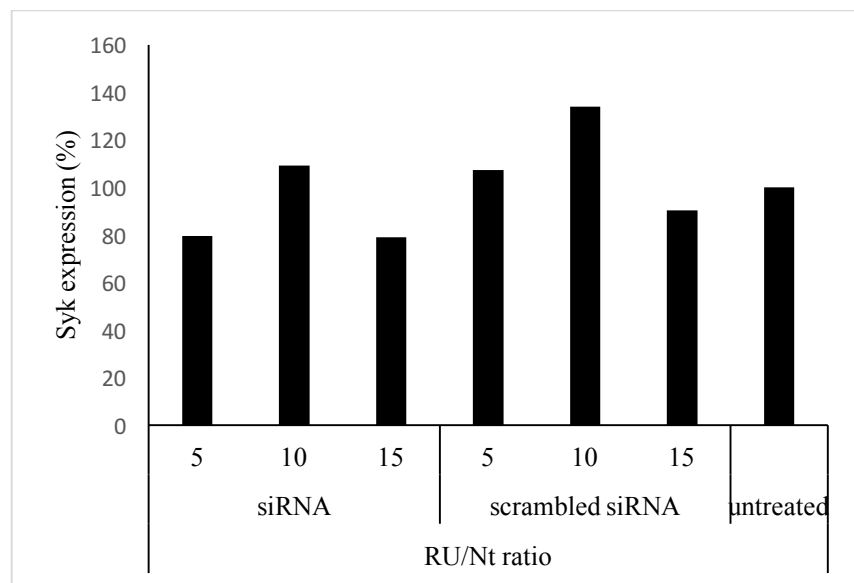


Figure 4.20. Percentage of Syk expression in the NPY-mRFP reporter cell line after 2 days of transfection with the AGMA29–Syk siRNA H or AGMA29–Scrambled siRNA H polyplex. The untreated cells represent 100% expression.

Table 4.2 Percentage of Syk expression in the NPY-mRFP reporter cell line after 2 days of transfection with the AGMA29–Syk siRNA polyplex

RU/Nt ratio	% of Syk mRNA compared to scrambled siRNA H	% of Syk mRNA compared to untreated cells
5	74	79
10	81	109
15	87	79

4.4 Conclusion

In this chapter, two reporter cell lines, NPY–mRFP and NFAT-DsRed, were evaluated for quantifying siRNA knockdown of Syk mRNA. Firstly, the optimisation of the concentration of calcium on the release of RFP in the NPY–mRFP cell line and the expression of DsRed in the NFAT-DsRed cell line was evaluated. Based on the results, a new method was established for measuring the release of RFP in the NPY–mRFP cells, which could be used for detecting allergic reactions. Thereafter, the optimal calcium conditions were used to evaluate the knockdown of Syk mRNA with the AGMA29–Syk siRNA H polyplex. The results show that AGMA29–Syk siRNA H polyplexes with lower polymer ratio have higher efficiency with regard to preventing the release of RFP in the NPY–mRFP cell line. the knockdown of Syk protein and mRNA was

evaluated by Western blot and RT-qPCR to validate the knockdown efficacy. The results shows that best knockdown of Syk mRNA was achieved by using a lower RU/Nt ratio of AGMA29/siRNA polyplex (5 RU/Nt). This could be because that at the lower ratio, siRNA can be dissociated from the AGMA29 in the cytosol to a higher extend than the higher ratio. However, low knockdown of Syk mRNA was observed from RT-qPCR. Therefore, the transfection efficiency could be enhanced with the use of cationic polymers that are more biodegradable such as polyester based polymers, as these may result in better release and dissociation of Syk siRNA H than AGMA29 which will be discussed in the next chapter.

CHAPTER 5: PHYSICOCHEMICAL CHARACTERISATION OF THE PGA-siRNA POLYPLEX

5.1 Introduction

In the previous chapters regarding the physicochemical and biological characterisation of the AGMA29/siRNA polyplex, AGMA29 was found to form a small polyplex size with siRNAs but did not show good transfection efficiency in the RBL-2H3 cell line. Therefore, in this chapter, the polyester-based polymer poly(glycerol adipate) (PGA) was evaluated, as it may have different physicochemical characteristics from AGMA29. Two novel cationic polymers synthesised from PGA were evaluated. The first polymer was PGA-50% lysine (PGA-50% Lys), in which 50% of the pendant hydroxyl groups of the polymer are covalently bound to a lysine moiety. The second polymer was PGA-20% histidine (PGA-20% His), in which 20% of the pendant hydroxyl groups of the polymer were covalently bound to histidine. Those ratios of lysine and histidine were selected as an initial evaluation to detect their efficacy on binding and forming a small polyplex size prior further modification that may be required. Those polymers have not been used previously in gene delivery and therefore, it is unknown whether they can be complexed with siRNA, form a small complex or transfect in vitro cell line successfully. Both the lysine and histidine moieties were protected by the *tert*-butyloxycarbonyl group (*t*-BOC) in α ammonium cation and ϵ ammonium cation of lysine and in α ammonium cation

of histidine. The presence of *t*-BOC groups to in the lysine and histidine amines were selected prior the synthesis of the PGA-50% Lys and PGA-20% His in order to guide the reaction to form ester bond between the hydroxyl group of PGA and carboxylic acid group in the lysine or histidine. Thereafter, the removal of *t*-BOC groups from PGA-50% Lys and PGA-20% His is required so that the protected amino groups become free primary amines that could have high electrostatic binding ability to siRNA.

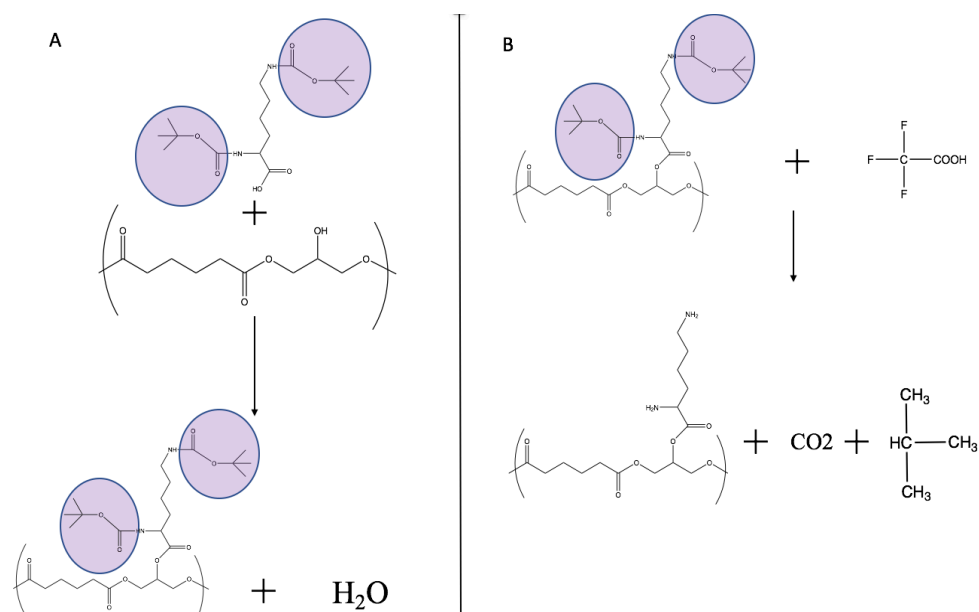


Figure 5.1 Schematic representation of the synthesis and deprotection process of PGA-50% Lys. A) Synthesis process of PGA-50% Lys. B) Deprotection process of PGA-50% Lys. Transparent purple circles highlight the *t*-BOC moiety.

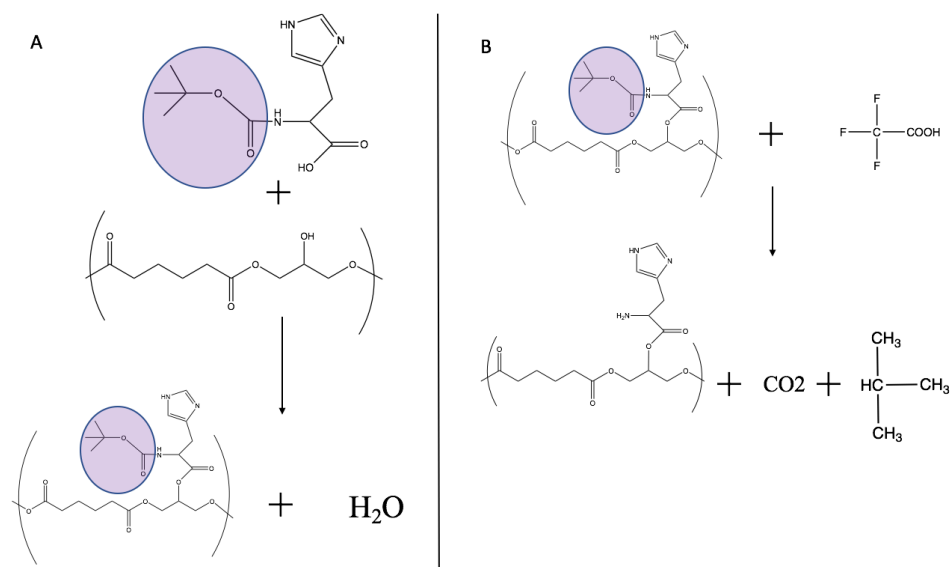


Figure 5.2 Schematic representation of the synthesis and deprotection process of PGA-20% His. A) Synthesis process of PGA-20% His. B) Deprotection process of PGA-20% His. Transparent purple circles highlight the *t*-BOC moiety

5.2 Aims and objectives

The aim of this chapter is to identify the optimum conditions for the formation of PGA-50% Lys/siRNA and PGA-20% His/siRNA polyplexes. The first step is to evaluate whether the de-protection assay can be used for the removal of *t*-BOC from the polymers without influencing their stability. The next step is to determine the pKa values of the amino functional groups of the lysine moiety in the PGA-50% Lys polymer. In the third step, the optimal amino acid

(AA)/nucleotide (Nt) ratio of the polyplex will be determined by the gel retardation assay and DLS. Finally, the optimal NaCl concentration for the formation of the polyplex will be determined.

5.3 Results and discussion

5.3.1 Chemical stability of the PGA polymers after the de-protection assay

In this section, it will be evaluated whether the deprotection conditions result in hydrolysis of the PGA polymer backbone. after the polymers were treated with trifluoroacetic acid (TFA) in the de-protection assay (section 2.2.1.1). The stability of PGA was evaluated because it could be highly biodegradable on account of the ester backbone. The molecular weight of PGA polymer can be evaluated by gel permeation chromatography (GPC). GPC is a size-exclusion chromatographic technique that can be used to separate different chemical compounds according to size (Lamba et al., 1998). In GPC, polymethylmethacrylate calibration polymers of standard sizes are eluted through a GPC column; the higher molecular weight polymers are eluted first as they have a lower retention time, while the lower molecular weight polymers are eluted later because they have a longer diffusion length in the GPC column beads and therefore have a higher retention time. The molecular weight of the PGA polymers can be determined by comparing their retention time to that of the

polymethylmethacrylate standards. The aim of the first experiment is to evaluate the stability of a PGA polymer that did not contain either lysine or histidine by comparing its Mn after treatment with TFA to the known Mn of PGA before treatment with TFA, which is 13 kDa, as reported by Taresco et al. (2016). As shown in Figure 5.3, PGA shows only one peak after TFA treatment, which corresponds to an Mn of 12.4 kDa. This result indicates that TFA does not result in degradation of the ester bond backbone, as the differences of Mn of PGA is minor before and after treatment with TFA. This minor differences can be mainly attributed to that the PGA was precipitated in petroleum ether which removes the higher and lower molecular weights impurities that was exist during the synthesis of PGA.

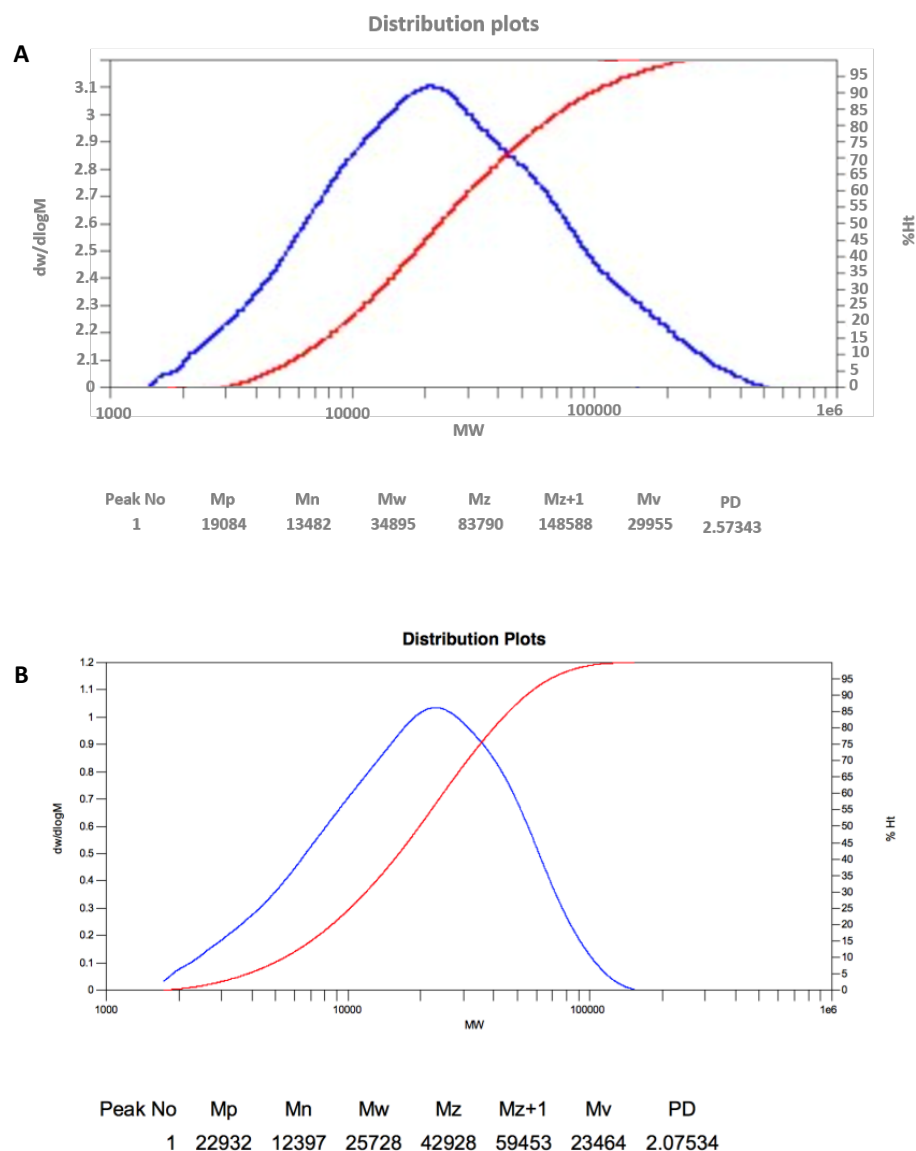


Figure 5.3 GPC result of Mn of PGA. A) Before the treatment with TFA. B) After 5 h of treatment with TFA

The size of PGA-50% Lys and the PGA-20% His cannot be determined after deprotection of the polymer from *t*-BOC by TFA because the lysine and histidine moieties in the polymer are highly protonated and can interact with the GPC column. However, it can be assumed that PGA-50% Lys and the PGA-20% His are stable after TFA treatment, because the previous results show that TFA does

not result in breakdown of the ester backbone and therefore does not affect the stability of PGA. Further confirmations of the presence of lysine moiety in the PGA-50% lys are determined by evaluating the pK_as on amine groups in lysine in PGA-50% lys in section 5.3.2 and by evaluating the binding of PGA-50% lys to siRNA in section 5.3.3.

The aim of the second experiment was to determine whether TFA can remove *t*-BOC from PGA-50% Lys and PGA-20% His. The removal of *t*-BOC was examined using ¹H-NMR. The signal of the methyl groups in *t*-BOC is expected to show a single peak at 1.43–1.44 ppm (Stella et al., 2000, Yang and Lopina, 2003). In this experiment, ¹H-NMR analysis of PGA-50% Lys and PGA-20% His was performed before and after TFA treatment. It can be seen from Figure 5.4 that in the ¹H-NMR spectrum of PGA-50% Lys, the methyl groups of *t*-BOC (numbered 43, 44, 45, 50, 51 and 52) peaked at 1.38 ppm. This peak completely disappeared after 5 h of treatment with TFA, which means that *t*-BOC was successfully removed from PGA-50% Lys.

For PGA-20% His, as shown in Figure 5.5, the methyl groups of *t*-BOC (numbered 45, 46, and 47) peaked at 1.42 ppm before TFA treatment. This peak disappeared after TFA treatment of PGA-20% His, which means that *t*-BOC was successfully removed from this polymer.

It can be concluded from the results in this section that TFA can be used for the removal of *t*-BOC from PGA-50% Lys and PGA-20% His without compromising the integrity of the polymers.

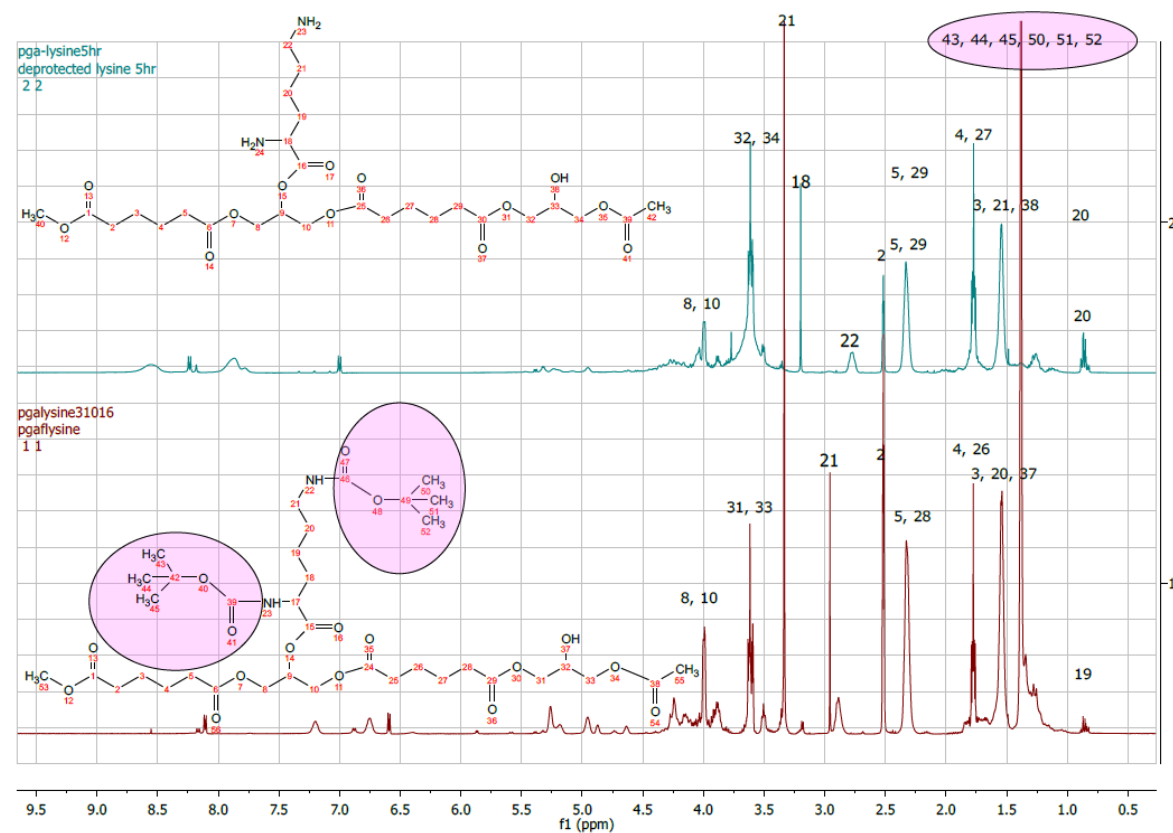


Figure 5.4 ¹H-NMR analysis of PGA-50% Lys. The red colour NMR trace is representative of *t*-BOC protected PGA-50% lys. The blue colour NMR trace was obtained after 5 h of treatment of PGA-50% Lys with TFA. Transparent red circles highlight the *t*-BOC moiety.

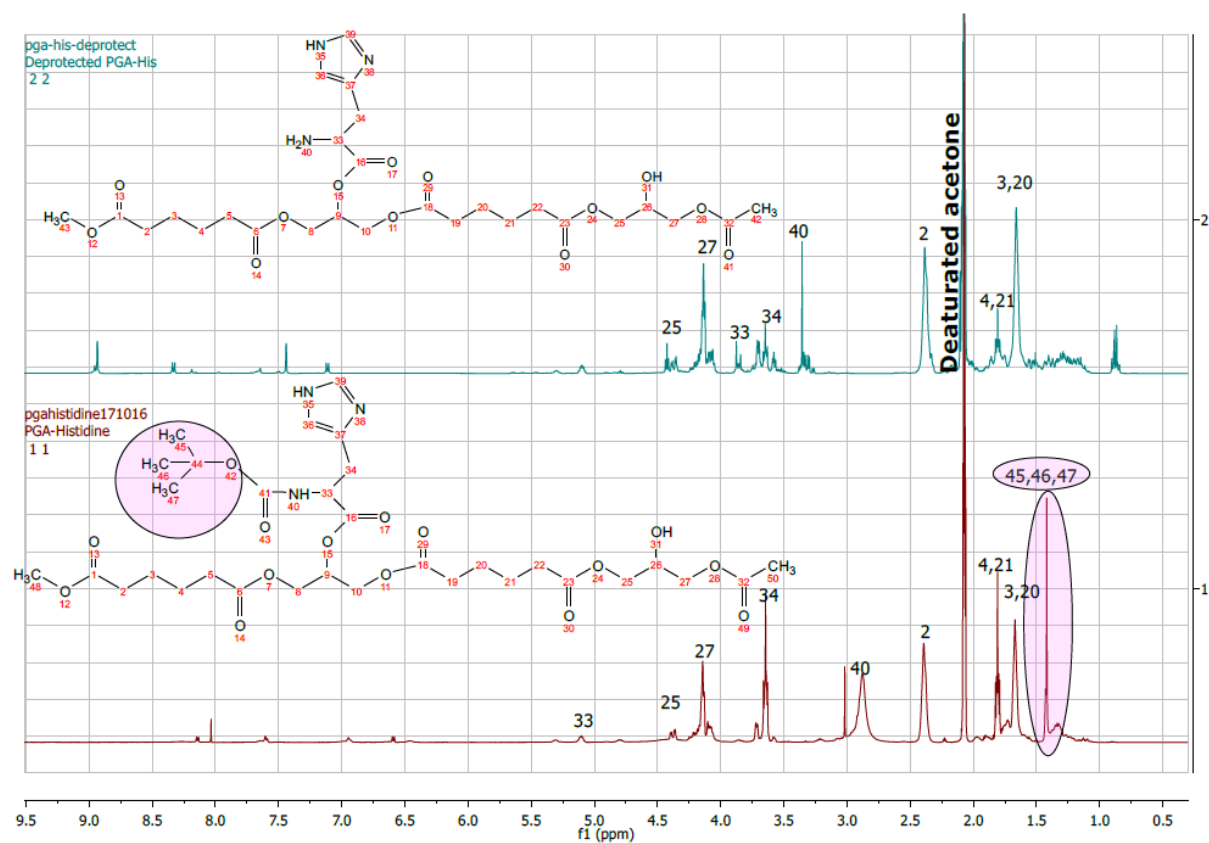


Figure 5.5 ^1H -NMR analysis of PGA-20% His. The red colour NMR peaks are representative of *t*-BOC protected PGA-20% his. The blue colour NMR peaks were obtained after 5 h of treatment of PGA-20% His with TFA. Transparent red circles are the *t*-BOC moiety.

5.3.2 Titration curve of PGA-50% Lys

In this experiment, the pKa values of the amine groups of lysine in PGA-50% Lys were determined by titrating the PGA-50% Lys solution under acidic conditions with 0.1 M NaOH and measuring the changes in pH. The pKa of an amine group can be detected in the area that shows resistance to changes in pH. It has been reported that the pKa of the α ammonium cation of lysine (which is attached to the same carbon atom as the carboxylic group of lysine) is 8.95, whereas the pKa of the ϵ ammonium cation of lysine is 10.52 (Dewick, 2006). Figure 5.6 shows the titration curve of the PGA-50% Lys solution under acidic conditions with 0.1 M NaOH. It can be seen from the results that the solution shows resistance to changes in the pH from pH 2.3 until 3.1, which is due to the reaction between the titrant NaOH and the HCl in the solution. Thereafter, the inflection points were observed at pH 3.1 and 8.9 and the equivalence point was approximately 5.9. Thereafter, resistance to pH changes was then observed from pH 8.9 until pH 11.3 and the highest change in pH that was observed at pH 9.8 which might be the second equivalence point. From the estimated two equivalence points, the pKa of α ammonium cation of lysine might be 9.05 which is the average point of the buffering region between the two estimated equivalence points. The end point of the titration that can show change in the slope was not observed so it is estimated that 11.3 is the end point. The pKa of the ϵ ammonium cation of lysine might be 10.9.

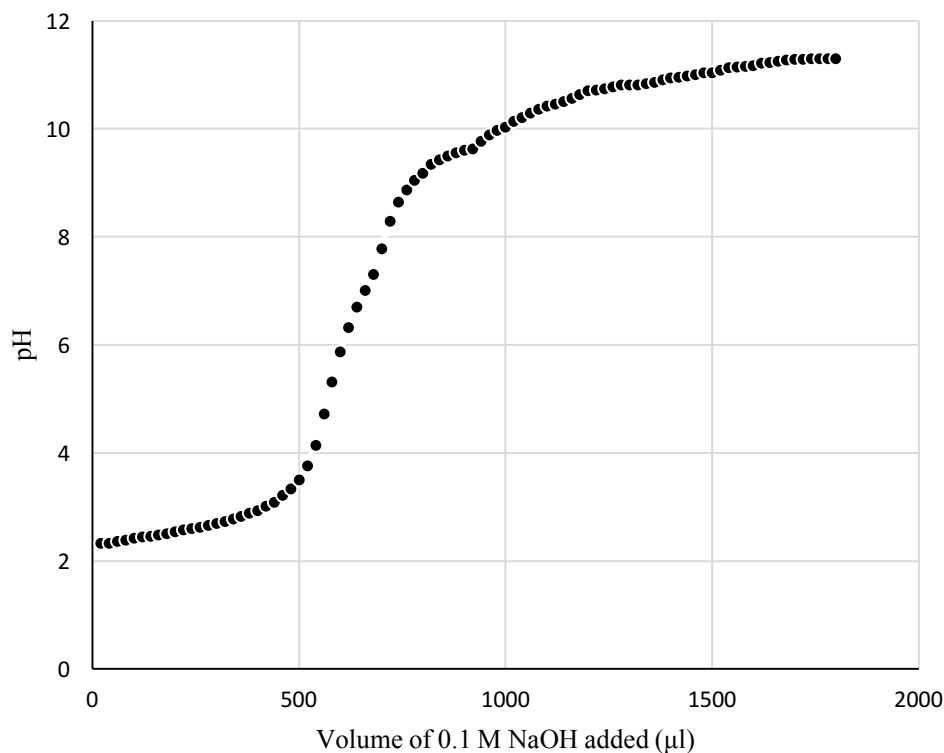


Figure 5. 6 Titration curve of PGA-50% Lys

5.3.3 Complexation behaviour and size of the PGA-siRNA polyplexes

In this section, the binding properties of de-protected PGA-20% His and PGA-50% Lys with siRNA were evaluated by a gel retardation assay, for which the polymers were dissolved in pure water and the pH was adjusted to 7. The ratio polymer/siRNA is expressed as amino acid per nucleotide (AA/Nt) ratio because PGA-50% Lys has one positively charged amino acid lysine per 2 repeating

units and PGA-20% His has one positively charged amino acid histidine per 5 repeating units. Figure 5.7 shows the binding of PGA-20% His to siRNA at different AA/Nt ratios. It can be seen from the results that the siRNA in all the lanes moved toward the anode. This indicates that PGA-20% His did not bind to the siRNAs even at high AA/Nt ratios. This is probably because only 20% of the RUs of PGA substituted with histidine and in which the PGA-20% His is less soluble in water than PGA-50% Lys. In histidine, only α ammonium has a high pKa value of 9.17, whereas the pKa of imidazole is 6.04 (Dewick, 2006, Garrett and Grisham, 2009). Therefore, it is expected that the histidine moiety has less electrostatic interaction to negatively charged siRNA than lysine moiety at neutral pH. However, the complete lacking of binding of PGA-20% His to siRNA was not expected because α ammonium of histidine moiety is highly cationic which can show at least a partial binding to siRNA. This may be due to that the integrity of the histidine moiety was affected by the deprotection process. The histidine moiety was used by Stevenson et al. (2008) to enhance the efficiency of their reducible polycation polylysine (called CK₁₀C) for siRNA and pDNA delivery, since the imidazole in histidine has a high buffer capacity in the low pH endosome, which protects the siRNA from degradation during endocytosis. Moreover, histidine was used by Midoux and Monsigny (1999) for the modification of polylysine for the delivery of pDNA, which has a higher transfection efficiency than polylysine alone. In their study, they conclude that the incorporation of histidine in polylysine enhances its transfection efficiency because it allows for escape from the endosome rather than enhancing the encapsulation and cellular uptake of the polyplex. Based on these previous works and the present result, it can be concluded that it is preferable to use

histidine to enhance the transfection efficiency than to use it as a main cationic moiety for complexation with siRNA.

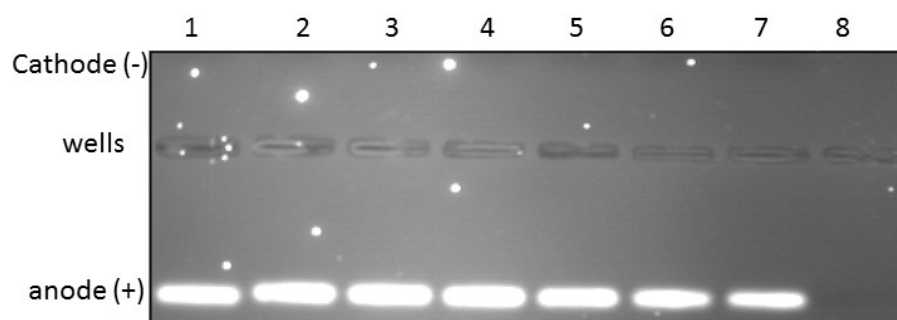


Figure 5. 7 The binding properties of PGA-20% His to siRNA as demonstrated by the gel retardation assay. Lane 1 contains free siRNA. Lanes 2, 3, 4, 5, 6 and 7 contain polyplexes with AA:Nt ratios of 1:1, 2:1, 3:1, 4:1, 5:1 and 10:1, respectively. Lane 8 contains free polymer.

Figure 5.8 shows the binding of PGA-50% Lys to siRNA in agarose gel. It can be seen from the figure that the binding of PGA-50% Lys to siRNA began to increase at an AA/Nt ratio of 3 (lane 4), as the ethidium bromide fluorescence intensity at the anode was low at this ratio. Complete complexation was observed at an AA/Nt ratio (lane 7) of 10, since at this ratio, ethidium bromide fluorescence was detected in the well and but not at the anode site. In Figure 5.9, the estimated percentage of free siRNA that did not bind to PGA-50% Lys compared to the siRNA alone in lane 1 was determined.

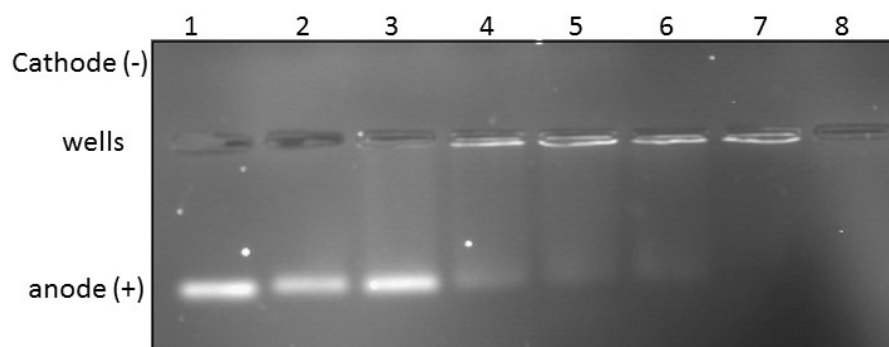


Figure 5. 8 The binding rate of PGA-50% Lys to siRNA was demonstrated by the gel retardation assay. Lane 1 contains free siRNA. Lanes 2, 3, 4, 5, 6 and 7 contain polyplexes with AA:Nt ratios of 1:1, 2:1, 3:1, 4:1, 5:1 and 10:1, respectively. Lane 8 contains free polymers.

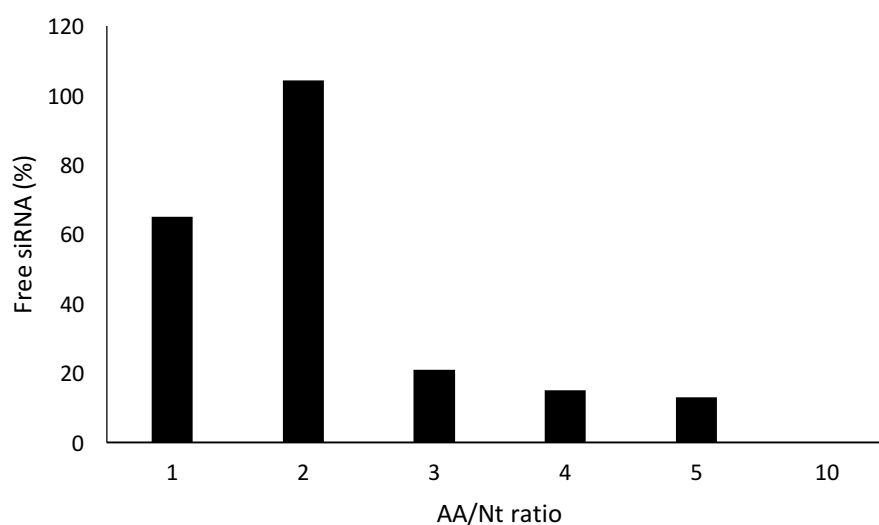


Figure 5.9 Agarose gel analysis of the percentage of free siRNA that did not bind to PGA-50% Lys at different AA:Nt ratios

The size of the PGA-50% Lys/siRNA polyplexes was evaluated as shown in Figure 5.10. The size was the greatest at an AA:Nt ratio of 3, with the diameter of the polyplex being 286 ± 39 nm. The size of the PGA-50% Lys/siRNA polyplex then decreased after further increase in the AA:Nt ratio, until it reached its lowest value at the AA:Nt ratio of 10, at which point the diameter was 191 ± 37 nm.

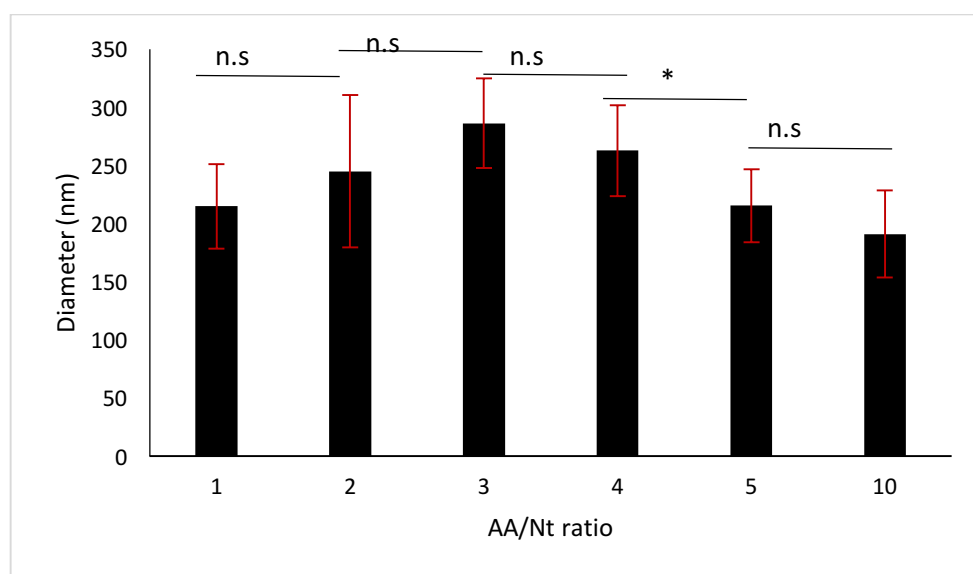


Figure 5.10 The size of the PGA-50% Lys/siRNA at different AA/Nt ratios. A two-tailed *t*-test with different variables was used to compare each ratio with the adjacent ratio. P value < 0.05 (*), n.s. = not significant ($p > 0.05$) ($n = 8$).

5.3.4 Effect of NaCl on the binding and size of the PGA-50% Lys/siRNA polyplex

In this section, in order to determine the optimal conditions for polyplex formation, the effect of different NaCl concentrations on the binding of PGA-

50% Lys to siRNA was evaluated. The formation of the PGA-50% Lys/siRNA polyplex at an AA/NT ratio of 3 was examined under NaCl concentrations ranging from 0 to 150 mM at pH 7. As can be observed in the agarose gel in Figure 5.11, the lowest ethidium bromide fluorescence intensity (which is considered to be correlated to the amount of free siRNA that did not bind to the polymer) at the anode site was unclear. Therefore, the band intensities were determined by measuring the band intensity at the anode site for each NaCl concentration and comparing it to the band intensity of siRNA alone in Lane 1 (as shown in Figure 5.12).

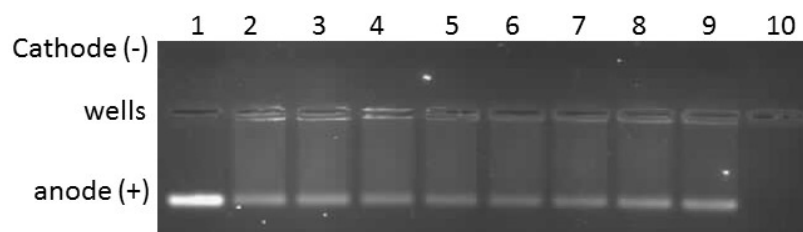


Figure 5.11 The effect of different NaCl concentrations on the binding of PGA-50% Lys to siRNA at an AA/NT ratio of 3. Lane 1 contains free siRNA. The NaCl concentration in lanes 2, 3, 4, 5, 6, 7, 8 and 9 are 0, 10, 20, 30, 50, 80, 110 and 150 mM respectively. Lane 10 contains free polymer.

It can be observed from Figure 5.11 that the percentage of free siRNA that did not bind to PGA-50% Lys decreases as the concentration of NaCl increases, until it reaches its lowest percentage (29%) at the NaCl concentration of 50 mM. Further increase in NaCl concentration results in an increase in the percentage of free siRNA, which becomes 50% at 150 mM NaCl.

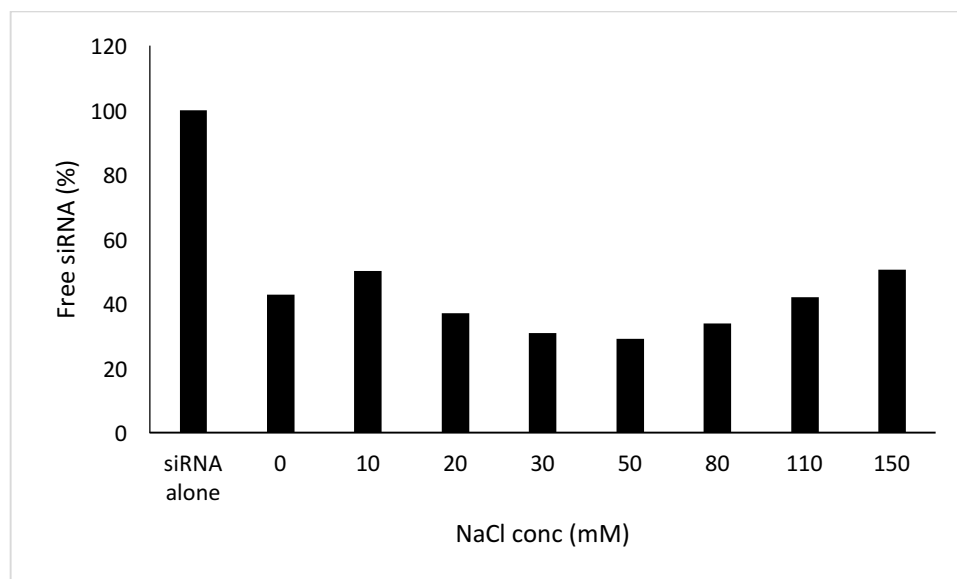


Figure 5.12 Agarose gel analysis of the percentage of free siRNA that did not bind to PGA-50% Lys at the AA:Nt ratio of 3 at different concentrations of NaCl.

Another experiment was performed at an AA:Nt ratio of 10, at which full complexation may be formed in the presence or absence of NaCl. Figure 5.13 confirms that at the AA:Nt ratio of 10, the PGA-50% Lys/siRNA polyplex can form a full complex at all NaCl concentrations ranging from 0 to 150 mM, since no ethidium bromide band was observed at the anode site. It can be observed from Figure 5.14 that the diameter of the PGA-50% Lys/siRNA polyplex at an AA:Nt ratio of 10 increased with increase in the NaCl concentration, from 202 nm in the absence of NaCl to 1000 nm in the presence of 150 mM NaCl. From this result, it could be concluded that the optimal condition for formation of the PGA-50% Lys/siRNA polyplex is deionised water, since an increase in NaCl concentration can lead to an increase in the diameter of the formed polyplex suggesting a negative influence the stability of the polyplex. While lower salt

concentrations up to 10mM have a small effect, higher concentrations have a much greater influence.



Figure 5. 13 Effect of NaCl on the binding of PGA-50% Lys to siRNA at an AA/Nt ratio of 10 according to the gel retardation assay. Lane 1 contains free siRNA. Lanes 2, 3, 4, 5, 6 and 7 contain polyplexes formed at NaCl concentrations of 0, 10, 20, 50, 110 and 150 mM respectively. Lane 8 contains free polymer.

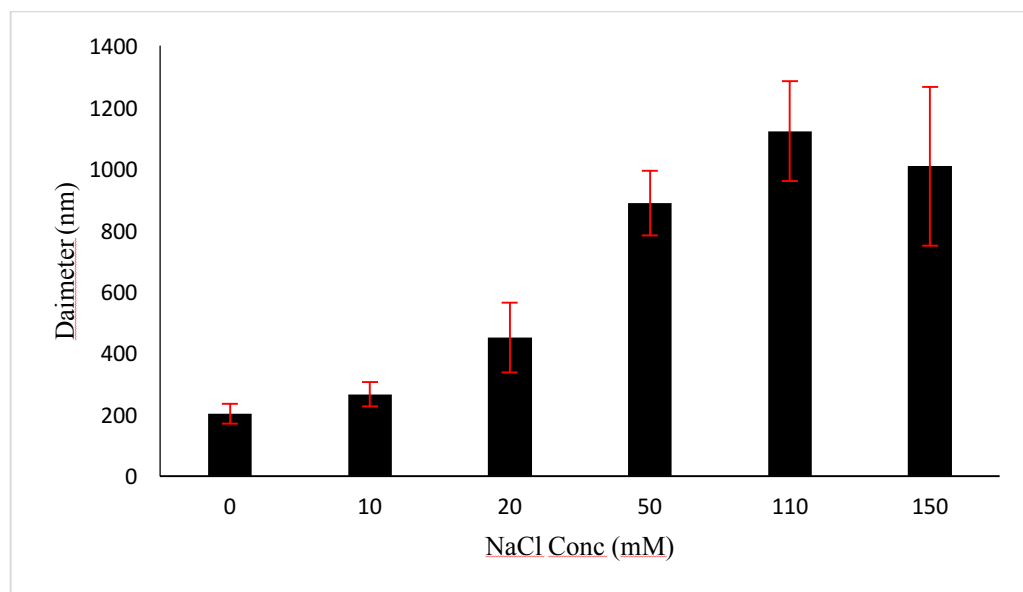


Figure 5. 14 Effect of NaCl on the size of the PGA-50% Lys/siRNA complex at an AA:Nt ratio of 10

5.4 Conclusion

In conclusion, physicochemical characterisation of PGA derived polymers were evaluated in terms of forming a stable polyplex with siRNA. The results show that TFA can successfully remove *t*-BOC from PGA derived polymers without causing significant cleavage of the polymer backbone. Further, PGA-50% Lys can bind to siRNA successfully and form a stable polyplex at an AA/Nt ratio of 10. However, PGA-20% His did not bind to siRNA, which was probably because integrity of the histidine moiety was affected by the deprotection process and the low percentage of histidine that is present in the polymer. Finally, the results showed that preparation of the PGA-50% Lys/siRNA

polyplex in deionised water results in the formation of more stable polyplexes than preparation in NaCl solutions, with significant effects above 10mM.

CHAPTER 6: SYK KNOCKDOWN ASSAYS OF THE PGA-50% Lys POLYMER

6.1 Introduction

In the previous chapter, it was observed that the PGA-50% Lys polymer can bind to siRNA more efficiently than PGA-20% His. Therefore, this chapter will focus only on PGA-50% Lys/Syk siRNA H knockdown of Syk mRNA and protein. Even though the AGMA29/siRNA polyplex has a smaller diameter (90 nm) than the PGA-50% Lys/siRNA polyplex (191 nm) at an AA/Nt ratio of 10, the PGA-50% Lys/siRNA polyplex could be more biodegradable due to the presence of an ester group, which may enable easy release of the siRNA to its target mRNA if there is degradation in the lysosomal compartment.

The disassembly of nucleic acids from their cationic polymer vectors is one of the major barriers to effective gene transfection (Read et al., 2003). For example, Schaffer et al. (2000) evaluated fluorescently labelled polylysines of different molecular weight (MW) stained with the Alexa 488 dye complexed with fluorescently labelled pDNA with Alexa 546. They concluded from their results that pDNA is probably localised in the nucleus and dissociates from lower MW polylysines more efficiently than it does from higher MW polylysines. In

addition, they found that the transcription of pDNA is significantly lower when higher MW polylysine is used or a higher AA/Nt ratio is used.

PGA-50% Lys might allow efficient siRNA delivery. This is because the PGA monomers that make up its backbone contain esters, and the carboxylic group of lysine covalently binds to the hydroxyl group of PGA to form a highly biodegradable ester bond that is hydrolysed to release the siRNA. In contrast, in polylysine, an amide group is formed by the binding of the carboxyl group in one lysine moiety to the α ammonium group of the adjacent lysine moiety.

In this chapter, we have used PGA-50% Lys/Syk siRNA H polyplex for the transfection of NPY-mRFP reporter cell line to address the question of the biodegradability of the polymer.

6.2 Aims and objectives

The aim of this chapter is to analyse the ability of the PGA-50% Lys/Syk siRNA H polyplex to knock down the level and function of the Syk protein in the NPY-mRFP reporter cell line. First, inhibition of RFP release in NPY-mRFP reporter cells transfected with the PGA-50% Lys/Syk siRNA H polyplex will be evaluated. Thereafter, the Syk protein level will be measured by western blot analysis. Finally, the changes in Syk mRNA levels in NPY-mRFP reporter cells

transfected with Syk siRNA H will be assessed by real-time quantitative PCR (RT-qPCR) and compared to that in untreated cells and cells transfected with scrambled siRNA H.

6.3 Results and discussion

6.3.1 Efficacy of the PGA-50% Lys/Syk siRNA H polyplex in the inhibition of Syk protein function

In this section, the efficacy of PGA-50% Lys/Syk siRNA H in inhibiting degranulation of NPY-mRFP cells by the Syk protein was evaluated by the fluorescence release assay (described in section 2.2.2.4). Moreover, the viability of these cells was evaluated by the resazurin toxicity assay (described in section 2.2.2.6). As reported in Chapter 4, 500 ng of Syk siRNA H seems to be optimal for transfection. Figure 6.1 shows the fluorescence and viability results obtained with the NPY-mRFP reporter cell line. The result shows that in the cells transfected with PGA-50% Lys/Syk siRNA H, the fluorescence was lower than that in the scrambled siRNA-transfected cells only at the AA/Nt ratio of 10 (69% vs. 92%). Moreover, the viability in all the groups was higher than 92%, which indicates that PGA-50% Lys was not cytotoxic. This means that the decrease in fluorescence observed with 10 AA/Nt PGA-50% Lys/Syk siRNA H is not caused by a decrease in cell viability. The decrease in the percent fluorescence with the 10 AA/Nt PGA-50% Lys/Syk siRNA H was consistent with the

previous result in section 5.3.3, full complexation can occur and small-sized polyplexes are formed at this ratio. However, further validation of Syk protein knockdown is required by western blot analysis.

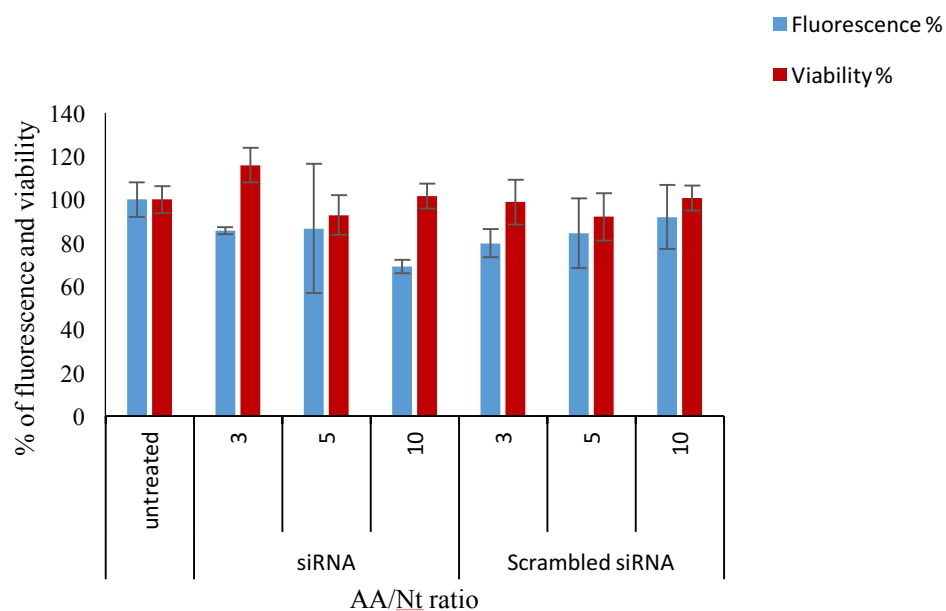


Figure 6.1. Percentage fluorescence and viability (mean $\pm$ SD values) in the NPY-mRFP reporter cell line after siRNA treatment for two days. PGA-50% Lys/Syk siRNA and PGA-50% Lys/scrambled siRNA were used at AA/Nt ratios of 3, 5 and 10. The amount of siRNA H or scrambled siRNA H per well is 500 ng. The cells were sensitised with DNP-specific murine IgE and then stimulated with DNP-BSA. The untransfected cells represent 100% fluorescence. This experiment has been performed in triplicate.

6.3.2 Validation of Syk protein knockdown by Western blot analysis

In this section, the level of Syk protein in the NPY-mRFP reporter cell line transfected with PGA-50% Lys/Syk siRNA H was evaluated by Western blot analysis. Previously, in Western blot analysis of the AGMA29/Syk siRNA H polyplex-treated cells described in section 4.3.4, the chemiluminescence bands of Syk in the nitrocellulose membranes appeared weak in general (Figure 4.12A and Figure 4.13A). Therefore, the concentration of the anti-Syk antibody SYK-01 that was used in this experiment was increased from 2 $\mu\text{g}\cdot\text{ml}^{-1}$ to 4 $\mu\text{g}\cdot\text{ml}^{-1}$. Figure 6.2 shows the chemiluminescence images of a nitrocellulose Western blot membrane that was treated with primary and secondary antibodies against Syk and GAPDH. NPY-mRFP reporter cells were transfected with the PGA-50% Lys/Syk siRNA H polyplex or PGA-50% Lys/scrambled siRNA H two days before they were lysed for protein extraction. It can be seen from Figure 6.2 that the GAPDH bands in lanes 2, 3 and 4, which represent AA/Nt ratios of 3, 5, and 10, respectively, are not clear. Therefore, only the 20 AA/Nt PGA-50% Lys/Syk siRNA H polyplex is evaluated. At this ratio, the percentage expression of Syk was 52% compared to the scrambled siRNA H-transfected cells and 50% compared to the untreated cells (Equation 2.2 from section 2.2.2.7.4 was used to determine the percentage expression) as shown in Figure 6.4.

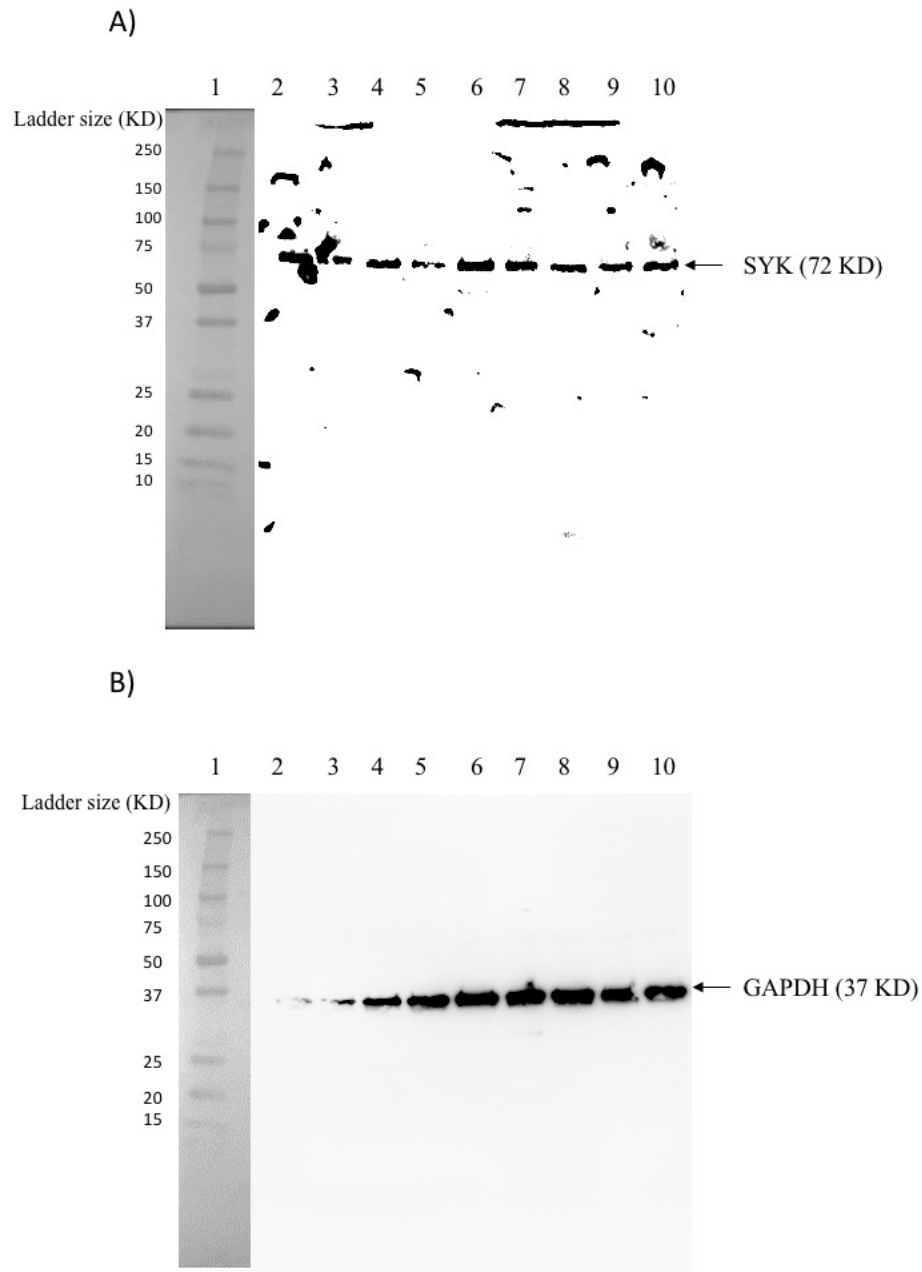


Figure 6.2. Western blot membrane chemiluminescence image analysis of the (A) Syk and (B) GAPDH proteins after siRNA treatment for 2 days. Lane 1 shows the kaleidoscope ladder; lanes 2, 3, 4, and 5 represent cell lysates treated with the PGA-50% Lys/Syk siRNA H polyplex at AA/Nt ratios of 3, 5, 10 and 20, respectively; lanes 6, 7, 8 and 9 represent cell lysates treated with PGA-50% Lys/scrambled siRNA H polyplexes at AA/Nt ratios of 3, 5, 10 and 20 respectively; lane 10 represents the untreated cell lysate.

Another Western blot experiment was performed to investigate the level of Syk protein 3 days after transfection of the NPY-mRFP cells with the PGA-50% Lys/Syk siRNA H polyplex. That is, this experiment was performed to determine whether the level of Syk protein decreases any further after three days of transfection as compared to two days. However, from the band intensities of the Syk and GAPDH proteins observed in Figure 6.3, it seems that there was no decrease in the level of Syk protein in the cells transfected with PGA-50% Lys/Syk siRNA H compared to the cells transfected with PGA-50% Lys/scrambled siRNA H at all AA/Nt ratios as shown in Figure 6.4. This is probably because the transfection efficiency was low, or Western blot is not ideal as a quantitative method for detection of the Syk protein level. A third reason could be that the Syk protein is highly stable in that even if the knockdown of Syk mRNA occurs, the existing Syk protein has a long half-life ($t_{1/2}$) – this will be discussed below. Therefore, further investigation into the Syk mRNA level after treatment of the cells with the PGA-50% Lys/Syk siRNA H polyplex is required.

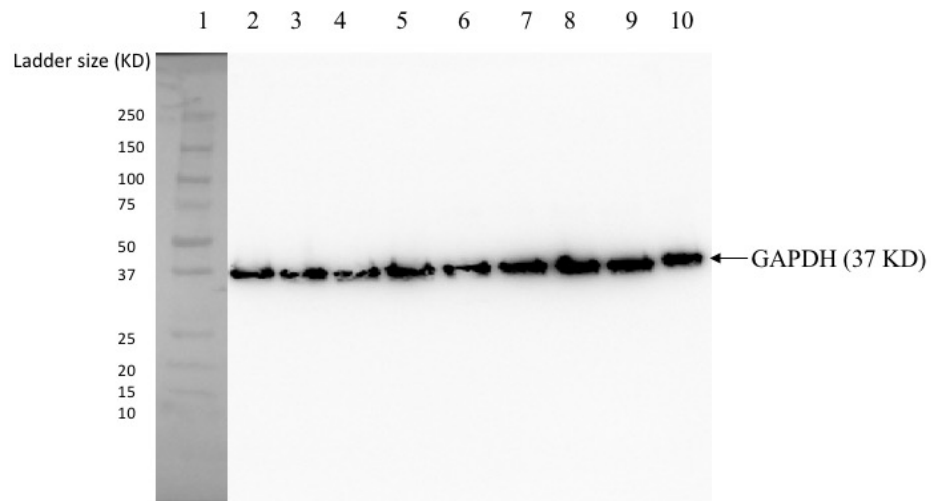
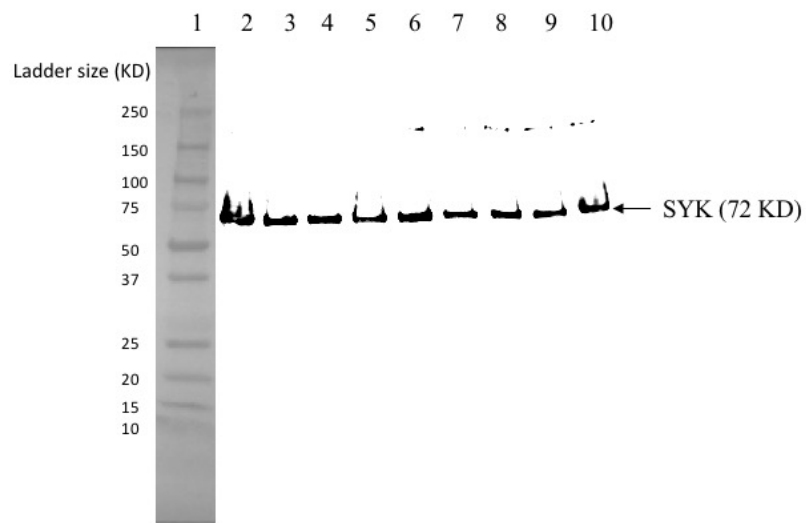


Figure 6.3. Western blot membrane chemiluminescence image analysis of the (A) Syk and (B) GAPDH proteins after siRNA treatment for 3 days. Lane 1 represents the kaleidoscope ladder; lanes 2, 3, 4 and 5 represent cell lysates treated with the PGA-50% Lys/siRNA polyplexes at AA/Nt ratios of 3, 5, 10 and 20, respectively; lanes 6, 7, 8 and 9 represent cell lysates treated with the

PGA-50% Lys/scrambled siRNA polyplexes at AA/Nt ratios of 3, 5, 10 and 20:1, respectively; lane 10 represents the untreated cell lysate.

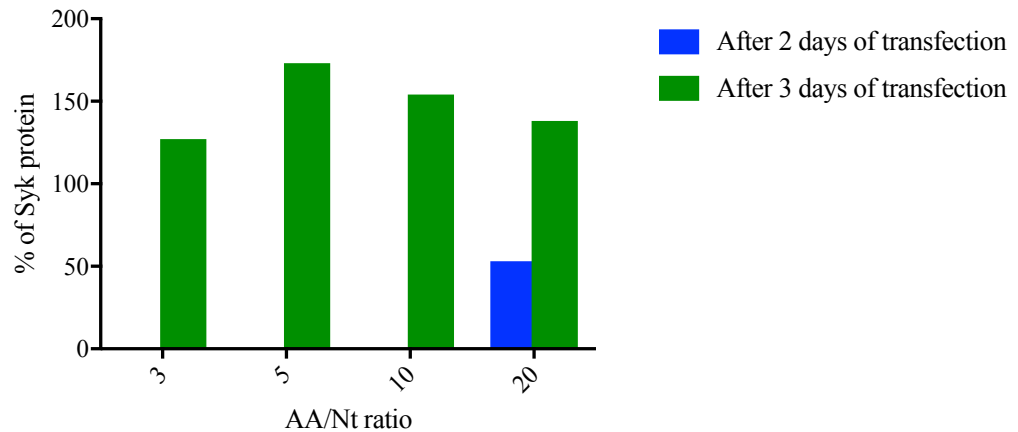


Figure 6. 4 Percentage of Syk protein after 2 and 3 days of transfection of the cells with the PGA-50% Lys/siRNA polyplexes compared to cells transfected with the PGA-50% Lys/ Scrambled siRNA H polyplexes at the same AA/Nt ratio and the same period of transfection. The Percentage of Syk protein after 2 days of transfection of the cells at AA/Nt ratios of 3, 5, and 10 was not measured because GAPDH bands in lanes 2, 3 and 4 (Figure 6.2, B), which represent AA/Nt ratios of 3, 5, and 10, respectively, are not clear.

6.3.3 Validation of Syk mRNA knockdown by RT-qPCR

Changes in the level of Syk mRNA after transfection of the NPY-mRFP reporter cell line with the PGA-50% Lys/Syk siRNA H polyplex was evaluated by RT-qPCR. The first experiment in this section was the evaluation of Syk cDNA after one day of transfection of the cells with the polyplex. A single copy gene (rat Gamma-glutamyl transferase) was used as reference for normalisation. As shown in the melting curve in Figure 6.5, a single defined melting point is present for each amplified Syk cDNA and each amplified Gamma-glutamyltransferase 1(Ggt1) cDNA; this indicates that only the targeted genes were amplified by the primers. Also, Ct for each Syk cDNA and ggt1 cDNA fragment has sigmoidal shape as shown in Figure 6.6. Figure 6.7 shows the RT-qPCR results after one day of knockdown of Syk mRNA in the NPY-mRFP reporter cells by PGA-50% Lys/Syk siRNA H. Interestingly, the level of Syk mRNA expression in the PGA-50% Lys/Syk siRNA H group was much lower than that in the untreated cells. However, scrambled siRNA H also seemed to have an off-target inhibitory effect on Syk mRNA expression compared to the untreated cells, although this is somewhat weaker than that of Syk siRNA H, as shown in Table 6.1.

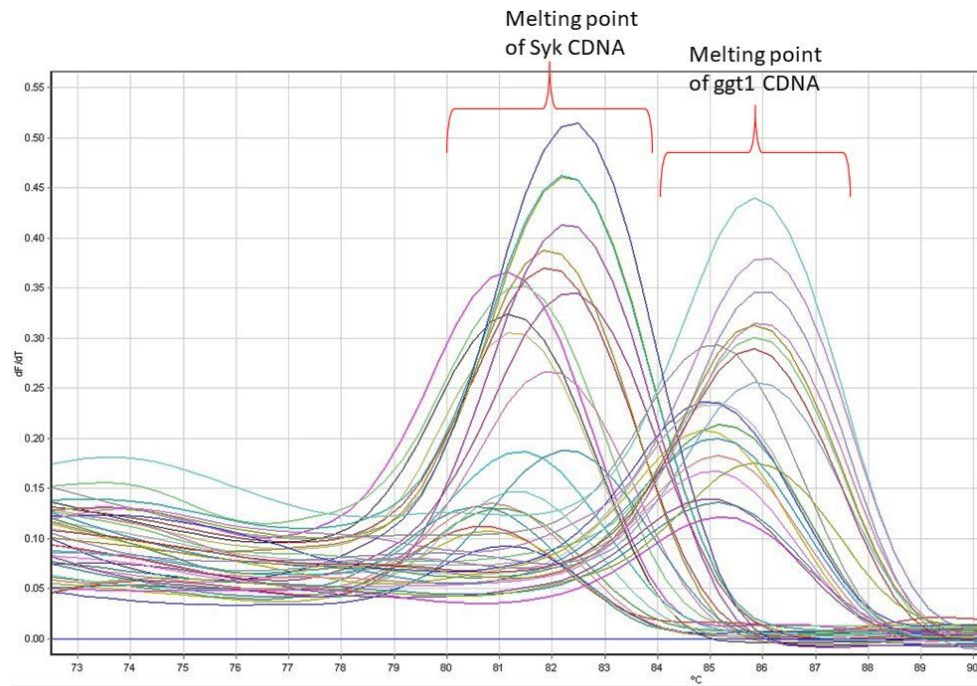


Figure 6.5. Melting points of the amplified Syk cDNA and ggt1 cDNA after one day of transfection with PGA-50% Lys/Syk siRNA H, PGA-50% Lys/scrambled siRNA H, and untreated cells.

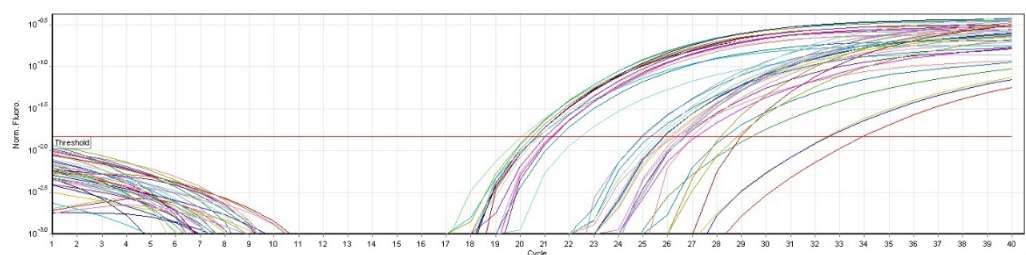


Figure 6. 6 Ct of the amplified Syk cDNA and ggt1 cDNA after one day of transfection with PGA-50% Lys/Syk siRNA H, PGA-50% Lys/scrambled siRNA H, and untreated cells.

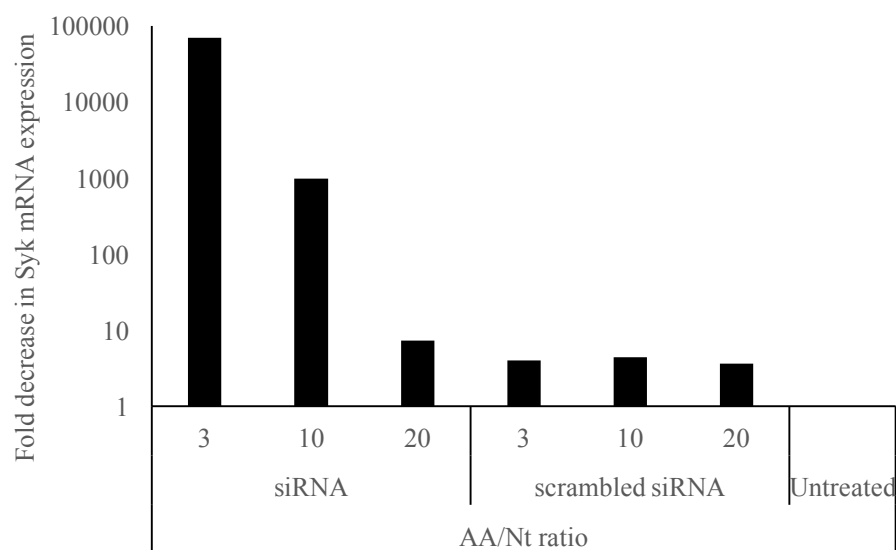


Figure 6.7. fold decrease of Syk mRNA expression in the NPY-mRFP reporter cell line after one day of transfection of the cell line with PGA-50% Lys/Syk siRNA H or PGA-50% Lys/scrambled siRNA H in comparison to the untreated cells.

Table 6. 1. Percentage Syk mRNA expression of the NPY-mRFP reporter cell line after one day of transfection with PGA-50% Lys/Syk siRNA H compared to PGA-50% Lys/scrambled siRNA H at the same AA/Nt ratio or untreated cells

AA/Nt ratio	% of Syk mRNA compared to scrambled siRNA H	% of Syk mRNA compared to untreated cells
3	6×10^{-3}	1.4×10^{-3}
10	0.4	1×10^{-1}
20	50	13.72

Another experiment was performed to evaluate changes in Syk mRNA expression after two days of transfection of the cells with the PGA-50% Lys/Syk siRNA polyplex to determine whether there was any further knockdown of the Syk mRNA in comparison with the previous one-day experiment. It can be observed from Figure 6.10 and Table 6.2 that almost full knockdown of Syk mRNA occurred when the cells were treated with the PGA-50% Lys/Syk siRNA H polyplex at AA/Nt ratios of 5, 10 and 20. Moreover, the off-target effects of PGA-50% Lys/scrambled siRNA H appeared to wear off after two days in comparison to the previous observation made after one day of transfection. The C_t for Syk cDNA was not detected even after 40 cycles with PGA-50% Lys/Syk siRNA H at AA/Nt ratios of 5, 10 and 20, whereas the C_t for Syk cDNA for the scrambled siRNA H or the untreated cells was observed mainly between 20 and 21 cycles. From these findings, it can be concluded that due to the full knockdown of Syk mRNA by PGA-50% Lys/Syk siRNA H polyplexes at all the ratios mentioned, there was no Syk cDNA left to be amplified by the Syk primers. As a result, Syk cDNA melting points were not observed for these cells while Ggt1 cDNA C_t and melting points could be detected for the same cells (Figure 6.8).

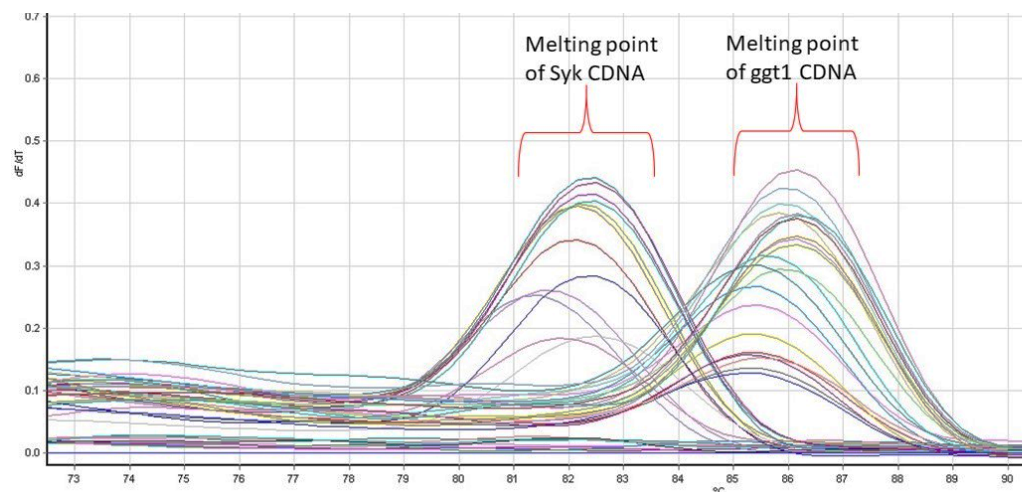


Figure 6.8. Melting points of the amplified Syk cDNA and ggt1 cDNA after 2 days of transfection with PGA-50% Lys/Syk siRNA H, PGA-50% Lys/scrambled siRNA H, and untreated cells.

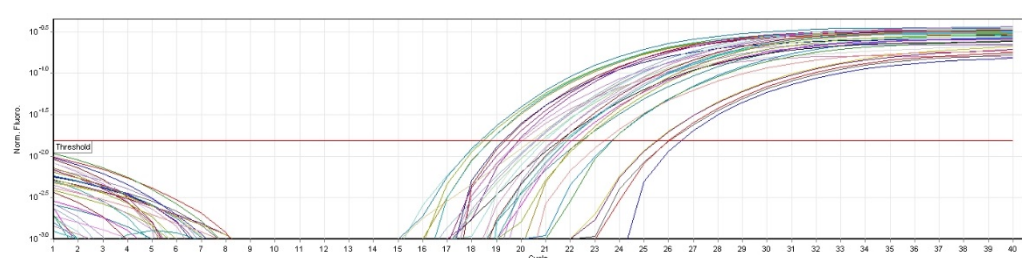


Figure 6. 9 Ct of the amplified Syk cDNA and ggt1 cDNA after two days of transfection with PGA-50% Lys/Syk siRNA H, PGA-50% Lys/scrambled siRNA H, and untreated cells.

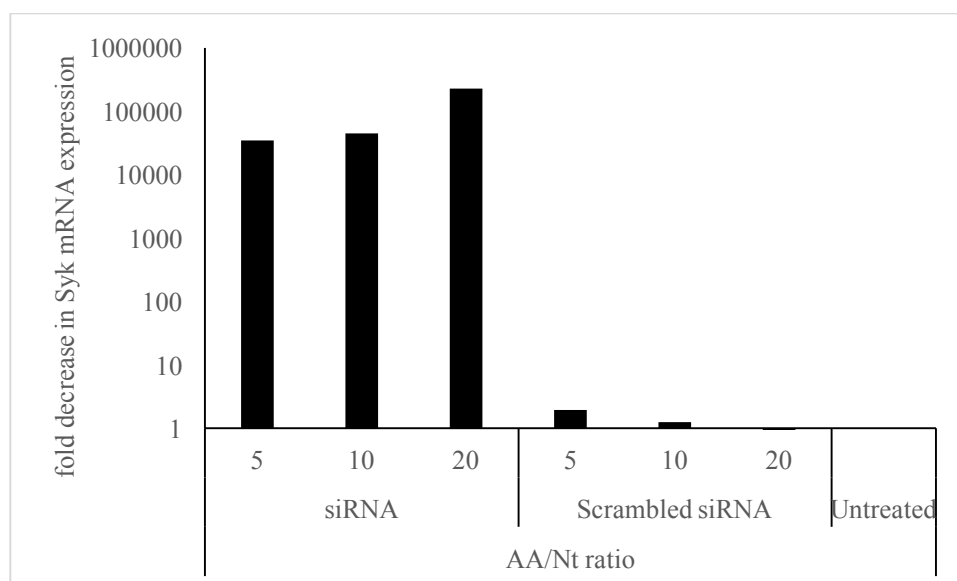


Figure 6.10. fold decrease of Syk mRNA expression in the NPY-mRFP reporter cell line after 2 days of transfection of the cell line with PGA-50% Lys/Syk siRNA H or PGA-50% Lys/scrambled siRNA H polyplex in comparison to the untreated cells.

Table 6.2. Percentage Syk mRNA expression of the NPY-mRFP reporter cell line after 2 days of transfection with PGA-50% Lys/Syk siRNA H compared to PGA-50% Lys/scrambled siRNA H at the same AA/Nt ratio or untreated cells

AA/Nt ratio	% of Syk mRNA compared to scrambled siRNA H	% of Syk mRNA compared to untreated cells
5	5.64×10^{-3}	2.9×10^{-3}
10	2.83×10^{-3}	2.3×10^{-3}
20	2.9×10^{-4}	4.4×10^{-4}

From the results of RT-qPCR and Western blot analysis, it can be deduced that the Syk protein is highly stable, as the Syk protein is detectable even after full knockdown of Syk mRNA in NPY-mRFP cells transfected with PGA-50% Lys/Syk siRNA. The stability of the Syk protein in human peripheral blood basophils was evaluated previously by MacGlashan (2016) using a pulse–chase experiment in which ^{35}S -methionine was used to label the Syk protein. The results of the study showed that the amount of labelled Syk protein was approximately the same after 4 h of the chase period, based on which it was concluded that the half-life of the Syk protein is more than 12 h. Another study evaluated Syk protein stability in human peripheral blood basophils with and without IgE-mediated stimulation of the cells (MacGlashan and Miura, 2004). In this previous study, cycloheximide was used to prevent the synthesis of Syk protein during the evaluation of its stability. The results showed that the Syk protein is stable in the absence of IgE stimulation even after 18 h, whereas IgE stimulation results in a decrease in the Syk protein level to less than 20% after 18 h. This is because stimulation of the cells by IgE causes phosphorylation of c-cbl, which is known to play a role in the ubiquitylation of the Syk protein and thereby leads to its degradation. It was also reported that the half-life of the Syk protein in the B cell lymphoma cell line DG75 is more than 72 h (Mazur et al., 2015). However, there is a lack of direct evaluation of the half-life of Syk in RBL-2H3. The rat and human cDNA of Syk was isolated (Benhamou et al., 1993, Law et al., 1994). It was observed that cDNA sequences of rat and human are highly similar and the highest differences were found in the sequences that is known for the translation of the interdomain B of Syk protein (Razin and Rivera, 1999). Moreover, amino acids similarity between rat and human Syk

was evaluated by Rowley et al. (1995) and the result shows that they are highly similar and the main variances were observed in interdomain B of Syk protein. The ubiquitylation of the Syk protein in RBL-2H3 was evaluated by Paolini et al. (2002). Their result shows that the phosphorylation of the Syk protein which occurs after crosslinking of the FcεRI receptors via multivalent antigens and aggregation of FcεRI receptors is required for ubiquitylation of the Syk. They confirmed that this is because the phosphorylation of Syk causes phosphorylation and activation of c-cbl which will cause the ubiquitylation of the Syk protein by forming Syk.cbl complex. Therefore, the mechanism of ubiquitylation of the Syk protein in rat and human is approximately similar. From the long half-life of the human Syk protein, similarities in the structure and mechanism of ubiquitylation of the rat and human Syk protein, and our finding results, it could be concluded that Syk protein in RBL-2H3 is highly stable.

Thus, based on these previous findings and the results in this chapter, it could be concluded that the transfection of NPY-mRFP reporter cell line by PGA-50% Lys/Syk siRNA H polyplexes can cause a high knockdown of Syk mRNA as shown from RT-qPCR. However, this knockdown of Syk mRNA does not show in general a decrease in the level or the function of Syk protein as shown from Western blot and fluorescence release assays which could indicate the Syk protein is highly stable. Therefore, Syk is not a suitable target for the evaluation of the inhibition of the degranulation in NPY-mRFP reporter cell line due to its long half-life. The NPY-mRFP reporter cell line can be a good model for quantitative evaluation of the change in the degranulation of the cells because it

can be observed that there is a reduction in RFP released in 10 AA/Nt ratio of PGA-50% Lys/Syk siRNA H in comparison to the scrambled siRNA and untreated cells. However, further investigation is required by finding a short half-life protein that influence in the signalling cascade of the degranulation to confirm the suitability of NPY-mRFP reporter cell line for quantitative evaluation of the degranulation.

6.4 Conclusion

In this chapter, the efficacy of PGA-50% Lys/Syk siRNA H in the inhibition of the degranulation of the NPY-mRFP reporter cell line and in the decrease in the function and level of Syk protein and level of Syk mRNA were assessed. It concluded from the results that PGA-50% Lys is effective in terms of delivering siRNA to the NPY-mRFP reporter cell line which was confirmed by diminishing Syk cDNA by RT-qPCR. However, Syk has long half-life so it is not a suitable target for this type of experiments. It could be concluded that NPY-mRFP is a good model for the quantitative evaluation of the degranulation since it was observed in this chapter and chapter 4 that the decrease in RFP release was in general lower when siRNA H was used in comparison to the untreated or scrambled siRNA. However, further investigation is required by finding a better target protein that has a shorter half-life.

CHAPTER 7: SUMMARY AND FUTURE WORK

7.1 Importance and general aim of the project

Allergic disease hypersensitivity syndrome is caused by the immune system. Allergic reaction can lead to several medical conditions such as asthma, rhinitis, and dermatitis. It is one of the most widespread conditions in the UK. The main cells that are responsible for allergic reactions are mast cells and basophils. In the allergic reaction, degranulation of the mast cells and the release of allergic mediators such as histamine cause bronchoconstriction and vasodilation occurs in addition to the synthesis of other allergic mediators such as cytokines and arachidonic acid metabolites (Saitō and Okayama, 2005, Razin et al., 1984, Siraganian et al., 2010).

There are many medications used for the treatment of allergic disease, but all have their limitations. For example, Antihistamines mainly antagonise H₁ receptors which cause inhibition of the activation of nuclear factor- kappa B (NF- κB, one of the transcriptional factors of inflammatory cytokine (Brown et al., 2008). The limitations of antihistamine are histamine receptor which is the late stage of allergic reaction instead of the inhibition of the release of histamine from mast cells and basophils. It also inhibits the effect of histamine only without influencing in the effect of other allergic mediators such as prostaglandins, leukotrienes, cytokines and chemokines that is released after the mast cells and basophils degranulation. Another medication that is used for the

treatment of allergic disease is the monoclonal anti-IgE antibody. This medication acts by binding to the Fc region of IgE and forms a complex which will prevent it from binding to the IgE receptor. The main limitation is that it does not bind to IgE molecules that are already bound to IgE receptor. Also, the use of Omalizumab has been shown to correlate with a higher risk of developing cancer (Soler et al., 2001). The cost of the treatment of allergic patient in the UK by Omalizumab range from £1,665 to £26,640 annually

Because of the limitations of the existing treatment strategies detailed above, there is clearly a continuing need for better treatment options. This project investigates an alternative treatment strategy targeting spleen tyrosine kinase protein (Syk) which is known to have a critical role in the early signalling cascade in mast cell and basophil degranulation resulting in the release of their allergic mediators and synthesis of allergic mediator cytokines (explained in detail in section 1.3).

10-20% of humans are so-called non-responders, i.e. they do not respond to IgE-mediated stimulation (Nguyen et al., 1990, Knol et al., 1992). It turns out that this status is caused by low Syk levels (Kepley et al., 1999). This seems to indicate that Syk is a good target for treatment.

Knockdown of Syk expression by small interfering RNA (siRNA) could be a useful way to prevent the release and induction of allergic mediators from mast

cells and basophils and induction of an allergic response. SiRNA binds to mRNA during the activation of RNA-induced silencing complex (RISC) which causes a degradation of mRNA. SiRNA cannot be delivered alone to its targeted cells for several reasons which were mentioned in detail in section 1.7. Therefore, it is important to use carrier vectors for delivery of siRNA such as cationic polymers.

In this project, two types of cationic polymers were used for the delivery of Syk siRNA. The first one was AGMA29. AGMA29 is an amphoteric polyamidoamine (PAA) having a carboxylic group that can be protonated at low pH and amine groups that can be protonated at high pH. The advantages of using AGMA29 are its variety of functional groups with different actions. The first functional group is a guanidine group that is highly cationic which is protonated even at high pH. The second is a carboxylic group which is protonated at low pH. This is because it was reported that amphoteric PAA is more biocompatible than the cationic polymer that has the same net average positive charge but without an acid functional group (Ferruti, 2013). Also AGMA29 mimics the tripeptide Arg-Gly-Asp (RGD) motif that can bind to different integrin receptor families which might enhance the cellular uptake of AGMA29. The second cationic polymer used was Poly (glycerol adipate) (PGA) polymer. PGA has an advantage as a cationic polymer in that it contains ester groups in its backbone which are more easily biodegradable than other cationic polymers used in nucleic acids delivery. This may allow an easier dissociation of the siRNA from the polymer so that it is more available for binding to target effectors in the

cytoplasm. The presence of the hydroxyl group in the PGA monomer (shown in Figure 1.5) has an advantage that it can be covalently attached to a carboxyl group of one of several positively charged amino acids such as lysine, arginine, and histidine. Therefore, two novel PGA derived polymer were evaluated in this project which are PGA-50% lysine (PGA-50% Lys) and PGA-20% histidine (PGA-20% His).

In order to evaluate the efficacy of these cationic polymers in the delivery of the Syk siRNA, the level of Syk protein function knockdown was evaluated using two genetically modified cell lines derived from RBL-2H3.

The first one, is stably transfected with a neuropeptide Y (NPY)–mRFP fusion construct that is expressed in the granules. Therefore, it is expected that the knockdown of Syk protein will cause the inhibition of the degranulation of the cells and so inhibit the release of mRFP protein during the allergic reaction. This pathway reflects the process which in activated mast cells and basophils results in the release of preformed mediators such as histamine.

The second one is RBL-2H3 that is stably transfected with the human FcεRI receptor α chain and a tetrameric synthetic NFAT binding site located upstream of a DsRed reporter gene. The knockdown of Syk protein indirectly can be expected to cause the inhibition of the translocation of NFAT transcription factor from cytoplasm to nucleus of the cells during the allergic reaction which causes

the inhibition of the expression of NFAT-DsRed. This pathway is reflective of the cytokine induction pathways which in basophils would result in the release of pro-allergic cytokines such as IL-4 and IL-13.

7.2 Optimisation and evaluation of the physicochemical characterisation of the polyplexes

The amine groups of PGA-50% Lys and the α ammonium of histidine in PGA-20% His are protected by *t*-BOC during the polymer synthesis need to be removed without degrading the polymer to allow the expression of the cationic character of the polymer. It was shown that the removal of *t*-BOC using TFA as described in section 2.2.1 can be successfully performed without affecting the molecular weight of the polymers (as shown in Figures 5.1, 5.2, 5.3, and 5.4) .

The formation of stable polyplex is between a cationic polymer and siRNA is essential for in vitro and in vivo transfection. Therefore, the polyplex formulation was optimised prior using the polyplex for the transfection of the genetically modified RBL-2H3 cell lines. At the beginning, the optimal repeating unit (RU) of AGMA29 or amino acid (AA) of PGA-50% Lys or PGA-20% His per nucleotide (Nt) of siRNA that shows a full complexation was evaluated by gel retardation assay. The full complexation of AGMA29/siRNA polyplex was observed at 4 RU/Nt ratio whereas a higher ratio of PGA-50% Lys/siRNA (10 AA/Nt) is required for full complexation. Regarding PGA-20%

His, binding with siRNA was not observed even at high AA/Nt ratio and therefore it was excluded from further evaluation.

The influence of NaCl in the complexation in order to determine the optimal conditions for polyplex formation was evaluated for AGMA29/siRNA and PGA-50% Lys/siRNA polyplexes. The presence of NaCl at 150 mM concentration can result in unstable polyplex formation. Use of this salt concentration resulted in diameters of 680 nm and 1 μ m for AGMA29/siRNA and PGA-50% Lys/siRNA polyplex respectively whereas their diameter in the deionised water were 90 nm and 202 nm.

7.3 Optimisation of the Syk knockdown assays

In order to use NPY-mRFP and NFAT-DsRed reporter cells for the evaluation of the functional knockdown of Syk protein, it is important to determine the optimal conditions for the release of mRFP from NPY-mRFP reporter cells and the production of DsRed in NFAT-DsRed reporter cells. This can be performed by evaluating the optimal calcium concentration in the media. This is because calcium influx to the cells after the sensitisation with IgE and stimulation with DNP-BSA is important for the degranulation and the release of RFP from NPY-mRFP reporter cell line. Also calcium influx is important for the translocation of NFAT transcription factor from cytosol to nucleus which cause production of DsRed in NFAT-DsRed. A new method was established for measuring the release of RFP in the NPY-mRFP cells that shows high signal-to-noise ratio (a

minimum 20:1 signal to noise ratio) by using MEM that does not contain FBS in the stimulation step with the DNP-BSA allergen (see Figure 4.2). Regarding the NFAT-DsRed, a low signal-to-noise ratio was observed when the NFAT-DsRed reporter cells were sensitised with human serum and stimulated with anti-human IgE whereas it became higher when the cells were sensitised with anti-DNP mouse IgE and stimulated with DNP-BSA. The optimum condition was observed when 10 mM of calcium was added to the MEM in the stimulation step with the DNP-BSA allergen.

When AGMA29/siRNA polyplex was used in the transfection of NFAT-DsRed reporter cells, the results were in contrast to the expectations, as the scrambled siRNA H resulted in a lower fluorescence percentage than the Syk siRNA H at all AGMA29/siRNA polyplex concentrations. Therefore, the red fluorescence protein production assay was not reliable for the quantification of Syk knockdown. This is due to that the viability cannot be assessed in the same cells used for the DsRed production assay. The evaluation of the viability in the same cells used for the DsRed production assay is important because the lower number of viable cells will cause lower level of DsRed that is produced. This limitation could be solved by using cell culture black well plates instead of using transparent well plates because the lysis of the cells will not be required and therefore the viability can be assessed in the same cells.

Regarding the evaluation of the decrease of the release of RFP in NPY-mRFP due to knockdown of Syk protein by AGMA29/Syk siRNA H polyplex, the variability between experiments was quite high, and taking an average of the

three results, the only significant differences between the Syk siRNA H and scrambled siRNA H could be seen in the 5:1 RU/Nt Syk complexes and the lipofectamine positive control. This could indicate that the cationic AGMA29 may have a strong electrostatic bond with the anionic siRNA, which could inhibit the release of siRNA in the cytosol and therefore the lower RU/Nt has a better transfection efficiency. For PGA-50% Lys, only 10:1 AA/Nt ratio of PGA-50% Lys/Syk siRNA H polyplex showed a decrease of the release of RFP compared to the scrambled siRNA H and the untreated cells.

The level of Syk protein was evaluated by Western blot analysis after 48 and 72 hrs of the transfection of the cells by AGMA29/Syk siRNA H or PGA-50% Lys/Syk siRNA H. This was performed in order to confirm if the reduction occurred at the protein level as well as the nucleic acid expression level, ie. if the decrease in the level of the release of RFP in NPY-mRFP was caused by Syk. The results show that the level of Syk protein after 48 and 72 hrs of the transfection of the cells with AGMA29/Syk siRNA H polyplex at 5 and 10:1 RU/Nt was lower than the cells transfected with scrambled siRNA H which were in general lower than 50 %. However, when the level of Syk mRNA was evaluated after 24 and 48 hrs of the knockdown of the cells by AGMA29/Syk siRNA H, the knockdown was not observed after 24 hrs and 48 hrs at 10:1 RU/Nt ratio whereas the percentage of Syk mRNA at 5 RU/Nt was only 74% compared to scrambled siRNA H after 48 hrs which is higher than the observed Syk protein in Western blot analysis.

The Western blot analysis in general has some limitations as a quantitative method. One of the main limitations is that the house keeping protein GAPDH is highly abundant. It was reported by Taylor et al. (2013) that GAPDH can be quantified accurately by western blot only when the total protein cell lysate from HeLa cell is lower than 0.5 μ g. Also, GAPDH level changes with the change of the confluence of the cells (Greer et al., 2010).

Regarding the Syk protein and mRNA level after the transfection of the cells by PGA-50% Lys/Syk siRNA H polyplex, the level of Syk protein did not in general change even after 72 h of knockdown whereas the mRNA had almost disappeared after 24 h and 48 h of transfection. Therefore, it is concluded that PGA-50% Lys/Syk siRNA H polyplex knocks down Syk mRNA successfully. However, Syk protein is highly stable, so that even though the Syk mRNA is successfully reduced, the Syk protein level does not change.

From the results obtained, it is hypothesised that the polyester based polymer could be highly effective for siRNA delivery for hard to transfect cells in comparison to PAA based polymer. It could be indicated from the polyplexes sizes that both AGMA29 and PGA-50% Lys can form small sizes polyplexes with siRNA which suggest that the cellular uptake for both AGMA29 and PGA-50% Lys can occur and the high transfection efficiency of PGA-50% Lys may not be because of the higher cellular uptake. Therefore, the high transfection efficiency of PGA-50% Lys could be due to siRNA easily dissociating from polyester based polymer in the cytosol to reach its target mRNA whereas PAA

based polymer might be strongly bound to siRNA which can hinder the siRNA from binding to its targeted gene. However, there are some limitations to this work in terms of lacking some experiments which could support this hypothesis. Firstly, the transfection efficiency of the polyplexes were not compared with the commercial vector lipofectamine in the RT-qPCR experiment. This comparison is important to determine whether the lipofectamine (or other positive control transfection reagents) can show similar efficacy in complete knockdown of siRNA to PGA-50% Lys or complete knockdown can only be observed in PGA-50% Lys that can be due to the distinctive physicochemical properties of PGA-50% Lys. Also, using positive control transfection reagents is important to perform indirect comparison between the polyester based polymer to PAA based polymer since by comparing the efficacy of knockdown of siRNA by those polymer to positive control transfection reagents. The other limitation of this work is that the mechanism of entry of the polyplexes into the cell and dissociation of the siRNA from the polymer were not characterised. This is important to detect whether the high efficacy of polyester based polymer is due to the easy dissociation of siRNA to the polymer whereas the low efficacy of PAA based polymer is due to high binding to siRNA that hinder its dissociation or there are other causes that need to be characterised. Also, it is important to know the cause of high efficiency of PGA-50% Lys in siRNA delivery which can contribute in exploring a mechanism of polyester based polymer. The characterisations of cellular uptake can be performed by using fluorescently labelled siRNA to be complexed with the polymer and measuring the amount of siRNA that enters the cell by fluorescence microscopy or flow cytometry. siRNA based Molecular Beacon technique in which the siRNA contains a

fluorescent dye and quencher can be used to investigate whether the siRNA dissociate from the polymer and binds to its target mRNA or not.

In conclusion, RBL-2H3 cell is notoriously difficult to transfect cell which is mainly transfected by electroporation method (Zhang et al., 1996, Sanderson et al., 2010, Willibald et al., 2012). Interestingly, PGA-50% Lys is effective in terms of delivering Syk siRNA to the NPY-mRFP reporter cell line as confirmed by RT-qPCR. Syk is not an ideal target for this type of experiments due to its long half-life. It can be observed a reduction of the release of RFP in the NPY-mRFP after the transfection with either AGMA29/Syk siRNA or PGA-50% Lys/Syk siRNA polyplexes in comparison to either scrambled siRNA or untreated cells. This can indicate that NPY-mRFP reporter cell line is a good novel model for the quantification evaluation of the change of the level of degranulation of the cells. However, because of the high stability of Syk protein, it may be required a further validation of the suitability of NPY-mRFP reporter cell line. Therefore, it is important to find a better protein target that has shorter half-life and involved in the degranulation signalling cascade to be knocked down for the assessment of NPY-mRFP model suitability for a quantification evaluation. For example, it was reported that Fyn protein's half-life is 8 h (Wolven et al., 1997). Fyn protein is phosphorylated after the ITAM phosphorylation during the crosslinking the FcεRI receptors via multivalent antigens and aggregation of FcεRI receptors (Parravicini et al., 2002).

Phosphorylation of Fyn protein cause activation of Gab2 then this will lead to signalling cascade that causes a degranulation of mast cell (Yu et al., 2006).

Regarding the use of Syk as a therapeutic target, there are several therapeutic approaches by inhibiting Syk for the treatment of allergy. For example, the level of Syk protein after 8 days of administration of PEI/Syk siRNA polyplex in the leg joint of mice that have arthritis and the efficiency on the diminishing of the joint swelling was evaluated by Huang et al. (2013). Their finding was that the swelling was reduced. Also, Syk protein level was decrease with other inflammatory cytokine mediators such as interferon- γ (IFN- γ) and interleukin-6 (IL-6). In addition, there are several synthetic Syk inhibitors that can be useful for the treatment of allergy. For, example, fostamatinib is an orally administrated Syk inhibitor that has therapeutic efficiency in the treatment of rheumatoid arthritis which is in clinical trial (Nijjar et al., 2013). However, it is required to be administrated twice per day and many adverse effects were reported which are gastrointestinal effects, hypertension, and neutropenia (Nijjar et al., 2013). Therefore, since PGA-50% Lys is effective in knockdown of Syk mRNA *in vitro*, it could be a useful therapeutic strategy to evaluate the PGA-50% Lys/Syk siRNA polyplex *in vivo*.

7.4 Future work

In our finding, PGA-50% Lys/Syk siRNA polyplex shows high transfection efficiency in knockdown of Syk mRNA in RBL-2H3 cells. Due to high stability of Syk protein and lack of published articles that directly evaluate the half-life of proteins that are involved in signalling cascade that leads to degranulation of

RBL-2H3 cells and cytokine induction, it would be useful to evaluate the half-life of those proteins to detect the lowest half-life protein. The stability of protein could be evaluated by several methods such as cycloheximide chase method and pulse-chase method. Cycloheximide chase method can be performed by blocking the synthesis of new protein in the cell by cycloheximide to evaluate the stability of the existed protein by quantifying the level of protein at different time intervals. In the pulse-chase method, the proteins are labelled with radioactive amino acid such as ³⁵S methionine in the pulse period then the level of the decrease of the radioactive labelled proteins at different time intervals.

Since the PGA-50% Lys/Syk siRNA polyplex shows high transfection efficiency for *in vitro* study, it is useful to evaluate the suitability of the polyplex for the *in vivo* siRNA delivery. The presence of free hydroxyl group in PGA-50% Lys is useful for incorporation of variety of amino acids or functional groups for further modifications if they are required. For example, cysteine can be added to the PGA-50% Lys which can lead to the formation of crosslinked bio-reducible PGA-50% Lys system due to the presence of sulphide group in cysteine.

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