

Bio-Reducible Polyamines for siRNA Delivery

by

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Declaration of Authorship

I do solemnly vow that this thesis is my own work. Where the work of others is referenced, it is clearly cited.

Abstract

Even though siRNA shows great promise in the treatment of genetic disease, cancer and viral infection; the lack of a suitable delivery vector remains a barrier to clinical use. Currently, viral vectors lead the field in terms of efficacy but are generally regarded as prohibitively dangerous. Synthetic alternatives such as cationic polymers could overcome this problem. Previous work in the group found that small, cationic, disulfide-containing, cyclic polyamines – despite being non-polymeric – were useful as vectors for pDNA transfection; this work focuses on adapting the material for siRNA.

A branched analogue of the cyclic compounds was prepared and the synthetic procedures investigated are discussed. The suitability of both compounds for siRNA delivery was studied in depth. Characterisation of their interactions with nucleic acids under various conditions was carried out using light-scattering techniques, gel electrophoresis and fluorescent dye exclusion assays. Results from these experiments were used to allow successful use of the materials as vectors and enable understanding of the mechanism of the template-driven polymerisation.

Early data concerning the efficacy of the materials as an siRNA delivery system *in vitro* was obtained using A549 lung carcinoma cells as a model system with siRNAs targeting the production of the enzyme GAPDH. Both compounds showed a hint of successful siRNA Delivery but the data was not overwhelmingly conclusive. Further experiments will be required to optimise the materials for maximum biological efficacy and to confirm they offer potential as a novel delivery system.

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List of Abbreviations

Ac - Acetyl

AD - Adamantyl

aq. - Aqueous

Bn - Benzyl

Boc -^tButoxycarbonyl

bp - base pair

bPEI -Branched poly(ethylenimine)

CB[n] - Cucurbit[n]uril

CBA - Cysteinebisacrylamine

CD - Cyclodextrin

CDP - Cyclodextrin-containing polymer

CFTR - Cystic fibrosis transmembrane conductance regulator

conc. - Concentrated

COSY - Correlation spectroscopy (NMR)

CPP - Cell Penetrating peptide

Da - Dalton

DBU - 1,8-Diazabicyclo[5.4.0]undec-7-ene

DCM - Dichloromethane

DIPEA - Diisopropylethylamine

DMEM - Dulbecco's Modified Eagles' Medium

DMF - *N,N*-dimethylformamide

DMMAn - Dimethyl maleic anhydride

DMSO - Dimethylsulfoxide

DNA - Deoxyribonucleic acid

EGF - Endothelial growth factor

EGFP - Enhanced green fluorescent protein

ESI - Electrospray ionisation

Et - Ethyl

Et al. - *Et alia* (and the others)

EtBr - Ethidium bromide

FBS - Fetal bovine serum

FFS - Fluorescence fluctuation spectroscopy

GAPDH – Glyceraldehyde 3-phosphate dehydrogenase

GFP - Green fluorescent protein

G_n - Generation number (of a dendrimer)

GSH - Glutathione (thiol)

GSSG - Glutathione (dimeric)

h - Hour

HA - Halyronic acid

HIV - Human immunodeficiency virus

HS - Heparin sulfate

i.e. - *Id est* (that is)

IR - Infrared

kDa – kilo Dalton

LDH – Lactate dehydrogenase

lPEI - Linear polyethylenamine

M - Molar

mAb - Monoclonal antibody

Me - Methyl

ME - Mercaptoethanol

Min - Minute

MRI - Magnetic resonance imaging

mRNA - Messenger RNA

MS - Mass spectrometry

MTT - 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

MW - Molecular weight

N/P - Ratio of nitrogen to phosphate

NLS - Nuclear localisation signal

Ns - Nosyl (2-nitrobenzenesulfonyl)

OEI - Oligo(ethylenimine)

Opti-MEM - Optimised Dulbecco's modified Eagles' medium

PAMAM - poly(amidoamine)

PBS - Phosphate buffered saline

PCI - Photochemical internalisation

PCS - Photon correlation spectroscopy

pDNA - Plasmid DNA

PEG - poly(ethyleneglycol)

PEI - poly(ethylenimine)

PES - Poly(ethylenesulfide)

PI - Polydispersity index

PLL - Poly-L- lysine

PLR - Poly-L- arginine

PMMA - Poly(methyl methacrylate)

PPA - Polyphosphoramidate

PPI - Poly(propylenimine)

ppm - Parts per million

PrI - Propidium iodide

Py - Pyridine

QD-FRET - Quantum dot Förster resonance energy transfer

RISC - RNA induced silencing complex

RNA - Ribonucleic acid

RNAi - RNA interference

RNP - Ribonucleic protein

RT - Room temperature

siRNA- Small interfering RNA

SOD1 - Superoxide dismutase 1

TEA - Triethylamine

TEM - Transmission electron microscopy

TF - Transferrin

TFA - Trifluoroacetic acid

TLC - Thin layer chromatography

TMC - Trimethyl chitosan

tren - Tris(2-aminoethyl)amine

Ts - Tosyl (4-methylbenzenesulfanyl)

VEGF - Vascular endothelial growth factor

Acknowledgements

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Secondly, I would like to thank my Grandma who – by providing me with a home for far longer than I originally asked (even though it remains contentious as to how long that actually was) – allowed more progress to be made on the project than would have otherwise been possible. I am also thankful to her – along with Pamela and Vera – for making my stay extremely entertaining. My parents also deserve approximately one ninth of the gratitude described above for the month that I spent living with them which allowed me to escape London and actually get some writing done. Of course, I am also grateful for their encouragement at every stage of everything.

Returning to the realm of science, I would like to thank Chris Drake for all his help – not to mention for providing such a solid foundation for this project to be built on – and also Steve Johns for his discussions (some of which were helpful). David Rees is deserving of acknowledgement for being an all-round excellent colleague as is Chirag Patel for providing (friendly) rivalry. I am also indebted to all of my other Whiffen lab colleagues past and present for their various contributions over the course of our time spent in that windowless place where the temperature plays by its own rules.

Finally, I would like to thank C. C. Cairns for reasons both numerous and complex.

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1.1. The History of Gene Therapy

Gene therapy is a general term encompassing any use of a variety of genetic materials as a means of treating a disease at the genome level. The first method of gene therapy to exhibit success is the introduction of DNA to a cell with a view to initiating the production of any protein that is not being produced correctly or in sufficient quantities for the cell to function properly. This is how it is envisaged that cystic fibrosis would be treated since this disease is caused by a mutation in a gene known as CFTR leading to formation of a malfunctioning variant of a key protein.¹ It also offers many different methods for treating cancer; faulty genes could be corrected, the production of naturally occurring anti-cancer proteins could be stepped up or cellular machinery could be harnessed in order to produce cytotoxic proteins or proteins capable of activating small molecule drugs in a controlled, cell-specific manner.¹

Gene therapy can also be employed to have the opposite mode of action to the potential treatments described above. RNA interference (RNAi) is a phenomenon – discovered in 1991² – that allows the selective knockdown of genes mediated by small interfering RNA (siRNA): a ~22 bp, double-stranded RNA species with a distinctive two base-pair overhang at the 5' position.³ The ability to knockdown genes in a selective fashion could potentially have a huge impact on how many diseases are treated. The most obvious application for RNAi would be to simply stop production of any proteins that are causing disease. This would be useful in treating genetic diseases such as Huntington's disease⁴ and spinocerebellar ataxia.⁵ A proposed treatment for motor neurone disease offers a good example of how such a therapy would work. The disease is caused by mutated SOD1 enzymes which increase the susceptibility of motor neurones to damage by molecules such as NO.⁶ It follows that preventing the production of the mutant would therefore reduce or cease neurone damage depending on the degree of knockdown.⁶ Of course, gene knockdown is not limited to human cells. Knockdown of the genes of a virus responsible for the production of essential proteins would also be possible and lead to a new generation of anti-viral drugs.⁷⁻⁹ For example, a treatment for HIV could be developed by targeting the gene that codes for the important structural polypeptide p24. Reducing

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or eliminating the production of such a vital protein would thereby inhibit the spread of the virus.¹⁰

Previously, the most widespread method of gene transfection was the use of viral vectors¹¹ though there are concerns regarding their viability. The major drawback to using viral vectors is that they are potentially very dangerous.¹² Another disadvantage is a matter of cost; viral vectors may prove to be too expensive to be used on a wide scale.¹ However, synthetic, non-viral vectors are not yet well-developed enough to compete with viral vectors in terms of efficiency. The most effective and widely used synthetic vectors are cationic liposomes, spherical structures formed by lipids under certain conditions.¹³ First reported in 1987 by Felgner *et al.*,¹⁴ transfection with liposomes – or “lipofection” – is based on the principle that lipids form a bilayer in an aqueous environment. A careful choice of lipids enables the formation of spherical vesicles and when cationic lipids are used, complexation with anionic nucleic acids takes place.¹³ The toxicity of these vectors is much lower than with viral vectors although there are concerns with the reproducibility of formulations and colloidal stability.¹ Quite simply, the fact that the vector itself is not held together by covalent bonds is a limiting factor. This is particularly troubling in environments such as the lung where there is a large amount of natural surfactant present.

1.2. The Mechanism of siRNA Interference^{15,16}

siRNA silences gene expression in a selective manner *via* an extremely elegant mechanism that makes use of various parts of the naturally occurring cellular machinery. The process begins when a complete siRNA forms what is known as an RNAi Silencing Complex (RISC) – a ribonucleic protein (RNP) complex with the Argonaute-2 (Ago2) protein. Once bound into this complex, one strand of the siRNA (the passenger strand) breaks away (Figure 1.1). The strand of siRNA that remains in the RISC (the guide strand) will ultimately act as the means of binding the RISC to the correct part of the target mRNA. The “decision” as to which strand becomes the passenger and which becomes the guide is made based on the stability of the base-pairing at the 5’ end of the strand.¹⁷ For example, the anti-sense strand is favoured if

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the 5' nucleotide is part of a C:G pair (three hydrogen bonds) compared to an A:U pair (two hydrogen bonds) at the 5' end of the sense strand.

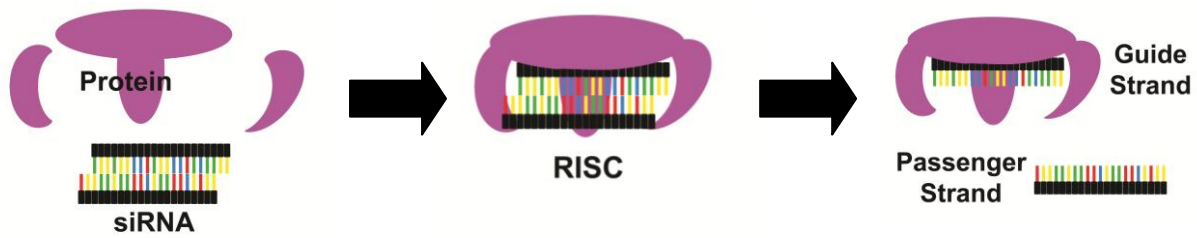


Figure 1.1 – Scheme depicting the formation of an RNA induced silencing complex (RISC).¹⁵

The RISC then binds to the target mRNA and cleaves it (Figure 1.2). The cleavage occurs in the middle of the guide strand. The cleavage occurs via hydrolysis of the phosphodiester backbone of the target mRNA and requires Mg^{2+} ions.¹⁶ The cleaved mRNA is identified by the cell and is destroyed. This prevents the expression of the gene that would have been coded by the mRNA.

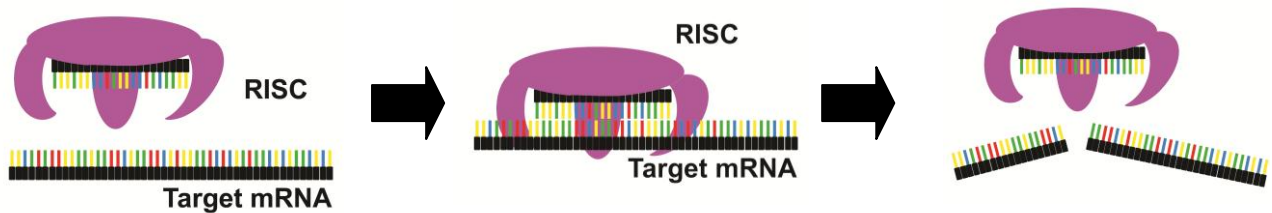


Figure 1.2 – Scheme showing the interaction of the RISC with mRNA and subsequent cleavage of mRNA.¹⁵

1.3. The Barriers to the Clinical Use of siRNA

In order to be able to deliver its cargo, the gene delivery vector must overcome many obstacles. These are identical for both pDNA and siRNA delivery with the only exception that the crossing of the nuclear membrane is not necessary for siRNA. The first of these barriers are encountered outside of the cell. An investigation by Burke and Pun into the extracellular barriers to *in vivo* delivery of pDNA to the liver showed that negatively charged FBS (fetal bovine serum), an anionic protein occurring in blood, would cause unpacking of polyplexes (a nanoparticle composed of cationic polymer and nucleic acid) due to electrostatic interactions.¹⁸ It was also shown that similar effect would result from interaction with anionic proteins in the extracellular matrix. As discussed in Section 1.4, the reduced binding forces between siRNA and cationic polymer relative to pDNA and cationic polymer suggest that this

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effect will be even more pronounced for siRNA delivery.¹⁹ Buyens *et al.*²⁰ found evidence for a direct correlation between the stability of siRNA polyplexes in serum and their effectiveness as vectors. They used a microscopy technique called Fluorescence Fluctuation Spectroscopy (FFS) in conjunction with fluorescently labelled siRNA to measure the amount of siRNA incorporated into polyplexes in solution. The principle of this technique is that at the focal point of the microscope imaging a sample of free siRNA the level of fluorescence would be relatively constant due to the homogeneity of the solution. However, in a solution containing siRNA in polyplexes the siRNA would be bunched together in polyplexes meaning the level of fluorescence observed would be prone to “spike” as polyplexes passed the focal point. By titrating different polyplex solutions with serum and monitoring dissociation a quantitative measurement of the resilience of the particles could be obtained. It was found that this related strongly to the ability to mediate gene knockdown and that stability in the presence of serum was vital for transfection efficiency.²⁰

Should the polyplex reach the cell, it must first adhere to the cellular membrane in such a way that it can pass through the cellular membrane. Cationic polyplexes are able to interact electrostatically with negatively charged macromolecules on the cell surface although, like serum proteins, they are capable of displacing the nucleic acid.¹⁹ Work by Schaffer and Lauffenburger used radiolabelled peptides to investigate interaction between polyplexes and the negatively-charged cell-membrane.²¹ They showed that increasing surface charge by using higher ratios of cationic poly-L-lysine to pDNA increased binding to the cell membrane and increased internalisation. It was also demonstrated that aiding binding by conjugation of EGF (epidermal growth factor) – a ligand that binds to EGF receptors on cells – to the polyplexes increased transfection.²¹ Both of these experiments showed that efficient adhesion to the cell surface is crucial if successful delivery of nucleic acids is to be achieved. The use of targeting ligands to improve adhesion to either all or specific cells is a broad area of research and discussed in Section 1.7.

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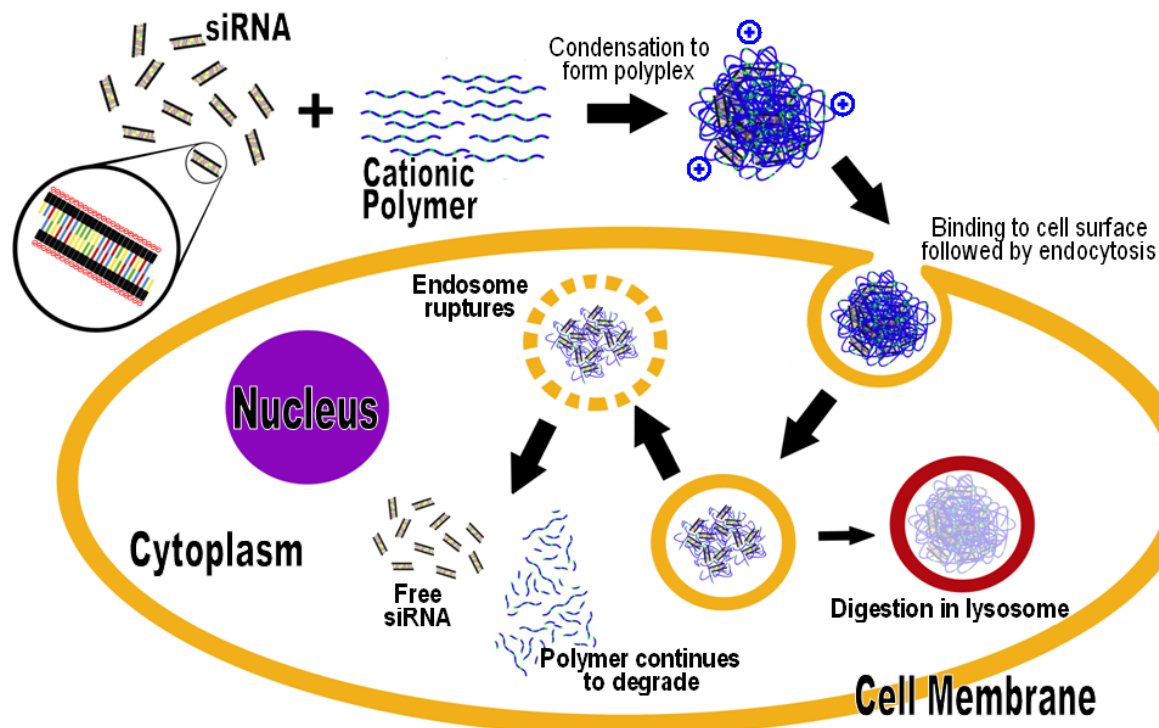


Figure 1.3 – Overview of the process of siRNA delivery using biodegradable polycations as the vector.

The exact mechanism of cellular entry of polyplexes is the subject of much debate and so far no definitive conclusion has been reached. Research by von Gersdorff *et al.*²² suggests that polyplexes can enter the cell *via* four main routes: clathrin-dependant endocytosis, clathrin-independent endocytosis, phagocytosis and macropinocytosis. The exact route taken depends on the formulation, polyplex size and cell type. Small PEI polyplexes (<200 nm) were found to enter the cell *via* clathrin-dependant endocytosis – the route where the “proton-sponge” mechanism is postulated to aid endosomal escape (discussed in Section 1.5.1). It was shown that inhibition of the clathrin-mediated pathway reduced gene expression to a greater extent than inhibition of clathrin-independent, caveoli-mediated endocytosis although the extent of this decrease varied according to cell-type. In particular, HeLa cells were found to have more caveoli-mediated endocytosis. In this instance, it was found that “proton-sponge” activity was not required and that disruption of endosomes occurred *via* another route.²²

Once the polyplex has been internalised and has reached the cytoplasm the journey is complete for siRNA as this is where it will mediate gene knockdown. It is here that strong-binding of the nucleic acid by a polycation becomes a disadvantage as in order

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to interact with the cellular machinery the nucleic acid must be unpacked. One of the most in depth studies of vector unpacking is by Chen *et al.*²³ and concerns pDNA polyplexes prepared using chitosan (see Section 1.5.2), PEI (see Section 1.5.1) and a proprietary polyphosphoramidate (PPA). They used quantum dot fluorescence resonance energy transfer (QD-FRET) to monitor the intracellular dissociation of quantum dot-labelled pDNA and Cy5-labelled polymer in a quantitative manner. It was found that polyplexes formed using PEI and PPA were more stable in the endosome and predominantly unpacked in the cytosol. On the other hand, chitosan polyplexes began to unpack in the endosome offering their cargo less protection and leading to lower transfection efficiency.²³ The tendency of polyplexes to unpack in the cytosol is beneficial to siRNA (assuming the results for pDNA polyplexes are replicated with siRNA) as trafficking to the nucleus is unnecessary. The integration of triggered degradability into a polymer is a common means of improving the unpacking efficiency of polyplexes as discussed in Section 1.6.

Though the journey for siRNA ends in the cytosol, it is worth mentioning that pDNA must ultimately reach the nucleus if it is to have any effect.¹ In this instance a polyplex must cross the cytosol, cross the nuclear membrane and enter the nucleus. It is vital at this stage that the pDNA remains protected as anti-viral defence mechanisms in the cytoplasm will degrade foreign DNA leading to a half-life of 50-90 minutes.²⁴ The entry of the vector into the nucleus is still not completely understood. Grosse *et al.*²⁵ reported the transfection efficiency of PEI with respect to cell division. It was found that most nuclear entry occurs passively when the nuclear membrane is temporarily broken during cell division. However, some transfection occurred in non-dividing cells. This was not due to entry through pores in the nuclear membrane and was independent of the presence of a nuclear-localisation signal (NLS) ligand indicating another route of entry.²⁵ This suggests that there is still more to be learnt about this stage of the gene transfection process.

1.4. The Differences Between siRNA and pDNA and Their Implications

So far, the discussion of polymeric vectors has been in general terms, and indeed, the similarity between pDNA and siRNA vectors within the context of extracellular behaviour, internalisation and unpacking is great; all else being equal, an siRNA

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polyplex and a pDNA polyplex will behave in much the same way. However, there are stark differences between the two nucleic acids in terms of their interactions with polycations.

Polymers that are positively charged are able to interact with nucleic acids such as siRNA and pDNA through electrostatic interactions with the anionic phosphate-backbone. For pDNA this results in a large reduction of the volume occupied by the DNA as it changes from a freely-moving polymer to a compact spherical structure of 30-300nm known as a polyplex (Figure 1.4).²⁶

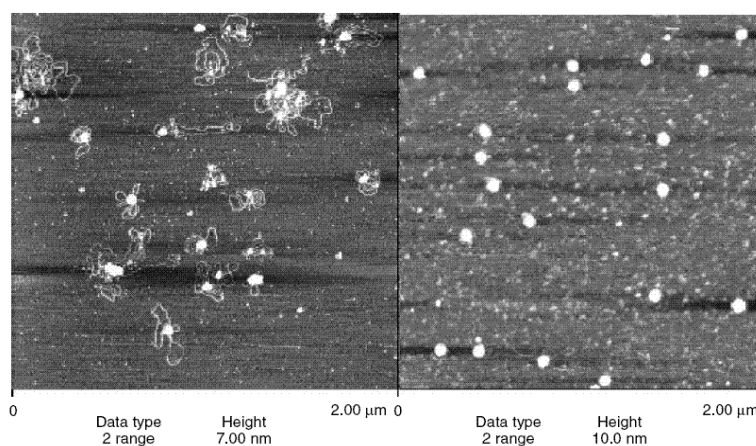


Figure 1.4 – Left: Loose particles formed between DNA and linear 22 kDa PEI Right: Compact nanoparticles formed between DNA and branched 25 kDa PEI²⁷ (Picture taken from Neu *et al. J. Gene Med.*, 2005, 7, 992-1009).

However, it is here that one of the primary differences between siRNA and pDNA is encountered. A 22 bp siRNA falls a long way short of the 260 bp persistence length of an RNA duplex.²⁸ This means that it can be regarded as a rigid rod as opposed to a flexible chain. The shorter chains also lead to reduced cooperativity of binding and electrostatic binding forces are one hundred times weaker.¹⁹ Also, according to computational work by Ou and Muthukamara²⁹ the driving force of the condensation of DNA with cationic polymers is entropic and due to the release of small molecule counter-ions (typically sodium chloride) upon complexation of the two polyelectrolytes. Following on from this, it could be envisaged that the size of siRNA – which is arguably closer to that of a small molecule than it is to a pDNA molecule – would reduce this entropic advantage.

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As a result of the differences the polyplexes formed between a cationic polymer and siRNA are larger and more diffuse than those formed with pDNA. For example, polyplexes made by Boeckle *et al.*³⁰ using 25 kDa branched PEI and pDNA were 130 nm and 116 nm at N/P ratios of 6 and 12 respectively. siRNA polyplexes made by Breunig *et al.*³¹ at the same N/P ratios were 1000 nm and 600 nm. This has two major implications. Firstly, the transfection efficiency of polymeric vectors has been found to be dependent on polyplex size³² and secondly loosely bound complexes would not offer the siRNA sufficient protection from enzymatic degradation to which it is especially prone.²⁸ Weaker binding also means there is possibility of exchange with other anionic, biological macromolecules (e.g. serum proteins) leading to the expulsion of the siRNA from the vector as discussed in Section 1.3.¹⁹

1.5. The Common Polymeric Agents Used For siRNA Delivery

The range of polymeric materials used for siRNA delivery is extensive although many of the examples found in the literature are based on only a few distinct polymer structures. These recurring motifs and their relative merits are discussed below.

1.5.1. PEI

The canonical example of a polymeric transfection agent is poly(ethylenimine) (PEI). One of the first polymers to be identified as an effective gene delivery vector for pDNA³³ PEI was later recognized as being appropriate for siRNA delivery.³⁴ The structure of the linear form of the polymer is shown in Figure 1.5. It is the protonatable nitrogens and more specifically the wide range of pKas – a result of the inter-nitrogen spacing being sufficiently small that protonation of one nitrogen dramatically alters the pKa of its neighbours – that makes it useful for gene delivery.

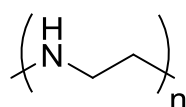


Figure 1.5 – The general structure of poly(ethylenimine) (PEI).

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These nitrogen atoms serve two purposes. Firstly, when protonated they provide the cationic character needed for interaction with nucleic acid. Protonatable amines are therefore an almost ubiquitous feature in all gene delivery vectors. The second function of the protonatable nitrogen atoms is that they appear to provide a means for endosomal escape which has been explained by the “proton-sponge” mechanism.³⁵ This as yet unproven mechanism suggests that upon exposure to the acidic environment of the endosome (see Section 1.3 for discussion of cellular entry) more nitrogens become protonated leading to the uptake of chloride counter-ions from the cytoplasm. The resulting influx of water due to osmosis causes the endosome to swell and eventually results in the bursting of the endosome and release of the polyplexes into the cytoplasm (Figure 1.6). Once in the cytoplasm, the nucleic acid must be released so it may traffic to the nucleus. Work by A. Akinc *et al.*³⁵ has provided good evidence for the “proton-sponge” theory by demonstrating that alkylated PEI that contained only unprotonatable quaternary amines was an ineffective vector.

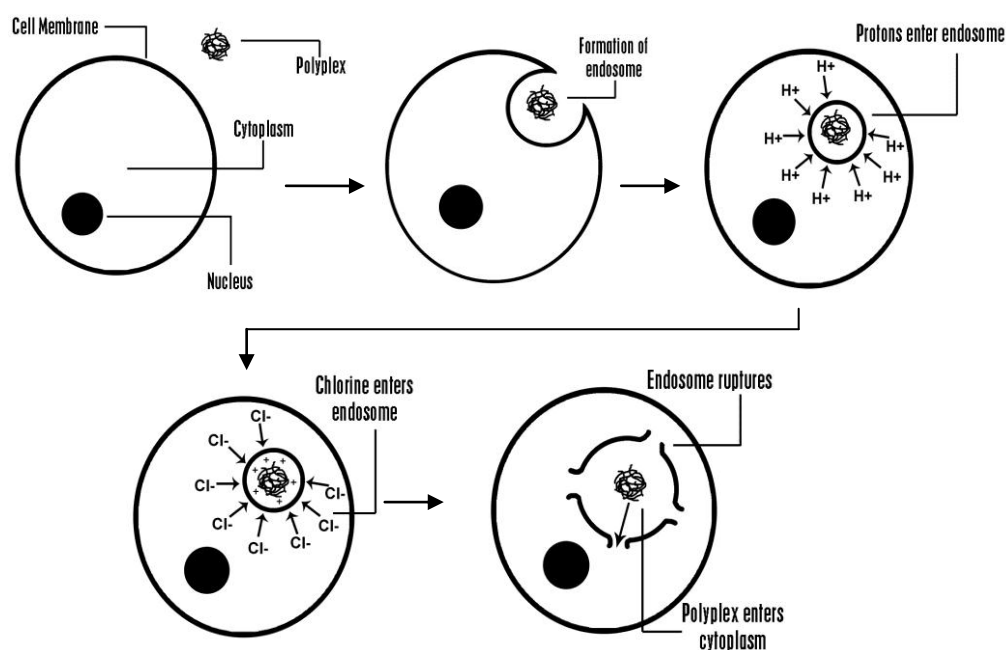


Figure 1.6 – A scheme showing the “proton-sponge” mechanism. The process begins when the polyplex undergoes endocytosis. Once inside an endosome the nitrogen atoms act as a buffer causing H^+ ions to enter the cell. This leads to the uptake of Cl^- counter-ions to balance the charge. The endosome grows to an unsustainable size and eventually bursts, releasing the polyplex into the cytoplasm.³⁵

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Following studies involving the delivery of pDNA, PEI has also been tested as a possible means of delivering siRNA³⁴. Hassani *et al.*³⁶ used 22 kDa linear PEI to attempt to deliver siRNA to mouse brains but it lacked efficacy compared to a liposome vector also tested. The group attributed this to the lack of release of the siRNA in the cytoplasm due to strong binding. A study by Grayson *et al.*³² looked at the differences in siRNA delivery capability between 800 Da and 25 kDa branched PEI and 22 kDa linear PEI. They found correlations between transfection efficiency, particle size and zeta potential suggesting that complexes that were small and positively charged underwent endocytosis more effectively. By their reckoning, the only suitable nanoparticles were formed by 25 kDa bPEI which was an effective, albeit toxic vector. 800 Da bPEI and 22 kDa lPEI showed lower binding affinities (according to EtBr exclusion assays) and typically formed larger particles. For example, at N:P 6:1 (the ratio at which all three polymers bound siRNA completely) 25 kDa bPEI formed particles of 59 nm compared to 800 Da bPEI and 22 kDa lPEI which formed particles of over 900 nm and 190 nm respectively.³²

Aside from branching, another method to make PEI more suitable for siRNA delivery is to incorporate hydrophobic sidechains as demonstrated by Creusat and Zuber.^{37,38} As shown in Figure 1.7, they coupled either leucine, tryptophan, phenylalanine or tyrosine groups to the primary amines in commercially available, 25 kDa branched PEI.

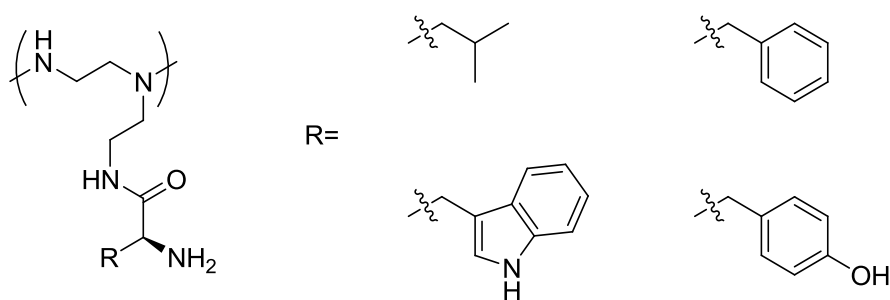


Figure 1.7 – The amino-acid side chains attached to PEI by Creusat and Zuber.³⁷

The reason for this modification was to overcome the inefficient condensation of siRNA described in Section 1.4. It was envisaged that *via* the hydrophobic effect, the incorporation of the chosen moieties would encourage particle formation. All of the

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polymers except for the leucine-containing material were more effective at delivering siRNA than 25 kDa bPEI (which they found to be ineffective). In fact, the use of the tyrosine sidechain allowed over 90% knockdown.³⁷ However, further work by the same researchers revealed that this was not due to improved particle-formation; in fact, the particles with the lipid side chains were between 570 nm (tyrosine) and 880 nm (phenylalanine). It was reasoned that the improved transfection was a result of the improved buffering capacity of the amines induced by the electron-withdrawing amides. Initial reduced protonation would also explain the larger particles observed.³⁸ Even so, the use of these side-chains represents a simple and effective means of improving PEI for siRNA delivery. Another issue raised by this paper is the lack of transfection by the 25 kDa bPEI. It is important to note that the transfection experiments in this study were carried out in the presence of serum (unlike the work by Grayson *et al.*³² discussed earlier in this section) which could have destroyed the polyplexes. This makes it more surprising that less protonated polymers would have higher transfection as they would be expected to be less stable in the presence of serum. No explanation for this is offered in the publication but it is possible that the presence of hydrophobic groups in the chain makes them less attractive to the binding by anionic serum proteins.

Despite its ability to deliver nucleic acids, PEI is cytotoxic. PEI is not biodegradable under physiological conditions and would therefore build up in the body over time.³⁹ This problem is particularly pertinent for siRNA delivery as siRNA therapy is envisaged to require repeated administration. Toxicity in PEI is thought to be due to two factors, namely, destabilisation of the cell membrane due to the formation of massive aggregates between cationic PEI and anionic serum proteins and interactions with anionic species inside the cytoplasm.³⁰ This effectively means that PEI has no potential as a clinically useful gene delivery vector. However, biodegradability can be incorporated into the polymer chain to mediate cytotoxicity problems while still allowing sufficient “proton sponge” behaviour and binding ability to be a useful vector. This is especially important since the use of a higher molecular weight version of the polymer increases transfection efficiency but also increases toxicity.¹⁸

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Triggered degradation would be doubly useful as there is also potential for it to act as a mechanism for release of the nucleic acid from the polyplex once it has entered the cell. In order for triggered degradation to occur a chemical moiety must be identified that will respond to the change in the environment between the interior and exterior of the cell. The principal changes that are of use in this context are the fact that the interior is more reductive³¹ and more acidic, especially if the polyplex enters via the endosome.³² The most widely used reduction-sensitive group is the disulfide which is reduced to form two free thiols. The scope for the pH-sensitive group is extremely wide and can include esters,⁴⁰ orthoesters,⁴¹ carbonate esters,⁴² acetals⁴³ and imines⁴⁴ which all breakdown at low pH. An important part of the design process is finding a group which does not undergo hydrolysis too rapidly as it is clearly desirable for a polyplex to remain intact in biological conditions long enough to reach the cell and undergo endocytosis.³³ The myriad of degradation triggers and appropriate functional groups is discussed in depth in Section 1.6.

1.5.2. Chitosan

Chitosan, shown in Figure 1.8, is a polymer consisting of partially-acetylated glucosamine repeat units that is obtained from the shells of crustaceans.⁴⁵ Its principal advantage for gene delivery is that it is known to be non-toxic and has already been widely used as a food additive,⁴⁶ in bandages and as a pharmaceutical excipient.⁴⁵ This innocuousness is a clear advantage over toxic polymers such as PEI and has led to some use of chitosan as an siRNA vector. However, unlike PEI, chitosan has no buffering which has led to problems with endosomal escape.⁴⁷

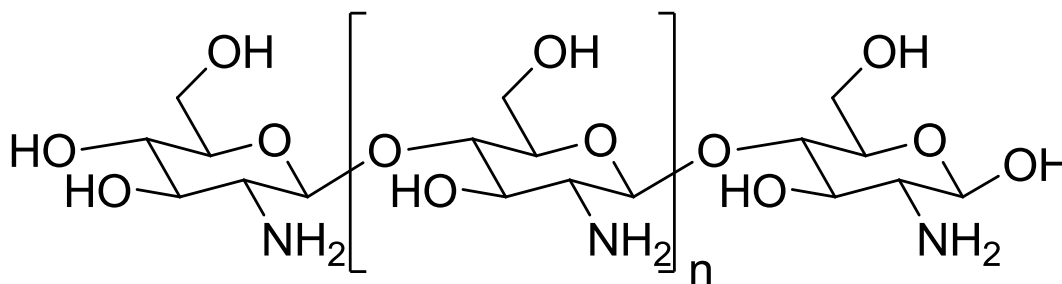


Figure 1.8 – The general structure of unacylated chitosan.

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In 2006, Howard *et al*⁴⁸ used Chitosan to deliver siRNA *in vitro* and *in vivo*. They used a polymer of 114 kDa and found that the polyplexes formed at an N/P ratio of 36 were able to transfect EGFP-expressing NIH 3T3 fibroblast cells to the same extent as a commercial lipid-based agent (*TransIT-TKO*). Knockdown of EGFP was about 80% and no toxicity was observed. Interestingly, they experienced none of the endosomal escape problems associated with the poor buffering capacity of chitosan as evidenced by the lack of improvement in transfection when chloroquine was used. *In vivo* knockdown in EGFP-expressing mice was also observed after nasal administration.⁴⁸ Unfortunately, no information concerning particle size of the N/P 36 particles is reported and across the various experiments N/P values are not comparable

Liu *et al*.⁴⁹ conducted an investigation into the relationship between chitosan structure and its transfection efficiency. They discovered that a chitosan of at least 12 kDa is required to form a stable particle with siRNA (as shown by gel electrophoresis). This is in contrast to the findings of a similar study using pDNA which suggested a minimum molecular weight of 4.7 kDa was required for stable polyplex formation.⁵⁰ Overall, it was found that – in common with PEI – higher molecular weight and greater deacylation (i.e. more amines) led to more stable polyplex formation. However it was also found that with identical degrees of acylation (84%) a 170 kDa chitosan (the highest M_w tested) is marginally less efficient than a 114 kDa material (the most effective). Since size and zeta-potential were found to be the same (~200 nm and ~20 mV) this is likely due to the higher molecular-weight material binding overly strongly resulting in ineffective release of the siRNA leading to inefficient delivery.⁴⁹

Chitosan has also been combined with PEI in an attempt to combine the excellent biocompatibility of chitosan with the transfection efficiency and buffering ability of PEI.⁵¹ A general structure and a summary of the synthetic procedure used to prepare the graft co-polymer is shown in Figure 1.9.

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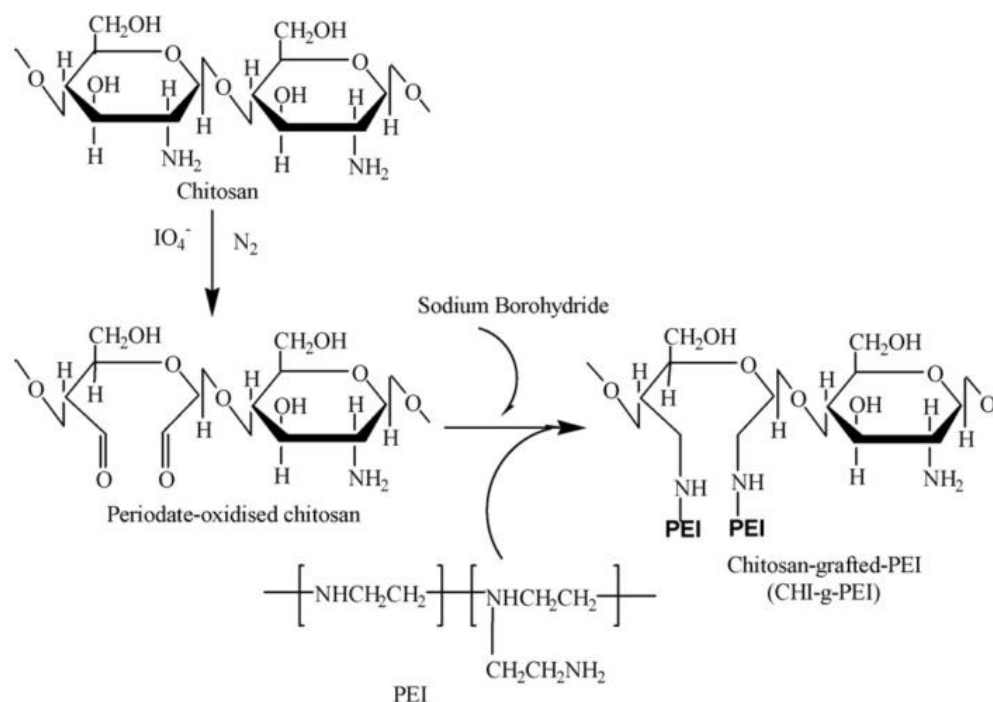


Figure 1.9 – Graft co-polymer prepared by Jere *et al.*⁵¹ (Picture taken from Jere *et al. Int. J. Pharm.*, 2009, 378, 194-200)

The co-polymers were prepared by grafting 1.8 kDa branched PEI to an oxidised 100 kDa chitosan chain *via* a reductive amination. Not only was the co-polymer 2.5 times more effective at *in vitro* gene knockdown than 25 kDa PEI but it also showed minimal toxicity.⁵¹ Unfortunately, the study did not compare the graft co-polymer with unmodified chitosan or branched PEI. The latter is a particular oversight due to the difference in siRNA transfection efficiency between linear and branched PEI reported by Grayson *et al.*³²

Another common modification to chitosan is trimethylation of some of the primary amines to form trimethylchitosan (TMC). This simple alteration imparts greater solubility at a range of pH values and improves buffering capacity.⁵² Varkouhi *et al.*⁵³ compared the siRNA delivery properties of TMC as both an unmodified material and as a thiolated one (Figure 1.10).

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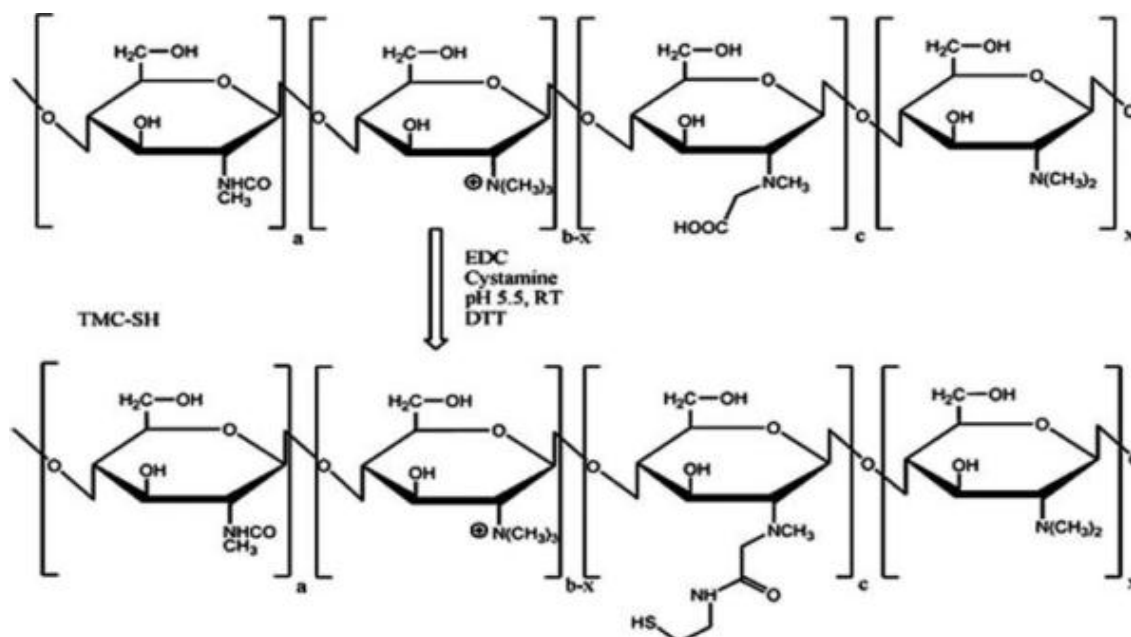


Figure 1.10 – Synthesis of thiolated TMC by Varkouhi *et al.*⁵³ (Picture taken from Varkouhi *et al. Bioconjugate Chem.*, 2010, 21, 2339-2346)

The incorporation of the thiol side-chains as an additional modification was envisaged to benefit transfection primarily by allowing disulfide bonds to form *in situ* and being able to act as a reversible means of improving particle stability. Unlike several of the examples discussed in Section 1.6.1 – where disulfide bonds are introduced to the polymers pre-formed – the use of thiols, which *in situ* can form disulfide bonds also allows several other advantageous properties to be imparted such as improved muco-adhesive potential and providing a means of attachment for targeting ligands or PEG.⁵³

The impact of the thiol groups was immediately apparent in an electrophoresis experiment when a 7%-thiolated TMC polyplex – but not an unmodified TMC one – was able to remain intact in the presence of heparin. The effect on knockdown was also dramatic with the thiolated material enabling approximately double the knockdown of the non-thiolated. Transfection was also attempted in the presence of hyaluronic acid – an anionic carbohydrate polymer that is known to adversely affect transfection with polyplexes.⁵⁴ The unthiolated polymer was unable to transfect the cells at all whereas the thiolated material could (albeit at a 25% lower level than

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before) suggesting that the additional stability observed in the electrophoresis experiments had a profound effect on transfection too.⁵⁴

1.5.3. Polypeptides

Cationic polypeptides are another class of materials well suited to gene delivery. The amino acids most widely seen as components in pDNA delivery are lysine,⁵⁵ arginine⁵⁶ and histidine.⁵⁷ This is not unsurprising given that they are the residues that contain protonatable amine groups (Figure 1.11). There is also another, unrelated use of peptides in gene delivery which is as ligands for targeting and as endolytic agents. This is discussed in Sections 1.7.

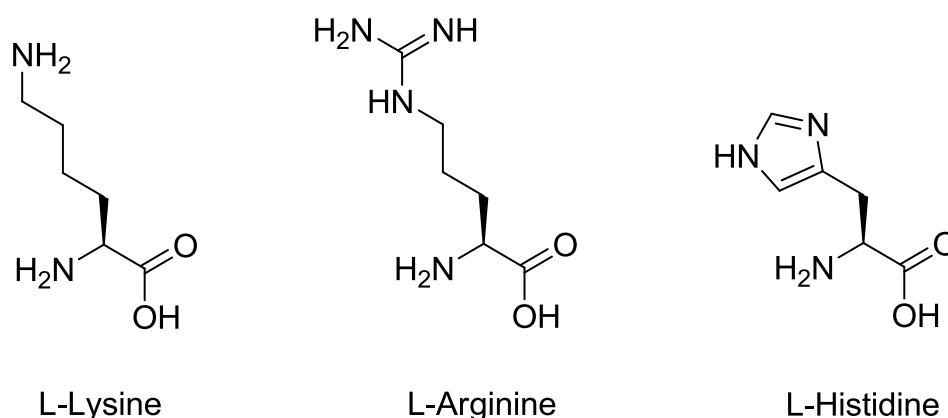


Figure 1.11 – The three cationic amino acids.

The use of polypeptides for siRNA delivery is relatively rare compared to the polycations already discussed although there are examples to be found such as the use of a reducible block co-polymer of histidine and lysine by Stevenson *et al.*⁵⁸ Their polymer was a material that had previously shown success at pDNA delivery and consisted of polypeptides of the formula Cys-His₆-Lys₃-His₆-Cys that had been cross-linked *via* oxidation of the cysteine residues to form disulfide bonds.⁵⁹ The reason for incorporating a combination of cationic amino acids is due to their different pK_a values. Lysine (pK_a 10.8) is almost entirely protonated at physiological pH and provides the necessary cationic charge for nucleic acid binding whereas histidine (pK_a 6.1) remains mostly unprotonated until endocytosis occurs at which point it aids endosomal escape through the proton-sponge effect.⁵⁹ It was found that

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knockdown of EGFP was possible using a 80 kDa polymer but not a 136 kDa polymer. For a 36 kDa polymer some knockdown was possible but only at a high N/P ratio of 40 (*cf.* N/P 4.6 for the 80 kDa material) which was attributed to there being a requirement for greater buffering capacity. The group also attempted delivery with polymer that had been conjugated (though a terminal thiol group) to a targeting peptide taken from *Plasmodium falciparum* – a parasite with a high affinity for liver cells. This led to an approximately three-fold increase in knockdown.⁵⁸

A paper published by Kim *et al.*⁶⁰ demonstrates an interesting approach to the delivery of siRNA using poly-L-arginine (PLR) in conjunction with hyaluronic acid (HA) an anionic carbohydrate macromolecule (Figure 1.12).

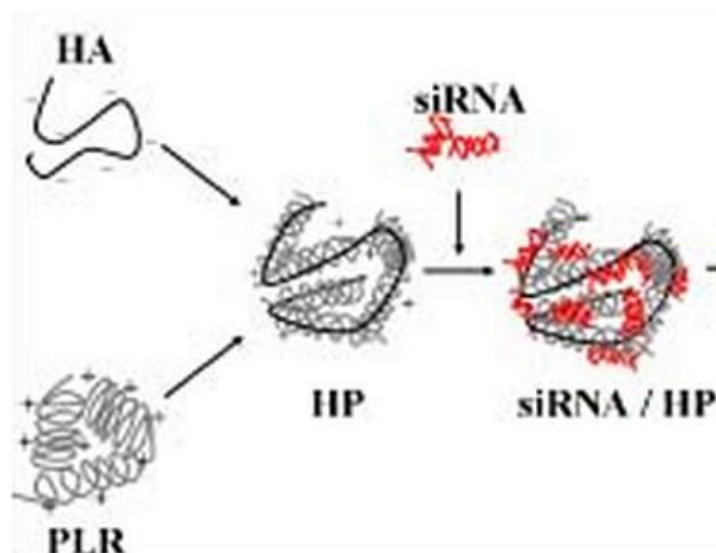


Figure 1.12 – A scheme depicting the dual-polymer strategy used by Kim *et al.*⁶⁰ The condensation of negatively-charged hyaluronic acid (HA) and the positively-charged poly(L-arginine) (PLR) is followed by electrostatic interaction with siRNA to form a nanoparticle. (Picture taken from Kim *et al. J. Gene Med.*, 2009, 7, 791-803)

Hyaluronic acid has an established biocompatibility and a history of use in pharmaceuticals⁶¹ and is used here due to its ability to aid in condensation as well as its ability to act as an aid to adhesion to cells via CD44 receptors. It was found that a delivery system composed of equal amounts of HA and PLR was effective and non-toxic compared to formulations of predominantly PLR, which were more toxic, or predominantly HA, which were not sufficiently cationic to enable the formation of stable polyplexes.⁶⁰

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1.6. The Incorporation of Degradability Into Polymeric Vectors

The incorporation of functional groups to allow triggered degradation of polymers has been alluded to several times over the course of the discussion of the principal cationic polymers used for siRNA delivery. In this section, it will be discussed in greater depth with a focus on the range of available functional groups and the means of triggering their degradation. Interesting degradable polymers that have been used to deliver pDNA will also be discussed since the advanced age of that area of research has meant a greater variety of polymer designs has been investigated than for siRNA.

1.6.1. Disulfides

One of the most common functional groups used as a bioresponsive trigger is the disulfide bond. As well as its widespread use in gene delivery it has also enjoyed success across a range of applications including theranostic nanogels,⁶² polymeric micelles for small-molecule drug delivery⁶³ and in chelates for gadolinium contrast agents for MRI.⁶⁴ In each of the scenarios the purpose of the disulfide bond is the same: to remain intact in the extracellular milieu and to break once inside the cell.⁶⁵

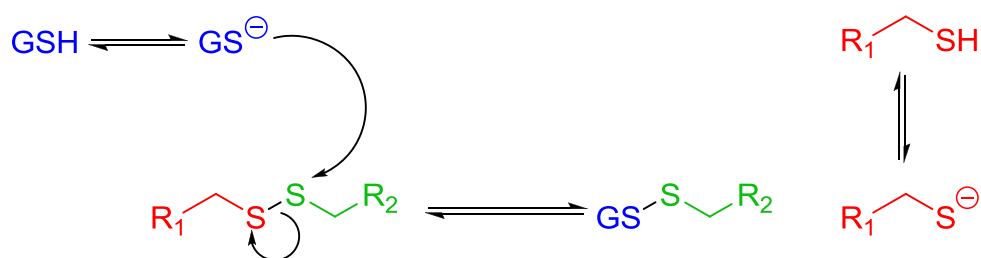


Figure 1.13 – The general mechanism for the reduction of disulfide bonds by glutathione (GSH) a low molecular weight biological thiol.⁶⁶

The reducing environment of the interior of the cell is principally a result of the high concentration of the thiol-bearing tripeptide glutathione.⁶⁷ These thiols act as reducing agent and break disulfide bonds through the mechanism shown in Figure 1.13. Although glutathione exists outside the cell, it exists predominantly as a disulfide (GSSG) with a concentration of the thiol form of only ~1.5 μM. The most reducing area of the cell interior is the cytosol where GSH concentrations can be as

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high as 20 mM and are maintained at that level by enzymes. Other areas of the cell interior such as the nucleus, the mitochondria and the endoplasmic reticulum are less reducing.⁶⁷ The clear contrast in the redox properties of the different cellular environments is what makes the disulfide bond an appropriate trigger.

The simplest way to incorporate the disulfide bond into a polymer is to use one of the many cross-linkers that are commercially available. This approach was used by Breunig *et al.*⁶⁸ who crosslinked low molecular-weight PEI (2.6 kDa) with Lomant's reagent as shown in Figure 1.14.

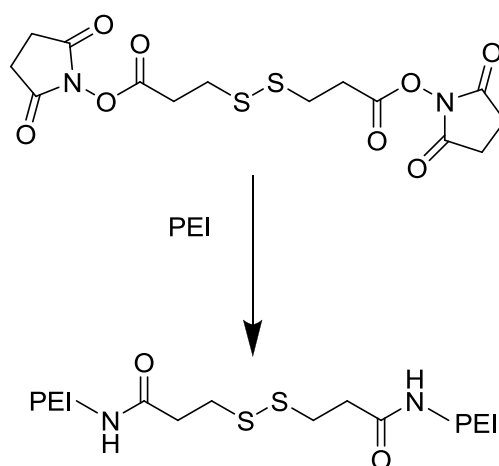


Figure 1.14 – Lomant's Reagent as used by Breunig *et al.* to cross-link low molecular-weight PEI.⁶⁸

The cross-linked polymer that they prepared was compared to 25 kDa branched PEI (similar to the cross-linked material) and 5 kDa linear PEI (similar to the reduced material). In terms of polyplex formation the branched and cross-linked materials were broadly similar: The bPEI fully complexed siRNA at N/P 2 and at an N/P ratio of 12 and higher formed particles of approximately 500 nm and the cross-linked PEI fully complexed siRNA at N/P 4 and formed particles of approx 500 nm at an N/P ratio of 6 and higher (the difference in particle size at N/P 6 was likely due to the strongly anionic zeta-potential of the particles formed with bPEI at this N/P ratio). It was found that polyplexes formed using the more branched bPEI were the most effective at entering the cell although when this difference was taken into account, the relative knockdown was higher using the disulfide crosslinked-polyplexes. This

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was suggestive of improved release of the siRNA from the bioresponsive polyplexes. The disulfide bonds also reduced toxicity of the cross-linked material to a negligible level in common with the low molecular-weight IPEI.⁶⁸

An alternative strategy of disulfide incorporation is to synthesise entirely novel alternating co-polymers. As shown in Figure 1.15, similar methods were used by both Kim *et al.*,⁶⁹ Rahbek *et al.*⁷⁰ and Hoon Jeong *et al.*⁷¹ who each prepared linear or branched polymers using cysteine bisacrylamide.

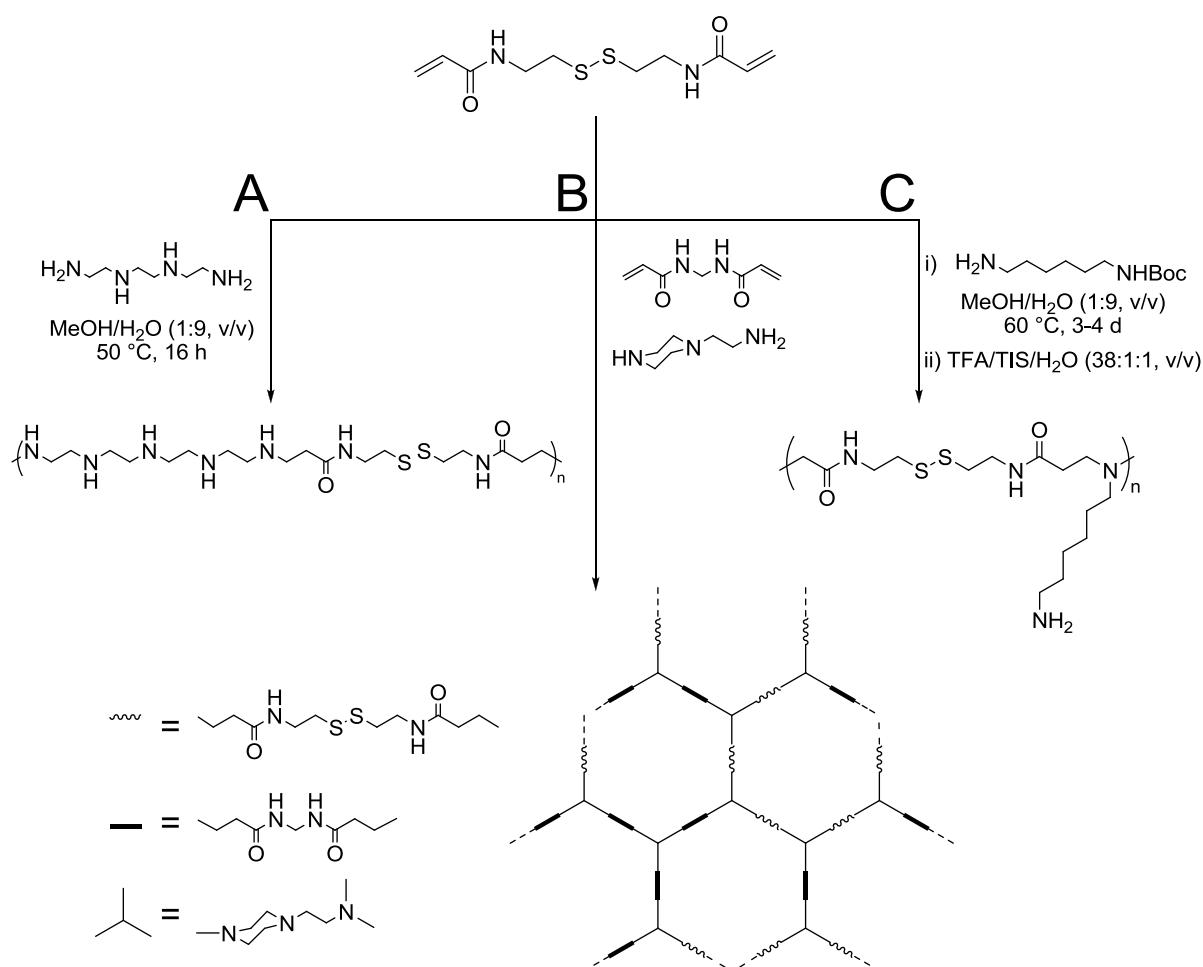


Figure 1.15 – Three different polymers prepared via Michael addition to cysteinebisacrylamide by A) Hoon Jeong *et al.*⁷¹ B) Rahbek *et al.*⁷⁰ and C) Kim *et al.*⁶⁹

Cysteinebisacrylamide is an attractive monomer as it reacts cleanly with amines and depending on the nature of the amine used, a variety of branched or linear materials

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can be prepared using mild conditions. The linear polymer prepared by Hoon Jeong *et al.*⁷¹ was prepared to a M_w of 3350 Da (by GPC) and compared to a much larger 25 kDa PEI. Fluorescently labelled siRNA was used to allow direct observation of the release of the siRNA in the cytoplasm. It was found that five hours after internalisation, the siRNA that had been delivered using the reducible polycation was more widely dispersed in the cytoplasm than that delivered using lPEI. This was attributed to more effective unpacking of the reducible polyplexes. However, since the lPEI used was of greater molecular weight than the reducible material it is unclear how much of this improved release was due to reduction and how much was due to the siRNA being more weakly held by a less cooperative, low molecular-weight polymer.⁷¹ The paper does not discuss branching of the polymer and since it is also mechanistically possible for secondary amines to react with CBA this is a particular oversight.

Hyperbranched material was prepared by Rahbek *et al.*⁷¹ by using CBA in conjunction with 1-(2-aminoethyl)piperazine which, able to react with up to three acrylate groups, forms a hyperbranched polymer as illustrated in Figure 1.15. In addition, non-reducible *N,N'*-methylene bisacrylamide was used to allow variation of the number of reducible cross-links without affecting the degree of cross-linking itself. However, this did lead to differences in molecular weight. As the weight ratio was the factor that was kept constant between polyplexes made with different materials there were some stark differences in the nature of the nanoparticles: specifically, more positively charged (+30 mV), larger (150 nm) particles prepared using a 17% reducible, 115 kDa material compared to those prepared using 40 kDa, 50% reducible material (+9 mV, 90 nm). It is reported that the material featuring only 17% reducible cross-links was the most effective at siRNA delivery *in vitro* and this is attributed to its increased zeta-potential enabling stronger interaction with the surface of cells and hence greater uptake. The most interesting observation that can be attributed to the difference in susceptibility to reduction of the polymers tested is the effect that the cross-links had on the release of siRNA over time. Counter-intuitively, it was the material with the greatest number of reducible cross-links that released siRNA the slowest – still releasing siRNA after 72 h. This is in contrast to the least reducible material which had reached a maximum level of knockdown after

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24 h. No explanation is offered for this result although it offers a potentially interesting method of siRNA dose-control.⁷⁰

The final method of reducible polycation synthesis to be discussed is the oxidative polycondensation. As shown by Won *et al.*⁷² this involves the use of monomers – in their case an 11-residue polypeptide – featuring terminal thiols that are oxidised by DMSO to form disulfide containing polymers (Figure 1.16).

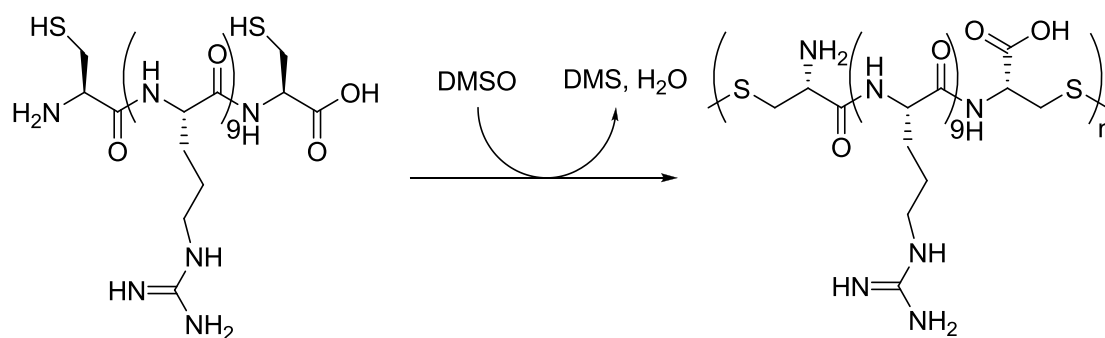


Figure 1.16 – Reducible poly-D-argininine prepared by Won *et al.* via an oxidative polycondensation reaction.⁷² (DMSO = dimethylsulfoxide, DMS = dimethylsulfide)

The advantages of the oxidative condensation are the mildness of the reagent used as well as the innocuous byproducts. Won *et al.*⁷² used their reducible poly-D-argininine (Figure 1.16) in an interesting study where they directly demonstrated the dependency of siRNA release on glutathione. They did this by showing a marked reduction in transfection when cells were treated with DL-buthionine-[S,R]-sulfoximine: an inhibitor of glutathione production. They also used their polymer in conjunction with siRNA targeted against vascular endothelial growth factor (VEGF) the knockdown of which has been shown to reduce tumour growth.⁷³ By directly injecting reducible polyplexes into the tumours of mice Won *et al.* were able to significantly slow the tumour growth over a period of 17 days and demonstrate the *in vivo* effectiveness of reducible polycations.

An unconventional manifestation of disulfide bonds is in conjunction with polymer/cyclodextrin supramolecular complexes.⁷⁴ Cyclodextrins (CD) are a species of cylindrical macromolecule which is hydrophobic on the inside and hydrophilic on

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the outside.¹ The CD is capable of reversibly threading onto a polymer chain creating a supramolecular complex called a polyrotaxane. Several polyrotaxanes have been synthesised for the purposes of gene delivery due to their low cytotoxicity and easy functionalisation with targeting moieties.⁷⁵ Triggered release of DNA from a polyplex takes place by harnessing the reversible nature of CD threading. Ooya *et al.*⁷⁴ created a polyrotaxane whereby cyclodextrins were covalently attached to cationic amine groups and subsequently threaded onto PEG-like chains. The chains were stoppered with bulky aromatic groups attached to the main chain with disulfide bonds. Upon entering the reductive cytoplasm, the stoppers are cleaved allowing the CD to de-thread leading to release of DNA (Figure 1.17) and successful transfection.⁷⁴ No information on this material being used as an siRNA vector has been published.

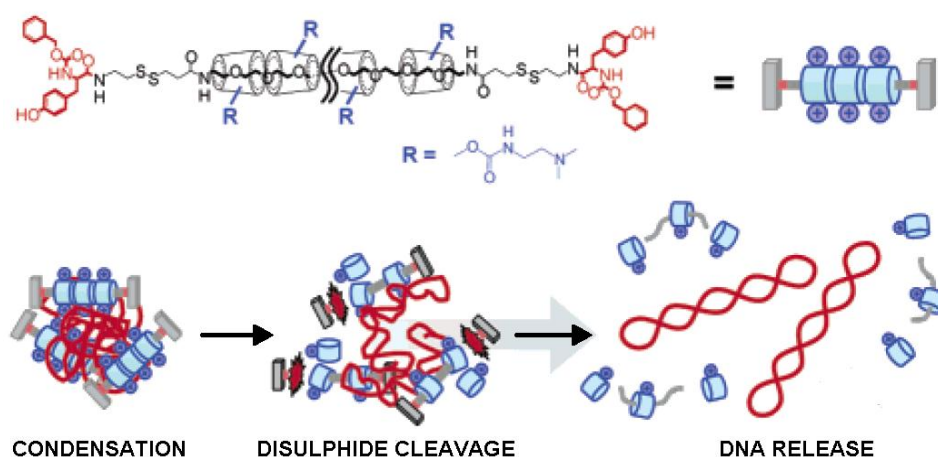


Figure 1.17 – Structure and degradation mechanism of disulfide rotaxane⁷⁴ (Picture taken from Ooya *et al.*, *J. Am. Chem. Soc.*, 2006, 128, 3852-3853.)

Disulfides also have another potential use of particular relevance within the context of polymer-mediated siRNA delivery. As will be discussed in Section 1.7, siRNA polyplexes may require a stealth layer to protect from blood serum protein adsorption, uptake by macrophages and RNase degradation *in vivo*. However, the steric shielding that offers the vector protection also works to undermine its interaction with the cell membrane. This problem was approached within the context of cationic liposome-mediated delivery (which has many parallels to polyplex delivery in the sense that both have to survive in the extracellular environment, undergo cell entry and unpack) by Kirpotin *et al.*⁷⁶ who attached the PEG chains via

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disulfide links with a view to exploiting the reductive nature of the surface of some cells. This could also allow a type of cell-specificity to be achieved as only certain cells have a reducing environment at the cell-surface.⁷⁶ While there is no precedent for this in polymer-mediated delivery of siRNA, this work demonstrates the overall usefulness of the disulfide bond in chemical biology applications.

1.6.2. pH Sensitivity

Another common method of incorporating triggered degradation is through the use of hydrolysis-sensitive functional groups. Esters in particular have been used as a component of pDNA vectors for many years^{77,78} and for the delivery of other drugs for even longer.⁷⁹ The principle behind such triggers is that the pH of the endosome is – at 5.5 – lower than the typical physiological pH; this leads to more degradation of any groups sensitive to acid-catalysed hydrolysis.⁴³ There is also precedent for pDNA vectors which incorporated amides sensitive to base-catalysed hydrolysis that were designed to be more hydrolytically stable at pH 5.5 than at physiological pH.⁸⁰ To have degradation triggered in this way is far less common though and on this occasion was done to lessen the impact of enzymatic pDNA degradation which is more prevalent at lower pH.⁸⁰

Overall, the purpose of hydrolysis triggers is broadly similar to that of reduction triggers. However, for the hydrolysis sensitive groups degradation is most rapid in the endosome unlike disulfide triggers which degrade quickest in the cytoplasm.⁶⁷ The other difference is that the trigger for the reduction of disulfides is more specific. As discussed previously, every environment that a vector would be exposed to *en route* to the cytoplasm is oxidising so a disulfide bond will remain stable. For a hydrolysis sensitive material, degradation can take place in any aqueous environment. This renders hydrolytic degradation far less specific. For example, a PEI featuring ketal bonds that was prepared by Shim *et al*⁸¹ only had a half-life of 20.6 hours at pH 7.4. While the implications of this from the point of view of drug delivery are not disastrous they would perhaps cause problems in terms of storage of the materials.

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The ketalised PEI mentioned in the previous paragraph is shown in Figure 1.18. In this case, the acid-labile linkage is used to facilitate triggered release of the siRNA and not full degradability.⁸¹

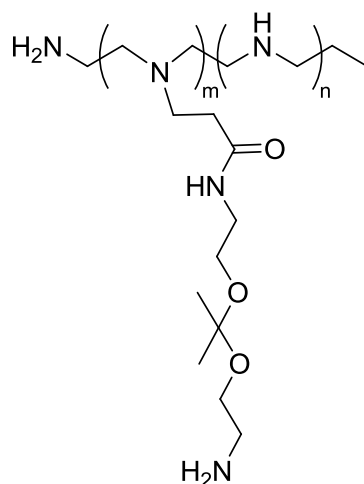


Figure 1.18 – The structure of the ketalized PEI prepared by Shim *et al.*⁸¹

Specific silencing of eGFP using this polymer is around seven times greater than unmodified linear PEI at their respective optimum N/P ratios and this is attributed to the ability of the polymer to release the siRNA when the side-chains detached. This was even the case at very high N/P ratios of 120. Another elegant feature of this polymer is that degradation of the polymer not only causes loss of amines, it causes a gain of a hydroxyl group. When formed, these neutral side-chains are believed to hinder complexation of siRNA to such an extent that the polyplexes swell and lessen trafficking of the polyplexes into the nucleus and increasing the amount of siRNA that is delivered to the cytoplasm.⁸¹

Shim *et al.* also used ketals in conjunction with esters to form a series of spermine-containing alternating co-polymers as shown in Figure 1.19.⁸² The inclusion of two different hydrolysis sensitive groups is important; the ketal linkage is intended to be degraded in the endosome whereas the ester bonds are susceptible to base-catalysed hydrolysis at pH 7.4 but relatively stable to acid-catalysed hydrolysis at endosomal pH. Interestingly, the presence of ester monomers slows down the degradation of the

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ketals and *vice-versa*.⁸² Though the reason for this behaviour is not discussed in the publication it is possible that this effect is similar to the one noticed by Kim *et al.*⁴⁰ while investigating hyper-branched poly(aminoesters) for pDNA delivery. They observed that an increase in the density of polymer slowed degradation based on a decrease in the accessibility of the ester-linkages. In the ketal-ester polymer it could be the case that at endosomal pH the presence of the ester bonds means the polyplex maintains its integrity for longer and make the ketals less accessible. The opposite would be true at pH 7.4 where the ketal bonds would hold the polyplex together.

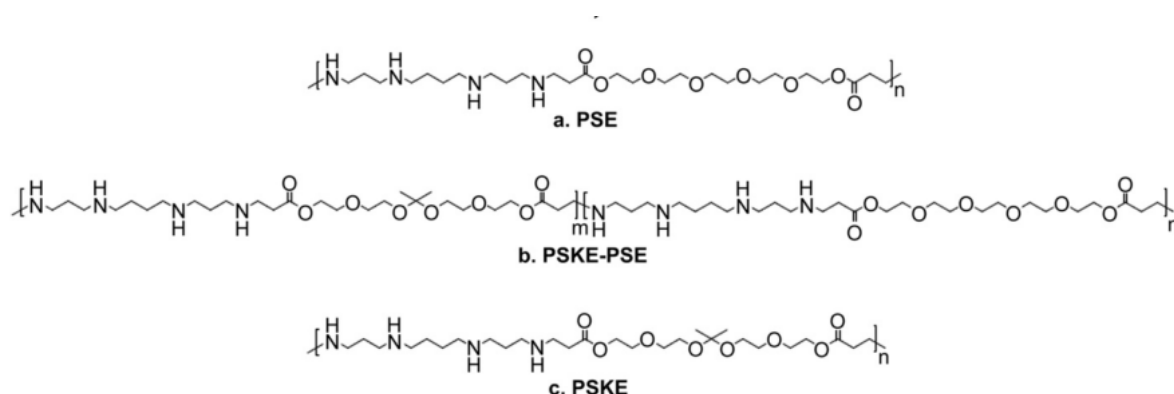


Figure 1.19 – The three polycations prepared by Shim *et al.*⁸² (Picture taken from Shim *et al.*, *Biomaterials*, 2011, 32, 4009-4020.)

By combining different proportions of ketal and ester it was suggested that greater control of the unpacking behaviour of the vector. Whilst this is more important from the point of view of ensuring that a pDNA vector would reach the nucleus it can also be envisaged that some degree of controlled release of siRNA could be induced. Also, in contrast to the previous example which had the ketal groups in the side chains, the location of the degradable linkers in the main chain meant that these polymers were less toxic than non-degradable analogues since hydrolysis led to a reduction in molecular weight. The ketal-only and ester-only (PSKE and PSE in Figure 1.18, respectively) were effective *in vitro* although performed worse than 25kDa bPEI. *In vivo* knockdown was also observed though no positive controls were used in that experiment.⁸²

An entirely different approach to pH sensitivity was taken by Dehousse *et al.*⁸³ They used two separate polymers: trimethylchitosan (see Section 1.5.2) and

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poly(methacrylic acid) (PMAA). The principle was that the PMAA would be anionic at pH 7.4 and aid polyplex formation through interaction with the cationic chitosan (see also Section 1.5.3 for a discussion of a similar system). At pH 6 however, it would be neutral and through the resulting destabilisation of the nanoparticles allow release of the siRNA and also disruption of the endosome. The drop in pH was measured to cause a change in particle size from 200 nm to 450 nm in the particles containing PMAA. For particles containing only TMC and siRNA the change was from 150 nm to 200 nm and was due to increased protonation of amines leading to some charge repulsion. Polyplexes containing PMAA were universally more effective than those that didn't due to this lack of pH sensitive behaviour.⁸³

1.6.3. Extrinsic Triggering

So far, the triggers that have been discussed are based on a response to naturally occurring conditions. Less common are triggers that respond to a controlled external stimulus. One example of this is the Photochemical Internalisation (PCI) technology used by Bøe *et al.*⁸⁴ in conjunction with a cyclodextrin containing polymer similar to the one discussed in Section 1.6.1. PCI technology has been used with lipid-vectors⁸⁵ and linear and branched PEI⁸⁶ to allow light-directed delivery of siRNA. The PCI technology is simply a means of allowing photo-initiated endosomal escape. The photosensitisers used are specifically designed to localise in the membranes of endosomes and lysosomes which they are then able to disrupt. This has great therapeutic implications as it allows direct control over the release of siRNA in terms of time and location.⁸⁴ It could be argued though that this strategy is far more wasteful than targeting ligands (Section 1.7) as one is delivering a greater dose than necessary.

Another example of extrinsic triggering is the use of temperature. Lee *et al.*⁸⁷ made nanocapsules using PEI and the amphiphilic polyether Pluronic. It is important to point out the distinction that these nanocapsules have more in common with liposomes than polyplexes consisting as they do of a roughly spherical "bubble" held together by the hydrophobic effect. The key property of these particles is that at room temperature they are around 350 nm in diameter yet at 37 °C they shrink to a more

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transfection-appropriate 100 nm. This transition is reversible and so it was envisaged that a cooling-induced expansion could be used as a means of endosomal escape. Transfection experiments showed that when the transfected cells were rapidly cooled to 15 °C, siRNA delivery on a par with 25 kDa bPEI polyplexes was obtained. Cells that were not exposed to the “cold-shock” trigger were only half as efficiently transfected. Presumably this was due to the PEI containing nanocapsules possessing a degree of proton-sponge capability. It is acknowledged that this technique has very little usefulness in terms of *in vivo* therapy although it is proposed that it does offer a convenient way of triggering endosomal escape in an *in vitro* context.⁸⁷

1.7. The Surface-Modification of Polyplexes

Another important aspect of vector design is the modification of the surface of the polyplexes in order to impart unto them extra abilities or allow them to overcome otherwise insurmountable obstacles. It is important to note that the reasons for and methods of surface-modification of polyplexes are independent of the cargo carried by the polyplexes and what is true for pDNA delivery is generally also true for siRNA. The following discussion uses examples from both fields.

The use of poly(ethyleneglycol) (PEG) “stealth layers” has been alluded to many times so far in the context of vector design although it has yet to be discussed in detail. Since the formation of polyplexes using cationic polyamines and anionic nucleic acid generally requires an excess of the cationic component, the polyplexes themselves are positively charged. This is problematic because it increases the chance of unwanted electrostatic interactions with anionic proteins in the extracellular environment.⁸⁸ The fact that the forces holding an siRNA polyplex together are weaker than in equivalent pDNA particles make these unwanted interactions even more disruptive.²⁸ The coupling of PEG chains to the surface of the particle effectively mediates charge and the coating also provides a steric barrier to interactions between anionic macromolecules and the cationic interior of the polyplex.⁸⁹ However, by shielding the positive charge, one is removing the ability of the particle to interact with negatively-charged proteoglycans on the surface of the cell.⁹⁰ This necessitates the introduction of an alternative means of encouraging

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interaction with the cell; this is typically in the form of a targeting ligand. Aside from simply overcoming the reduced interaction with cells, targeting ligands bring with them several other advantages too. The ability to prepare polyplexes which interact only with the desired cell type would be extremely useful not least if the target is an organ that the vector cannot be injected into directly. Such precision would also reduce the chance of side-effects and make therapies more financially viable as lower doses could be used.

Targeting is achieved by attaching to the polyplex a particular ligand that is complementary to a receptor on the target cell. The most widely used types of targeting ligand are those that are naturally occurring in the body (endogenous ligands) because they are well characterised and also well tolerated by the immune system.⁹¹ An example of this is transferrin: a serum glycoprotein with affinity for a receptor that is overexpressed in tumour cells.⁹² Covalent attachment of transferrin to PEI/pDNA polyplexes led in some cases to a 1000-fold increase in transfection efficiency and also reduced the minimum N/P ratio required for successful delivery.⁹²

Work by Fajac *et al.*⁹³ tested both mannose and lactose conjugated to PEI/pDNA polyplexes. Sugars are among the simplest targeting ligands; receptors called lectins present on cell membranes bind sugar molecules and thereby assist entry of the polyplexes attached to them. It was found that lactose was very effective at encouraging transfection although since lectins are not especially unique to any cell this should be considered a transfection aid as opposed to a targeting method.⁹³

Exogenous ligands are ligands that are not naturally found in the body and as a result can offer greater specificity than endogenous ligands. The downside is that, as foreign entities, they are also more likely to trigger an immune response.⁹⁴ A good example of exogenous ligands – common to all types of drug delivery – are the monoclonal antibodies (mAb).⁹⁵ MAbs are antibodies that are produced from a cloned cell and can be created for any binding site by introducing the ligand specific

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to the binding site into a mouse and harvesting the antibodies produced. Chiu *et al.*⁹⁶ attached Herceptin® mAbs to a 25 kDa lPEI chain since Herceptin® has great affinity for human epidermal growth factor receptor-2 (HER2), an 185kDa glycoprotein that is over-expressed on the surface of tumour cells. Her-PEI showed a 20-fold increase in transfection efficiency over PEI. The polyplexes also showed the expected selectivity when cell-lines with different levels of HER2 expression were transfected.⁹⁶

Another type of endogenous ligand is copied directly from viruses. Cell Penetrating Peptides (CPP) are used by viruses to facilitate cell entry via a poorly understood mechanism.⁹⁴ HIV possesses a CPP called HIV-1 Tat which was attached to 25 kDa bPEI polyplexes by Suk *et al.*⁹⁷ It was found that the use of the Tat protein increased transfection by a considerable amount although this was thought to be mostly through improved endosomal escape.⁹⁷ However, this is a non-cell specific targeting method and like the glycosyl ligands mentioned above serves to increase transfection to all cells.

Attaching PEG chains and ligands to a polymer also requires yet another design choice. The method of attachment needs to be synthetically viable, to not interfere with nucleic acid complexation and to not mask the targeting action of the ligand. Various strategies have been developed to do this. According to a study by Ogris *et al.*⁹⁰ there are three principal methods of ligand/PEG attachment (Figure 1.20).

The least effective method is reported to be direct attachment of the ligand to the polycation followed by PEGylation once a polyplex is formed (A in Figure 1.20). This is because the bulky ligand interferes with DNA condensation and hinders polyplex formation. The procedure designated B in Figure 1.20 is thought to be particularly effective since all PEG chains end up on the outside of the polyplex and the introduction of ligands after polyplex formation prevents them from interfering with binding. The only reported problem with this method is the number of steps required. The final method actually turned out to be the most effective since it resulted in the

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same product as B but in only one step. The desired arrangement, with PEG chains located at the surface of the polyplex, is claimed to form spontaneously due to the PEG chains not mixing with the PEI chains due to their ostensible difference in hydrophobicity.⁹⁰

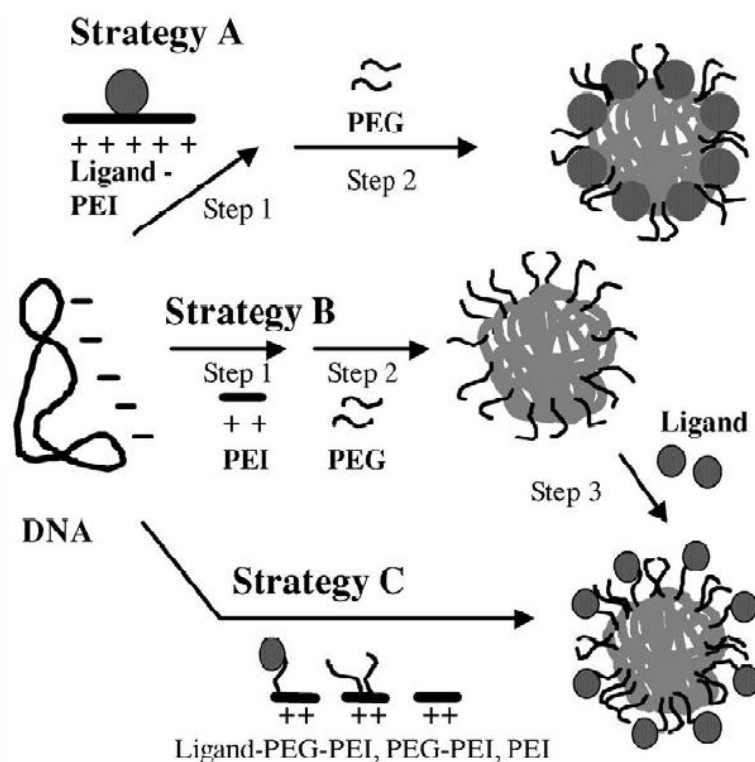


Figure 1.20 - A scheme showing the three methods of PEG/ligand conjugation identified by Ogris *et al.*⁹⁰ A) Ligand is attached directly to PEI. The polyplex formed with DNA is PEGylated. B) Polyplex is PEGylated followed by attachment of ligand to PEG. C) A polyplex is formed between DNA and a mixture of separately prepared PEI, PEI/PEG and PEI/PEG/ligand.

(Picture taken from Ogris *et al.*, *J. Control. Release*, 2003, 91, 173-181.)

1.8. The Use of Dendritic Vectors

Dendrimer architectures have also proved useful for gene-delivery. The principal advantage that dendrimers have over linear or branched polymers is that they have a clearly defined and reproducible size, shape and structure.⁹⁸ This could make them more viable as a clinical treatment since reproducibility is vital for a drug to gain approval from regulatory bodies. By far the most commonly used dendrimer type is poly(amidoamine) (PAMAM)⁹⁸ (Figure 1.21).

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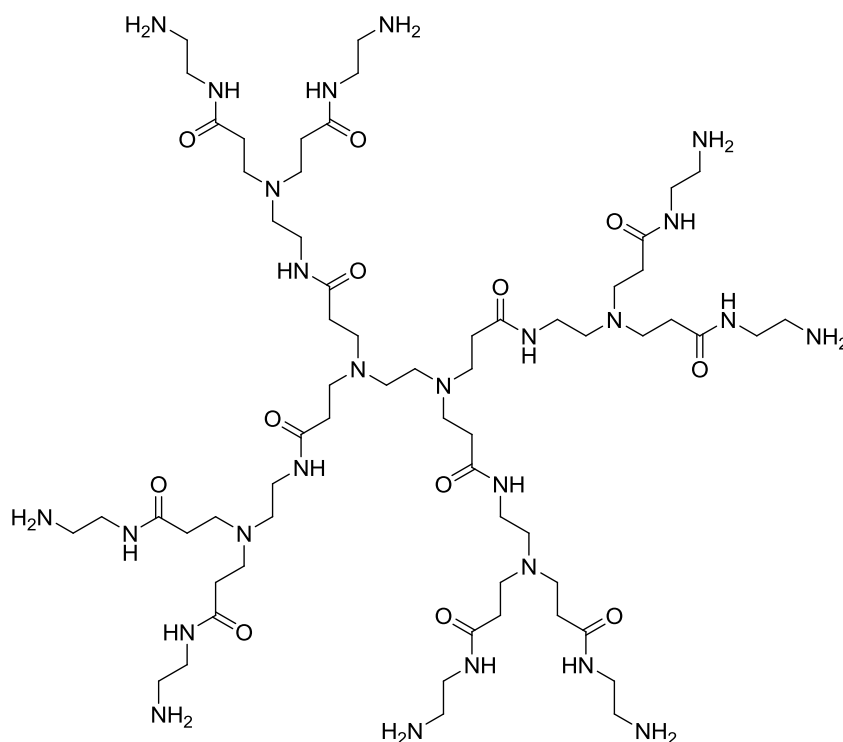


Figure 1.21 – First generation of PAMAM dendrimer with an ethylene diamine core⁹⁹
(Picture taken from Braun *et al.*, *J. Pharm. Sci.*, 2005, 94, 423-436.)

PAMAM is an effective vector due to its large number of protonatable amine groups suitable for binding siRNA in an entropy-driven manner¹⁰⁰ similar to the condensation of nucleic acids by linear polycations described in Section 1.5. Similar to PEI, PAMAM possesses a number of amines conducive to “proton sponge” behaviour.⁹⁸ Due to their low cytotoxicity,⁹⁸ triggered degradation is a lower priority in dendritic vectors than in linear polymers. The most useful feature of dendrimers with respect to siRNA is that particle size is predominantly dependent on the physical size of the dendrimer and as a result, inefficient “condensation” is not a concern. Particle size can therefore be tightly controlled *via* variation of dendrimer generation. However, it has also been reported that observed particle size depends greatly on the ionic strength of the media and that high salt concentrations (150 mM NaCl) could cause G₄ particles to form large aggregates of greater than one micron.¹⁰¹

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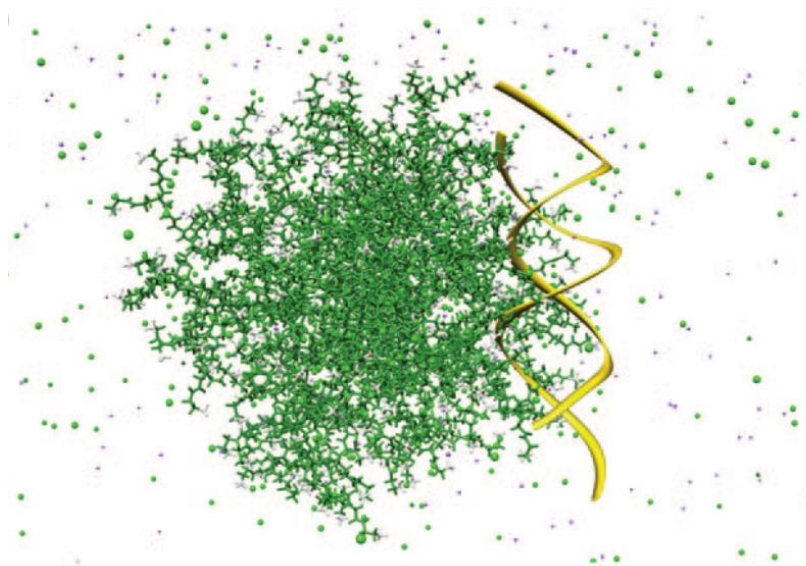


Figure 1.22 – Snapshot from a molecular dynamics simulation of a complex between G₆ PAMAM (green) and siRNA (yellow) at pH 7.4. Sodium and chloride ions are represented as green and purple spheres¹⁰² (Picture taken from Pavan *et al.*, *Chemistry*, 2010, 16, 7781-7795.)

One of the earliest reports of PAMAM dendrimers being used as siRNA transfection agents was from Zhou *et al.*¹⁰³ They compared dendrimers – made with a triethanolamine core – from generation one (G₁) to generation seven (G₇) and found that transfection efficiency increased with higher generation number and siRNA concentration. The toxicity of the materials was observed to be dependent on the amount of nanoparticle used. Enough material to elicit 80% gene knockdown also led to 50% cell death.¹⁰³ Zhou *et al.* proposed that the increase of transfection efficiency with increasing generation number was due to the increased number of primary amines on the surface of the dendrimer with a G₇ dendrimer (384 primary amines) able to form stable complexes at N/P ratios as low as 2. The idea that siRNA was being bound to the surface of the dendrimers was corroborated by an experiment whereby the surface amine groups were replaced with esters; this prevented stable complex formation at even N/P 20.¹⁰³ Computational studies by Pavan *et al.*^{102,104} have also shown this to be the most likely mode of interaction as shown in Figure 1.22. They observed that even at pH 5 – where increased amine protonation would not only be expected to increase the strength of electrostatic interactions with siRNA but to also drive the dendrimer branches apart due to electrostatic repulsion – the siRNA remained predominantly on the surface.

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Patil *et al.*¹⁰⁵ demonstrated that it was possible to use G₄-PAMAM to form nanoparticles of around 100 nm with siRNA. Their work involved modifying the dendrimers in two ways. Firstly, the interior tertiary amines were alkylated to form cationic quaternary amines. This was done to encourage the siRNA to bind beneath the surface of the dendrimer. The second modification was to acylate the surface primary amines to reduce toxicity by reducing non-specific interactions between the particles and the cell. They report that at doses where unmodified G₄-PAMAM causes about 50% cell death, the acylated material causes only 20%. They also used fluorescently labelled siRNA to enable the tracking of particles using confocal microscopy and found that the quaternized, acylated dendrimers were internalized by the cell to a far greater degree than the unmodified PAMAM.¹⁰⁵ No quantitative transfection data was reported and while successful internalization of the siRNA is an important step towards knock down, research into using PAMAM to deliver pDNA reports that it is possible to bind nucleic acid so strongly that it is not released from the complex.⁹⁹ The fact that the siRNA is held deep within the quaternized PAMAM could be a potential problem in this regard.

Overly strong binding was an apparent concern of Waite *et al.*¹⁰⁶ who used varying degrees of acylation of PAMAM as a means of controlling the interaction with siRNA. G₅-PAMAM dendrimers were acylated such that 20, 40, 60 or 80% of primary amines were affected and all but the most acylated were able to form stable complexes. Transfection was unaffected by 20% acetylation though it diminished to almost nothing by the 60% acylated sample which also performed poorly in a heparin competition assay suggesting binding may have simply been too weak. Though quantitative transfection experiments suggested that knockdown was unimproved by the acylation, confocal microscopy did show increased siRNA unpacking. The reason that this was not reflected in the transfection assay is attributed to the diminished endosomal escape ability of the acylated dendrons brought about by reduced buffering capacity.¹⁰⁶

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One of the most interesting dendrimer siRNA delivery agents was developed by Taratula *et al.*¹⁰⁷ They took a complex formed between siRNA and a poly(propyleneimine) (PPI) dendrimer and used amine-reactive, disulfide containing cross-linkers to “cage” the siRNA. This was an attempt to overcome the problem of needing a level of binding strong enough to protect the siRNA in the extracellular environment but sufficiently weak to allow the siRNA to be released in the cytoplasm. As shown in Figure 1.23, they also incorporated PEG chains and a targeting ligand for a receptor over-expressed in tumours.

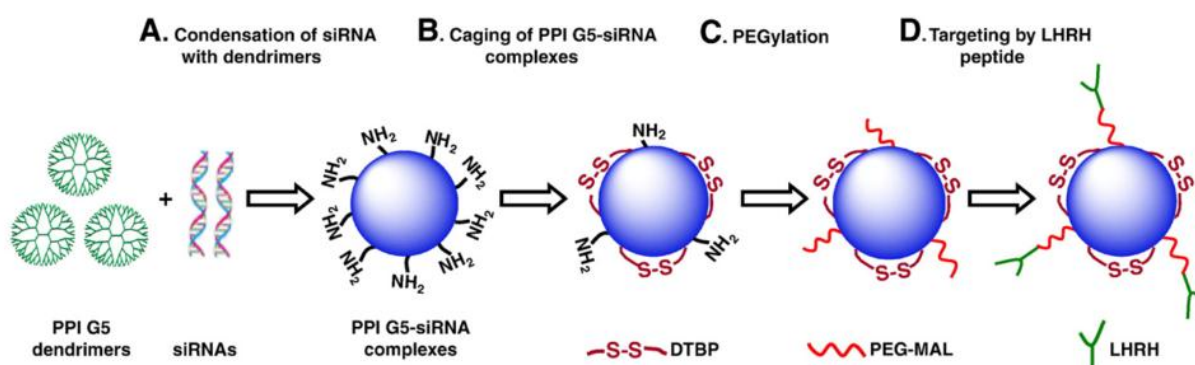


Figure 1.23 – Formation of “caged” dendrimers reported by Taratula et al.¹⁰⁷ DTSP is Dimethyl-3,3'-dithiobispropionimidate-HCl, a disulfide containing cross-linker; PEG-MAL is maleimide-terminated PEG; LHRH is Luteinizing Hormone-Releasing Hormone, a targeting ligand.

The principal behind the disulfide cross-linking is that in the extracellular environment the siRNA is sterically prevented from unbinding from the dendrimer. Equally, serum proteins are prevented from binding as shown by a competition assay where the amount of siRNA in the cross-linked particles is left relatively undisturbed at concentrations where the unmodified particles have shed their entire cargo. Once inside the cytoplasm, the disulfide bonds are reduced and the release of siRNA is no longer impeded. Knockdown experiments performed with this material were impressive with almost total *in vitro* knockdown observed with minimal toxicity. *In vivo* experiments were conducted and showed that due to the targeting ligand the dendrimer complexes delivered siRNA to the tumour target in preference to any other location.¹⁰⁷

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Another creative approach to dendrimer design was published by Deng *et al.*¹⁰⁸ who, as shown in Figure 1.24, combined PAMAM dendrons with β -cyclodextrin using azide-nitrile click chemistry to form an architecture similar to those used by Srinivasachari *et al.* for pDNA delivery.¹⁰⁹ As well as providing a convenient, defined core for the dendrimer it is also proposed that – commensurate with cyclodextrin's ability to host hydrophobic molecules – a co-drug could be delivered in tandem with siRNA.¹⁰⁸

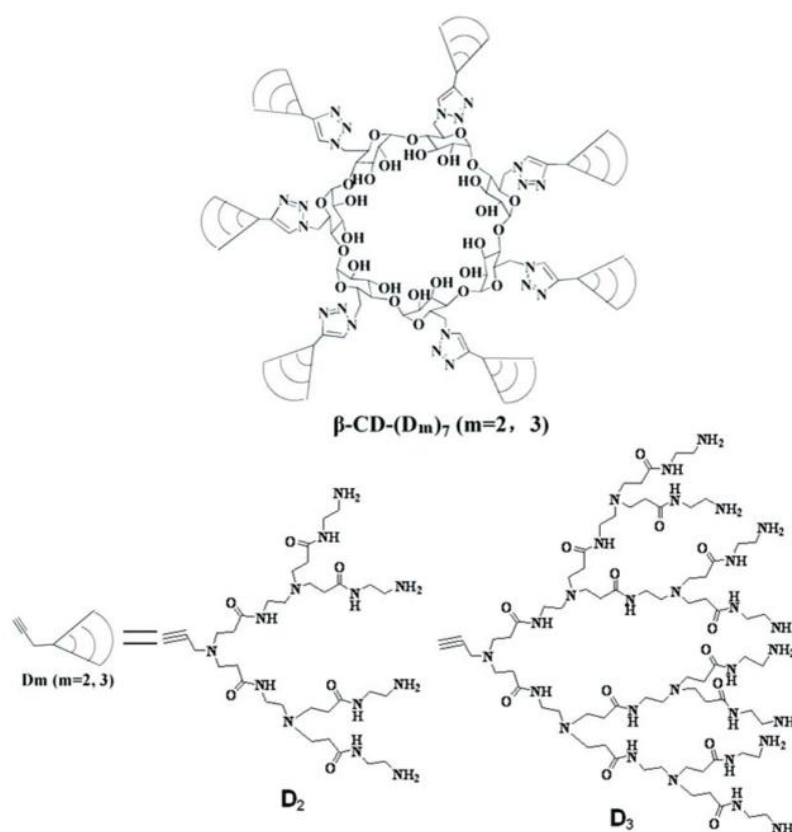


Figure 1.24 – The cyclodextrin-PAMAM dendrimers prepared by Deng *et al.*¹⁰⁸ (Picture taken from Deng *et al.*, *J. Mat. Chem.*, 2011, 21, 5273-5281.)

Complexes of approximately 100 nm were formed between the G₃ species and siRNA and as expected transfection efficiency was high with low cytotoxicity. Transfection efficiency was also relatively unaffected by the presence of serum – a characteristic of very stable particles.¹⁰⁸

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Although they have yet to be used for siRNA transfection, dendrons have been used for pDNA binding and offer another interesting structural possibility. This is demonstrated by Kostiainen *et al.*¹¹⁰ who synthesised and tested various generations of a pseudo-dendrimer containing a spermine motif (Figure 1.25).

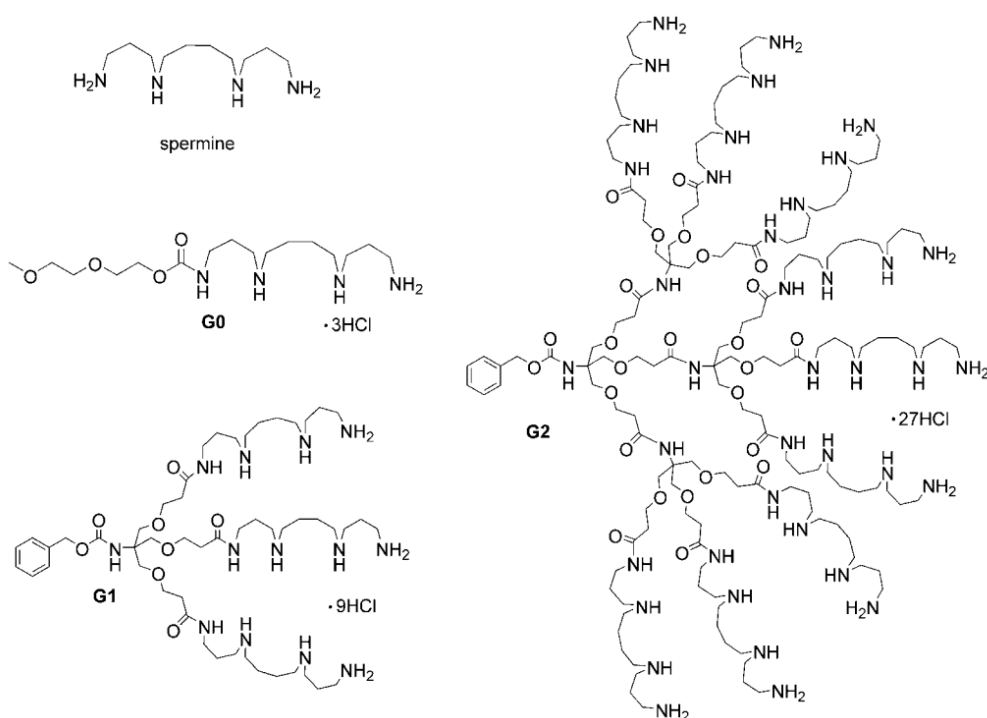


Figure 1.25 – Dendrons prepared by Kostiainen *et al.*¹¹⁰ (Picture taken from Kostiainen *et al.*, *Angew. Chem. Int. Ed.*, 2005, 44, 2556-2559.)

The increased multivalency of the higher generation dendrons increased the strength of binding to pDNA as measured by ethidium bromide displacement assays. Specifically, the binding of the G2 dendron was an order of magnitude higher than that of G1 and in the case of the former, independent of salt concentration. It was found in transfection studies that the G1 dendron was the superior vector due to lower toxicity compared to the G2 dendron at high concentrations.¹¹⁰ One major drawback is that, similar to PLL and other “non-proton sponge” polymers, transfection was only observable in the presence of an endolytic agent (chloroquinone). This was believed to be due to the poor buffering ability of spermine at physiological pH.¹¹⁰

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The final dendritic structure that will be described was used as a pDNA vector by Russ *et al.*¹¹¹ which combined the advantageous multivalency effect of dendrons with ester linkages (See Section 1.6.2). They started by functionalising 800 Da oligo(ethylenimine) with dioldiacrylates (with spacers of lengths between 2 and 6 carbons). This gave a highly-branched core to which oligoamines such as spermine could then be attached (Figure 1.26).

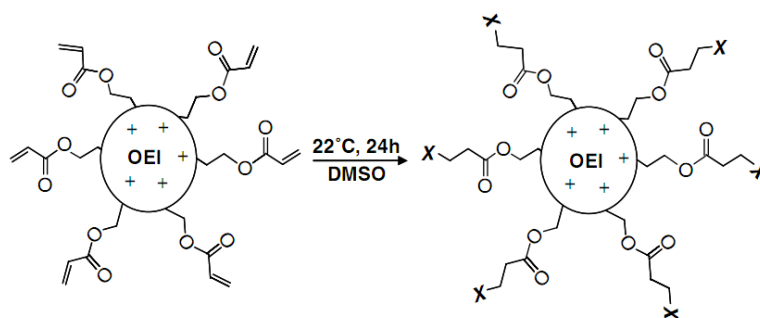


Figure 1.26 – Scheme showing the reaction of pseudo-dendrimer with an oligo(ethylenimine) (OEI) core and acrylate ester functionalised surface with an oligoamine (X). X = Spermine, spermidine, ethanolamine or more OEI¹¹¹ (Picture taken from Russ *et al.*, *Gene Ther.*, 2008, 15, 18-29.)

It was found that longer spacers between the esters of the cross-linkers gave more favourable binding to siRNA and increased toxicity due to increased hydrophobicity of the core. It was also found that OEI was a better surface group due to its high multivalency and low toxicity compared to the other oligoamines. A combination of the linker with the longest spacer between esters and OEI gave a vector as effective as PEI *in vitro* (though around an order of magnitude less effective in the presence of serum) and also displaying *in vivo* activity.¹¹¹

1.9. The Chemical Modification of siRNA

Unlike pDNA which is manufactured biologically by bacteria, siRNA is a synthetic molecule. This means that modifications can easily be made to alter its properties at the chemical level. An example of this is the “sticky siRNA” (ssiRNA) prepared by Bolcato-Bellemin *et al.*¹⁹ and shown in Figure 1.27. Here, many siRNAs are joined together in a non-covalent fashion creating a longer molecule and attempting to overcome the problems associated with the small size of siRNA (Section 1.4).

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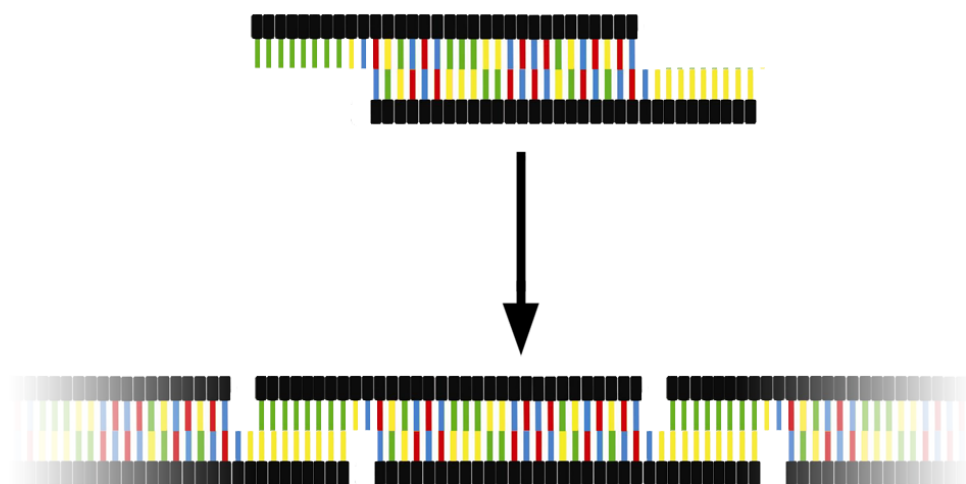


Figure 1.27 – Cartoon depiction of how the “sticky siRNA” prepared by Bolcato-Bellemin *et al.*¹⁹ is believed to form concatamers.

This was done by creating siRNAs with short chains of A or T nucleotides at the 3' ends. Base-pairing between the stretches of A and the stretches of T resulted in siRNA concatenating together in the form of a long chain. The effect of this was clearly seen in a gel electrophoresis experiment where polyplexes were prepared and incubated with different amounts of heparin sulphate (HS) – an anionic macromolecule typically found at the cell surface and able to bind to polycations and displace siRNA (see Section 1.3). It was found that the sticky siRNA polyplexes remained intact at even the highest concentrations of HS (40 eq) compared to unmodified siRNA particles which began to collapse at low concentrations (5 eq) and appeared entirely destroyed at 20 eq of HS. The result of the increased stability of the particles was that PEI polyplexes containing ssiRNAs showed ~10-fold higher silencing than those containing unmodified siRNA. In addition to this, they showed none of the non-specific silencing characteristic of long dsRNA. The effectiveness of the ssiRNA showed that being stuck together does not impact on the RNAi mechanism and that this could be used to improve polymer-mediated siRNA delivery across the board.¹⁹

Another means of modifying siRNA to circumvent the problem of weak interaction between the vector and cargo is to covalently attach a macromolecule directly to the

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siRNA helix. This was demonstrated by Rozema *et al.*¹¹² who had previously found the poly(vinyl ether) shown in Figure 1.28 to be effective at pDNA transfection.

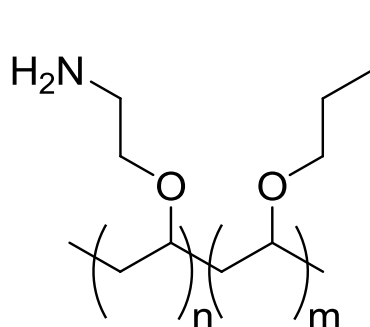


Figure 1.28 – The backbone of the polymer modified for siRNA delivery by Rozema *et al.*¹¹³

They made several alterations to the material for the purposes of siRNA delivery.¹¹³ Firstly they attached an siRNA bearing a single thiol to the polymer which had too been modified to incorporate a single thiol per chain. Finally, acid-labile PEG groups and targeting ligands were also attached to some of the pendant amines further modifying the polymer. Upon endocytosis it was envisaged that the PEG coating and targeting ligands would detach followed by detachment of the siRNA once the complex reached the cytoplasm. Evidence for greater cellular accumulation was found for the polyplexes formed with the covalently attached siRNA compared to that which was merely electrostatically attached. The polyplexes formed were also effective at targeted delivery of siRNA *in vivo* to mouse livers following *i.v.* injection.¹¹³

The approach taken by Mok *et al.*¹¹⁴ could be regarded as a combination of the two previously discussed. They took the separate sense and antisense strands of an siRNA and using disulfide containing cross-linkers made so called multimers of siRNA. When the two portions were brought together the strands self-assembled to form longer helices as shown in Figure 1.29. The relative populations of the structures formed were: dimer, 19.9%; trimer, 15.9%; tetramer, 10.3%; pentamer, 8.8% with 41.4% comprising hexamers and higher. Polyplexes were formed with 25 kDa PEI and it was found that the particle size of those made with the multimers was 81.8 nm compared to 463.9 nm for the particles formed with ordinary siRNA. As would be

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expected, the particles formed using the multimers were also more effective at mediating gene knockdown both *in vitro* and *in vivo*. Non-degradably cross-linked siRNA was also used and although it initially appeared to mediate knockdown, it was found to be non-specific and likely to be the result of the multimer being processed into a variety of different siRNAs by the enzyme Dicer.¹¹⁴

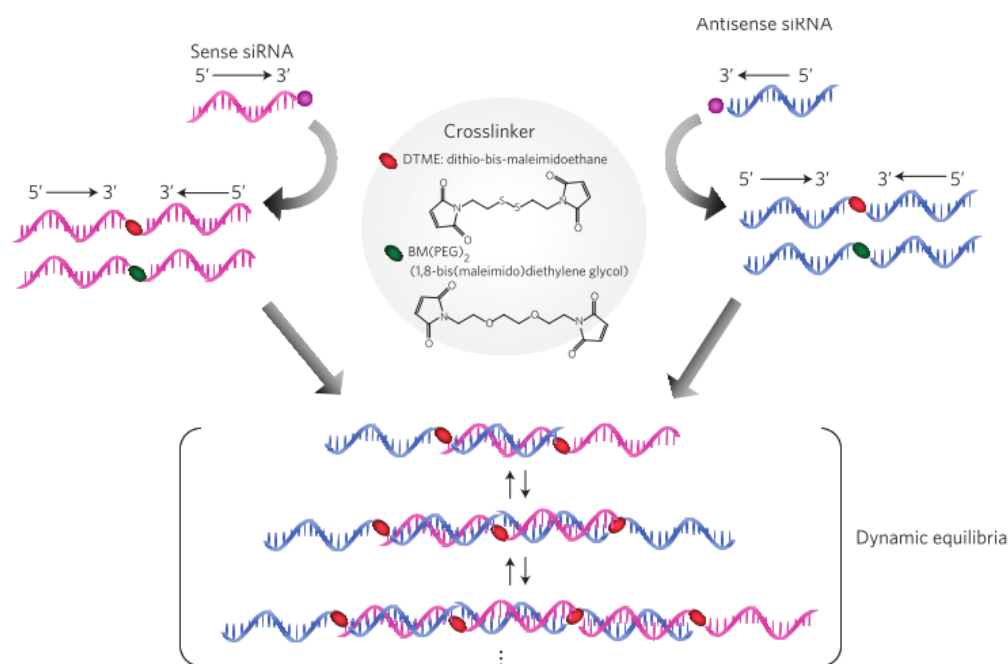


Figure 1.29 – A depiction of the formation of multimers by Mok *et al.*¹¹⁴ Also shown are the reducible and non-reducible cross-linkers used. (Picture taken from Mok *et al.*, *Nat. Mat.*, 2010, 9, 272-278.)

The work was continued by Chung *et al.*¹¹⁵ who investigated only dimeric siRNAs prepared by only cross-linking the sense strands. The dimers were found to be as effective as any other multimer and the uniformity of the sample in this case was considered advantageous. The strategy of siRNA dimerisation was also found to be amenable to the delivery of two different siRNAs at the same time which could have positive implications for future therapeutics.¹¹⁵

1.10. The Current State of the Art

The polymer prepared by Meyer *et al.*¹¹⁶ manages to incorporate almost all of the aspects of siRNA vector design discussed above and as such is worth a special

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mention. As shown in Figure 1.30, it includes disulfide bonds, pH sensitivity, PEG, an endosomolytic peptide and chemically-modified siRNA.

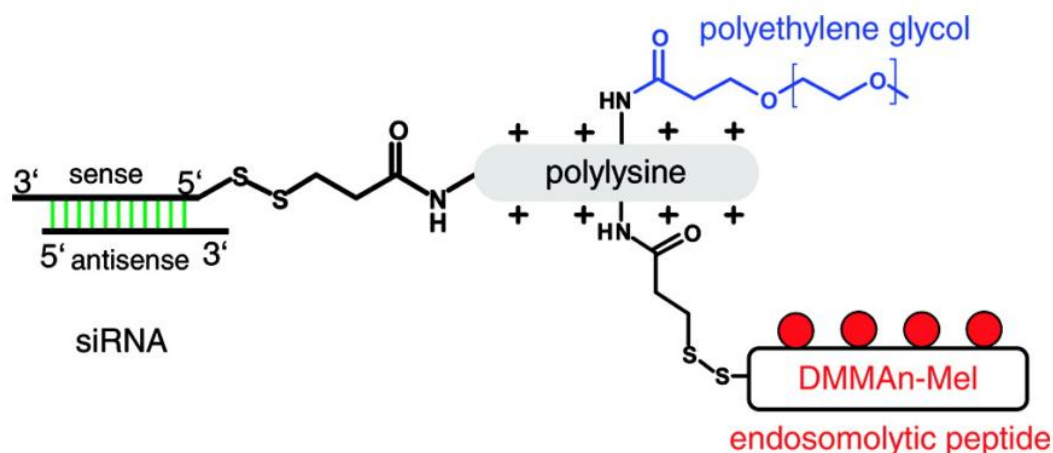


Figure 1.30 – Structure of the siRNA vector designed by Meyer *et al.*¹¹⁶ (Picture taken from Meyer *et al.*, *Mol. Pharm.*, 2009, 6, 752-762.)

The vector was based on poly-L-lysine which, as mentioned in Section 1.5.3, has no intrinsic buffering capacity. This necessitated the incorporation of the endosomolytic peptide which was masked using acid labile dimethylmaleic anhydride (DMMAn) groups to reduce the toxicity of the peptide in the extracellular environment.¹¹⁷ PEG chains were coupled to the PLL to prevent aggregation and additionally the siRNA was reversibly coupled to the polycation to overcome the weak interactions between cationic polymers and siRNA (see Section 1.4). The particles showed good stability, were resistant to heparin competition and showed almost total knockdown of luciferase at concentrations that exhibited minimal cytotoxicity. No *in vivo* data has yet been published for this material although the *in vitro* results point to the possibility of it being potent.¹¹⁶

Finally, the principal indicator of the viability of siRNA delivery with polymers has to be their successful use in a human clinical trial by Davis *et al.*¹¹⁸ This success did not only represent the first time that a polymeric material had been used to deliver siRNA in humans; it was the first use of any synthetic material. This includes liposomes which for many years were the most common non-viral gene therapy vector by a considerable margin.¹¹ The particles were used to treat melanoma which

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due to its physical accessibility is a suitable target for direct injection. The polymer used displays many of the key technologies and aspects of the state-of-the-art in siRNA vector design discussed so far.

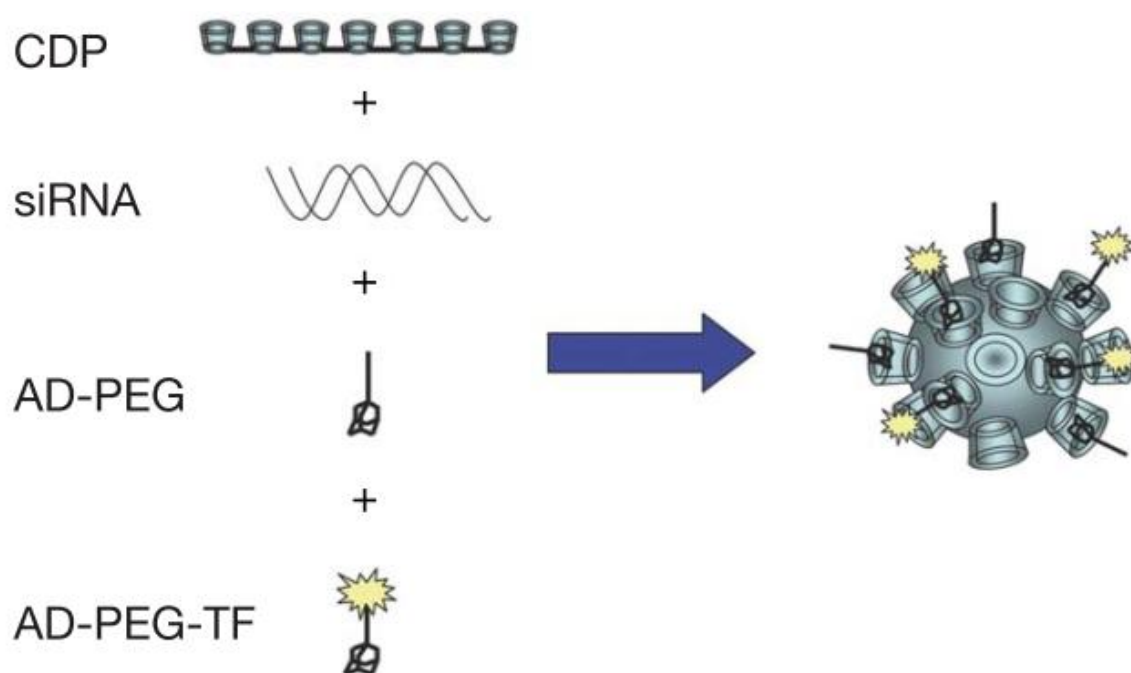


Figure 1.31 – Diagram showing the four components of the vector designed by Davis *et al.*¹¹⁸ CDP is the cyclodextrin-containing polymer (defined in Figure 1.32), AD-PEG represents a PEG chain attached to adamantane and AD-PEG-TF is a PEG chain with adamantane attached at one end and a transferring targeting ligand attached to the other. (Picture taken from Davis *et al. Nature.*, 2010, 464, 1067-1070.)

As shown in Figure 1.31, the nanoparticles consist of four components: siRNA, a cyclodextrin-containing polymer, a PEG chain attached to an adamantane molecule and another PEG-adamantane assembly attached to a transferring targeting ligand. The polymer shown in Figure 1.32 contains protonated amidine groups to enable electrostatic interaction with the siRNA. The cyclodextrin groups act as hosts for the adamantane groups allowing the non-covalent attachment of the PEG chains and targeting ligands. The principle advantage of this method of attachment is that the adamantane groups are only able to attach to the surface of the particle.¹¹⁹

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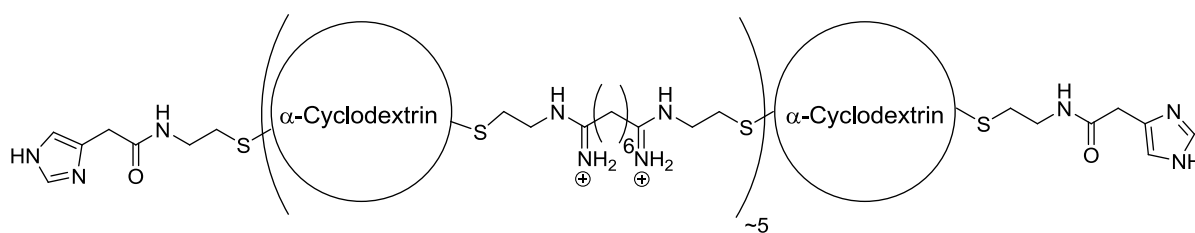


Figure 1.32 – Structure of the cationic component of the siRNA vector developed by Davis *et al.*¹¹⁹ (Picture taken from Davis, *Mol. Pharm.*, 2009, 6, 659-668.)

Particles formed are small-enough ($10 - 75 \pm 25$ nm) that they are able to access tumours and large enough to escape renal clearance. None of the components are inherently toxic and after disassembly can be cleared safely from the body. Another attractive feature of this system is that its multi-component and self-assembling nature enables formulations to be tuned without the need for the development of additional synthetic methodology.¹¹⁹ Interestingly, the cyclodextrin also allowed detection of the particles. When gold-nanoparticles capped with thiol-PEG-adamantane units were introduced to tumour cells that had been treated with the siRNA nanoparticles, they could interact with the CD groups enabling detection by TEM.

1.11. Project Focus

Previous work in the group produced a series of pDNA vectors (Figure 1.33) that could compete with PEI in terms of transfection efficiency while being significantly less toxic. The vector consisted of cyclic polyamines containing a disulfide link.¹²⁰

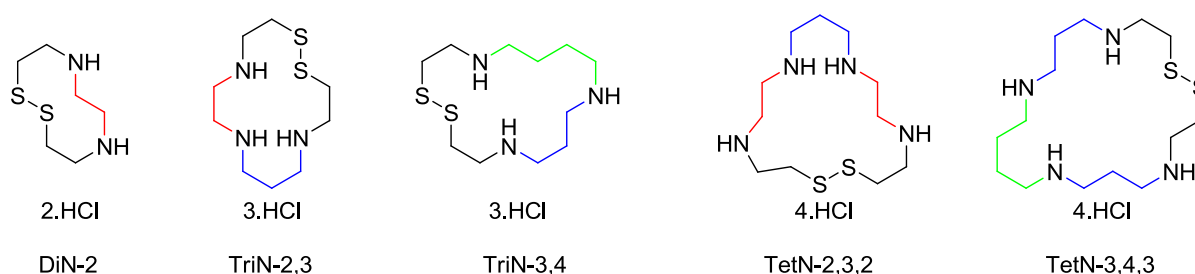


Figure 1.33 – Selection of cyclic disulfides synthesised as pDNA vectors. The most effective, based on spermine, is shown on the far right.¹²⁰

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When mixed with pDNA, electrostatic interactions between the cationic amines and the anionic pDNA, combined with thiol-disulfide exchange leads to the *in situ* formation of a cationic polymer, “caging” the pDNA.¹²⁰ The disulfide linkages are then reduced in the cytoplasm allowing triggered release. These cyclic disulfides proved to be effective vectors with cytotoxicity significantly less than PEI. The spermine derived cyclic was the most effective and transfection efficiency was to within an order of magnitude of PEI. They were also able to mediate pDNA delivery *in vivo*.¹²⁰

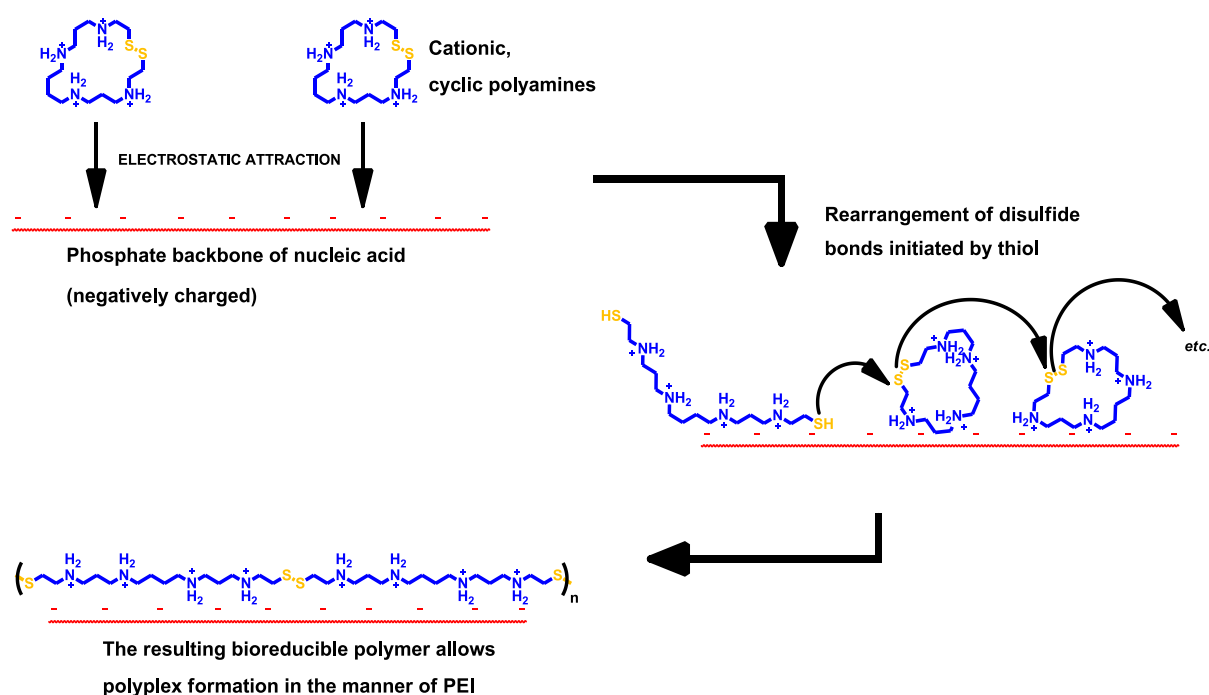


Figure 1.34 – Possible mechanism for the *in situ* polymerisation of cyclic disulfides in the presence of nucleic acid.¹²⁰

The exact mechanism for the formation of the polymer has not yet been conclusively proven though it is thought that it proceeds in the manner shown in Figure 1.34. The reaction is potentially initiated by the small amount of thiol that is likely to be present. Thiol-disulfide exchange between the cationic monomers that have been drawn into close proximity by the anionic backbone of the nucleic acid then leads to the formation of the polymer. Though polymer was never directly observed, the characteristics of the polyplexes formed were consistent with those formed between pDNA and a polycations such as PEI.

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The focus of this project is to apply and modify this work to the delivery of siRNA. One of the main beneficial features of this vector is that it is synthesised as a small molecule, avoiding the reproducibility problems that are of concern when attempting to use polymers as therapeutics. One of the main trends encountered in the literature with regard to the differences between pDNA and siRNA vectors is the higher viability of branched polymers for delivery of the latter²⁴ (Section 1.1.5). However, the structure of the cyclic disulfides means that it is only possible for linear polymers to form and it was anticipated that this could hamper their effectiveness as siRNA vectors. In light of this hypothesis, the synthetic focus of the project will be to synthesise a “branching unit” consisting of a monomer containing at least three thiol groups and a quantity of amines in such an arrangement that the monomer takes part in the *in situ* polymer formation giving rise to a branched polymer (Figure 1.35). The presence of “branching units” would also allow the formation of a greater variety of polymer structures during the *in situ* polymerisation.

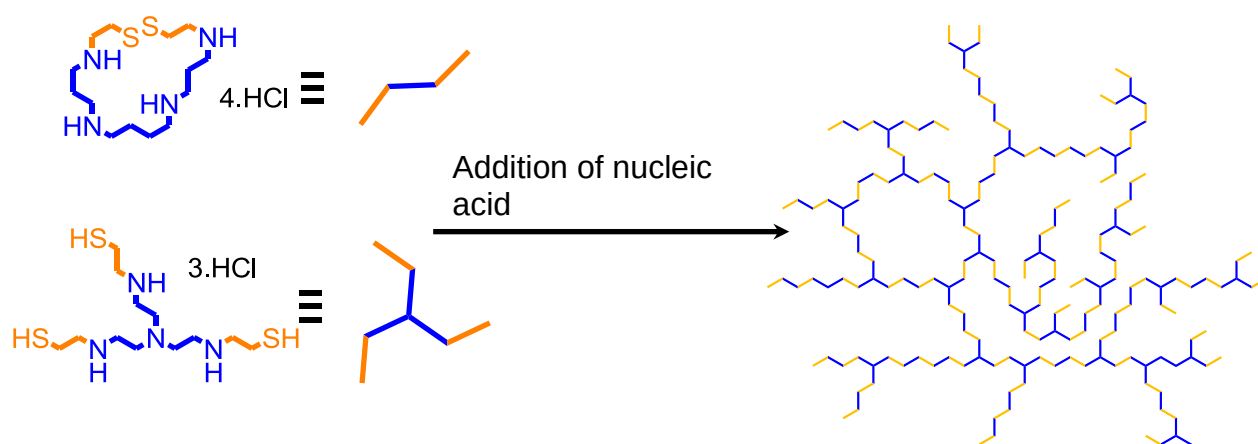


Figure 1.35 – Proposed formation of a branched polymer through use of cyclic disulfide and trithiol.

Figure 1.35 shows the proposed *in situ* formation of the branched polymer by templating with nucleic acid (not pictured) and the general structure of the products that would be expected.

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The two goals of the project are therefore the investigation of the cyclic materials from the earlier pDNA delivery work as well as the synthesis of a “branching unit” to allow the formation of branched structures. This will be used to investigate its effect on polyplex formation. Ultimately, a general synthetic scheme that allows the formation of branching units containing a variety of amine motifs will be developed so the effect that this has on transfection efficiency and cytotoxicity can be investigated.

There are two principle features that the “branching unit” will require: first of all, three or more thiol groups must be present or the formation of a branched polymer is impossible. Initially, three or four armed “branching units” will be investigated but there is a possibility that “branching units” with many more thiol groups could be useful. Secondly, a quantity of nitrogen atoms arranged in such a way as to allow interaction between the branching unit and siRNA allowing incorporation of the “branching unit” into the polyplex structure. The exact nature of the amine groups will need to be investigated though initially any amine spacing and number that has been shown to be effective at binding nucleic acids be it ethylene spacers (as in PEI) or more complex spermine or spermidine motifs which were found to be successful in the context of the cyclic disulfides. Initial work will use tris(2-aminoethyl)amine (tren) since it is commercially available and analogous to the branching points in branched PEI.

2. Preparation of Novel Compounds for siRNA Delivery Systems

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2.1. Background

In the previous work, a method was developed that allowed the addition of thioethyl groups onto primary amines. The reaction scheme for the preparation of an example cyclic disulfide is shown in Figure 2.1.

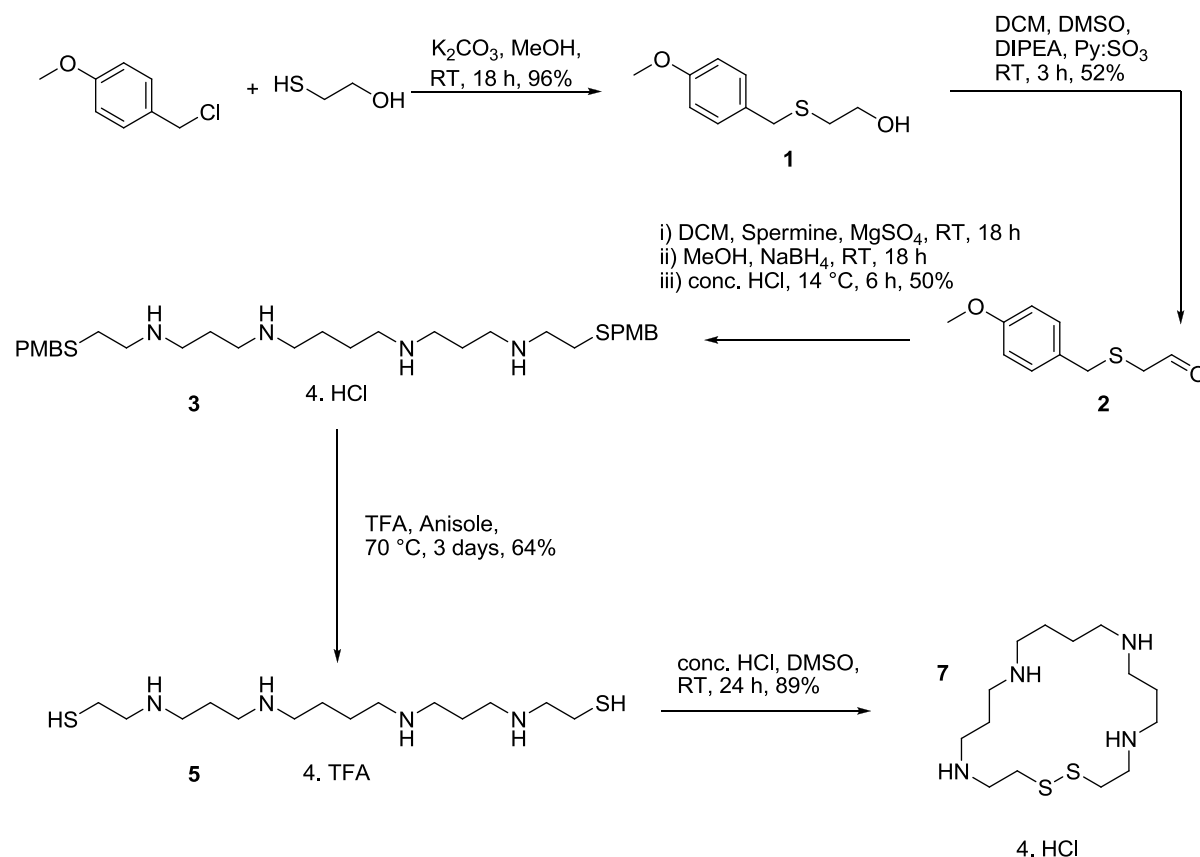


Figure 2.1 – A scheme showing the preparation of cyclic disulfides by Drake *et al.*¹²⁰

As shown in Figure 1.35, our initial synthetic target was a trithiol comprising of a tris(2-aminoethyl)amine core. Since the method used to prepare the cyclic disulfide had been effective in adding thioethyl groups onto primary amines it was decided to attempt to use the same method for our brancher materials.

2.2. Attempted synthesis of methoxybenzyl-protected trithiol **10** using reductive amination reaction.

It was thought that a branching unit containing several amine groups and three thiol groups could be made by subjecting tris(2-aminoethyl)amine (tren) to the same

2. Preparation of Novel Compounds for siRNA Delivery Systems

chemistry that had been used to add thiol groups to spermine in the previous work (Figure 2.2).

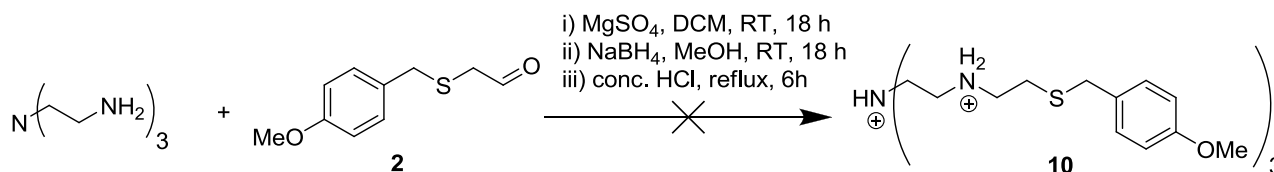


Figure 2.2 – Attempted reaction of aldehyde 2 with tris(2-aminoethyl)amine to give target compound 10.

When the reaction was first attempted and the solvent was removed after the final step, there was the expected white solid but also reddish-coloured oil. A ^1H NMR of the white solid showed that around two thirds of the primary amines had been substituted based on comparison of the aromatic methoxybenzyl protons with those alpha to the amine. The oil, soluble in d_6 -DMSO, showed the characteristic peaks of both the amine and methoxybenzyl groups along with poorly-defined triplets at $\delta = 9.47$ and 8.3 ppm which were attributed to aldehydes and imines respectively. This was especially surprising as one would have expected both of the groups to have been reduced by the sodium borohydride (present in a ten times excess). It was thought that perhaps neither the imine formation nor the reduction were being allowed to go to completion so the reactions would be followed in detail with NMR and be left longer than 18 h each. During a repeat of the experiment aliquots of the reaction mixture were taken 18 h into the imine formation. However, this gave a poorly defined spectrum on which no imine or aldehyde resonances could be observed, despite the aldehyde being observable on TLC. The reason for no aldehyde being shown in the NMR is perhaps due to concentration. The reaction was left for a further 24 h then reduced for 24 h only to give similar results to the previous attempt. One further attempt also showed that around two-thirds of amines had been substituted although mass spectrometry showed peaks at $m/z = 685.33$ (93%), 507.28 (100%) and 326.21 (48%) representing the tri-substituted, di-substituted and mono-substituted amines respectively. This showed that it was, in principle, possible for the substitution to go to completion.

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It was found later during an alternative synthesis of the free-base of the same material (Section 2.5) that the target compound was only very sparingly soluble in water or aqueous acid. It is possible that the work-up and purification strategy (formation of the HCl salt using methanolic HCl followed by recrystallisation from methanol) that was eventually used for the alternative synthesis may have been appropriate here too.

2.3. Nosyl protection of tris(2-aminoethyl)amine (**11**)

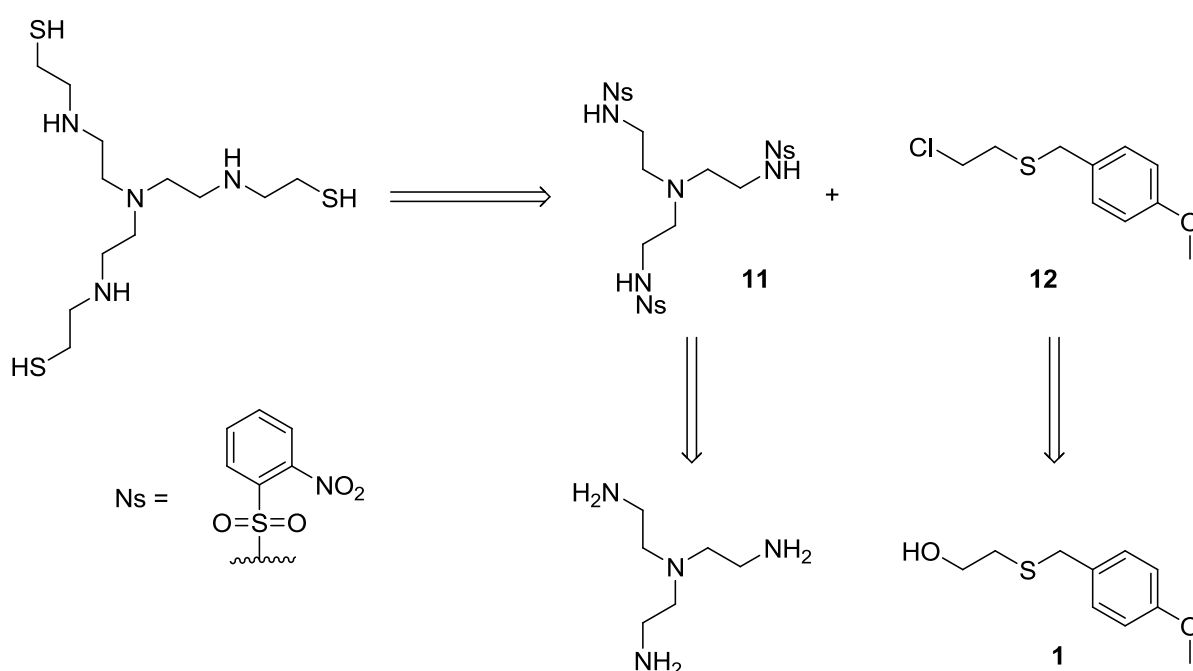


Figure 2.3 – Retrosynthetic analysis demonstrating the synthesis of a trithiol from protected tris(2-aminoethyl)amine (11**).**

Since the reductive amination chemistry was apparently not viable for the formation of the methoxybenzyl-protected trithiol, it was decided that an alternative synthetic route should be investigated – a path that would ultimately arrive at the freebase of compound **10**. This procedure involved protection of tren, reaction with 1-[(2-chloroethyl)sulfanylmethyl]-4-methoxybenzene, the chloride analogue of compound **1** and removal of the nosyl group (Figure 2.3). This had been used as an alternative route to some thiol containing compounds in the previous work.¹²⁰

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The nosylation of tren was carried out using previously reported nosylation conditions¹²¹, i.e. reaction of the amine with 2-nitrobenzenesulfonyl chloride in DCM with TEA (Figure 2.4).

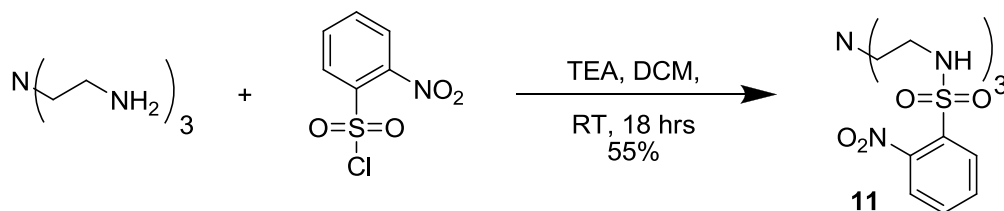


Figure 2.4 – The reaction to form nosylated amine 11.

After purification with column chromatography, material was obtained in fair yield (55%) and characterisation was consistent with that previously reported.¹²²

2.4. Alkylation of nosyl-protected tris(2-aminoethyl)amine

The next step was to react compound **11** with 1-[(2-chloroethyl)sulfonyl]methyl-4-methoxybenzene (**12**). This was carried out in dry DMF using caesium carbonate as the base¹⁰⁵ (Figure 2.5).

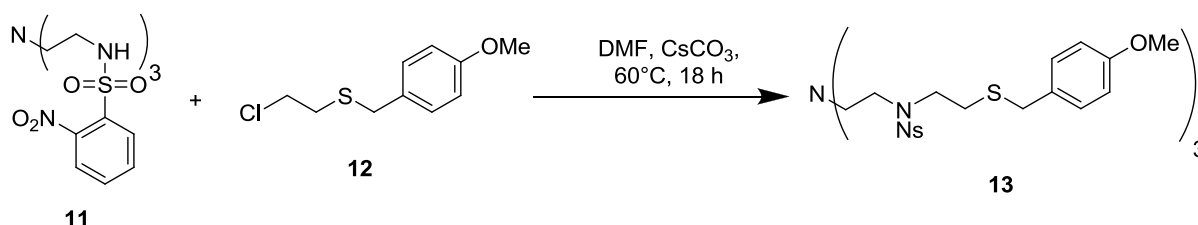


Figure 2.5 – Reaction to give nosyl and methoxybenzyl protected trithiol 13.

The reaction was first attempted using 200 mg of the amine. The work-up commenced with the addition of water followed by extraction with DCM. The resulting organic phase was evaporated to dryness under reduced pressure. It was decided that the best method of judging the degree of substitution would be to use proton NMR and compare the relative integrations of the two different sets of resonances due to the aromatic protons of the methoxybenzyl (two doublets at $\delta = 6.84$ and 7.22 integrating to 6H apiece) and nosyl groups (two multiplets at

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$\delta = 7.60\text{--}7.75$ and $7.89\text{--}7.95$ integrating to 9H and 3H respectively). On the NMR of the residue obtained, there was evidence for chloride **12** starting material still being present which meant this comparison was not possible and it was unclear whether the reaction was complete. The reaction also produced a low quantity of material (~20 mg). It was thought that the pH of the aqueous phase (~8.0) would have been sufficiently low that the tertiary amine (expected pKa ~10) would be protonated and therefore given a water-soluble salt. To eliminate this, 1M NaOH was added to the reaction mixture in place of water prior to extraction with DCM. This proved to be a more effective work-up though chloride **12** was still present. A TLC of the crude material with ethyl acetate as the mobile phase gave one spot running at $R_f = 0.8$ and one spot that remained on the baseline. Following purification over a short column of silica, NMR confirmed that the eluted fraction contained the product based on a comparison of the integrals of the aromatic protons from the nosyl and methoxybenzyl groups. The NMR also showed the presence of other, unidentified impurities. Since evidence for product formation had been obtained, and purification – in particular precipitation – would be easier on a larger scale the reaction was scaled up.

It was decided to scale up to using 2 g of amine. After three precipitations from DCM with ether, 1.5 g of amorphous orange solid was obtained. The NMR of this material confirmed full substitution based on comparison of the integrals of the two different types of aromatic proton. Another characteristic change in the spectrum upon substitution was the upfield shift of the resonance due to the proton *ortho* to the nitro group in the nosyl from $\delta = 8.12$ ppm to 7.93 ppm. Lack of a resonance above 8.0 ppm was therefore a good indicator that no unsubstituted sulfonamide remained. However, the resonances due to the methylenes α - to sulfonamides or amines were not sharp and other errant resonances were also observed in this region ($\delta = 2.5\text{--}3.5$ ppm, illustrated in Figure 2.6) suggesting impurity. Additional resonances due to the aromatic, methyl and benzylic protons of a methoxybenzyl group could also be seen though they were not due to chloride **12** as none of the other resonances characteristic of this compound were present (specifically 1:1:1 triplets at $\delta = 2.78$ and 3.55 ppm). This presence of impurity proved that the precipitation did not give completely pure material and that further purification would be necessary.

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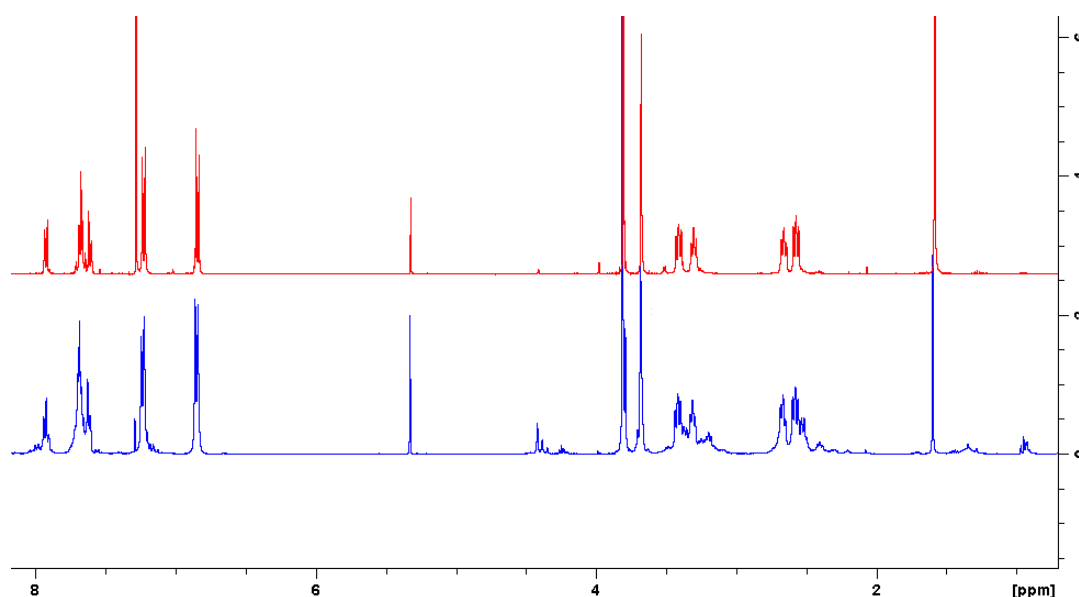


Figure 2.6 – A region of the ^1H NMR spectrum of compound 13 after various stages of purification. After flash column chromatography, 3% EtOAc and 0.1% (v/v) TEA in DCM (blue) and after flash column chromatography, 5% EtOAc (v/v) in DCM (red). The “extra” resonances in the region $\delta = 2.5\text{--}3.5$ ppm can clearly be seen. Assignment can be found in Section 6.2.13.

Further purification of the precipitated material was attempted using flash column chromatography. Through TLC experiments it was found that rather than simply two spots as observed previously, there were in fact three. When eluted with 3% EtOAc and 0.1% TEA (v/v) in DCM it was revealed that the moving spot in fact consisted of two spots, at $R_f = 0.6$ and 0.5. A column was run eluted with 1.5% EtOAc and 0.1% TEA in DCM in order to elute slower and therefore increase separation. However, it was not possible to completely separate the two components (Figure 2.6) and identification of the impurity was not possible spectroscopically.

It was believed that the lack of success of the column chromatography in providing pure material was due to streaking of the material which would have been exacerbated by the long run time caused by the choice of solvent system. To solve this, a more polar solvent system could be used. The collection of smaller fractions would also be appropriate. The proton NMR of the material obtained from a column using 5% EtOAc in DCM (v/v) and no TEA gave material of approximately 90% purity (Figure 2.6). It was decided that this material was appropriate for an attempt at deprotection with further purification to take place after removal of nosyl groups.

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2.5. Removal of nosyl groups to afford methoxybenzyl-protected trithiol **14**

An attempt was made to deprotect the crude compound **13** using standard nosyl deprotection conditions (thiophenol in acetonitrile with base at room temperature for three days)¹²¹. The work-up included an acid extraction and it was envisaged that the hydrochloride salt could be collected and recrystallised as for the spermine and spermidine derived protected-dithiols **3** and **4** (Section 1.11). An orange solid was collected and crystallisation from water was attempted as for compounds **3** and **4**. Similar to the earlier attempts to recrystallise compound **10** (the product of the reductive amination) this was ineffective. As discussed in Section 2.1, it was realised at this point that the free-base of the trisubstituted amine is only very sparingly soluble in distilled water. If the material would not dissolve in aqueous acid then the salt would be unable to form.

The work-up that proved to be successful used 1.5 M HCl in methanol to form the hydrochloride salt. After three days of reaction, the acetonitrile was removed under reduced pressure and the remaining residue was dissolved in a combination of DCM and water. After basic aqueous work-up to remove the potassium carbonate followed by evaporation of the DCM an oil was obtained. The oil proved to be entirely soluble in 1.5 M HCl in methanol and after dissolution followed by solvent removal a yellow solid was obtained. The principal byproduct of this reaction is the aromatic compound shown in Figure 2.7 separation of which was made simple by its lack of solubility in water.

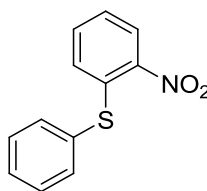


Figure 2.7 – The byproduct expected from the denosylation reaction.¹²³

Shaking the yellow solid in the presence of copious amounts of water followed by filtration allowed collection of the bright yellow byproduct. The product amine was obtained as an amorphous residue following the removal of the water under reduced pressure. Washing with acetone removed any remaining yellow byproduct and

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crystallisation from hot methanol allowed isolation of yellowish crystals. Further recrystallisation and acetone washing eventually led to off-white crystals. The proton NMR (Figure 2.8) showed no evidence of other contaminants suggesting that the incomplete purification of **13** in the previous step had no adverse effects.

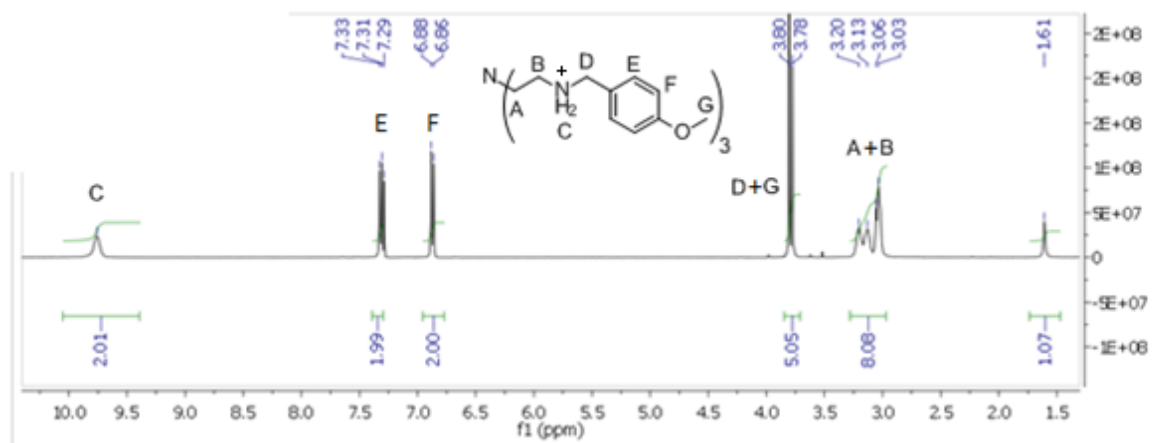
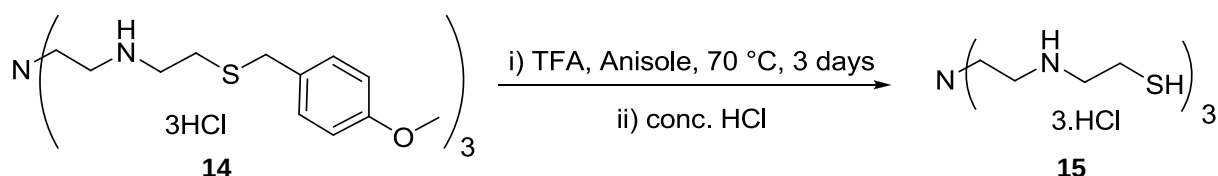


Figure 2.8 – NMR of compound 14.

Proton NMR of the material in CDCl_3 also indicates that a trihydrochloride salt is formed based on the relative integrals of the NH_2^+ protons. This is consistent with expectation as electrostatic repulsion from the three protonated secondary amines would make the pK_a of the central tertiary amine extremely low. Mass spectrometry indicated the presence of the desired compound. Unfortunately, solubility of the compound in CDCl_3 was too low to obtain a clear carbon NMR.

2.6. Removal of methoxybenzyl groups to give trithiol **15**



Following successful isolation of the protected trithiol, deprotection using TFA and anisole was attempted. A procedure identical to the one used for the dithiols was used though the material obtained from the methanol/ether crystallisation was incompletely soluble when redissolved in methanol and the NMR showed broad

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resonances. Due to the high probability of oxidation and the questionable value of isolating the TFA salt *en route* to the desired hydrochloride salt it was decided to take the crude product of this reaction, wash with degassed diethyl ether (to remove the anisole and byproduct) and dissolve the resulting residue in concentrated hydrochloric acid and then attempt precipitation with acetone. This also led to partial oxidation.

NMR of the crude residue after the ether wash suggested that recrystallisation would be unnecessary aside from producing an easier to handle substance. Fortunately, upon scaling the reaction up to 0.5 g of starting material (double previous attempts), it was possible to produce crystals simply by dissolving the ether-washed crude material in HCl, decanting to remove any insoluble material (*cf.* the spermine cyclics which are insoluble in HCl once oxidised) and removing the solvent under reduced pressure. After exposure to high vacuum, scraping the residue with a spatula afforded a colourless solid in a yield of 62.0%. NMR in 20% DCl in D₂O showed that minimal oxidation had taken place (<2% based on the methods described in detail in Section 2.8). Mass spectrometry also confirmed the formation of the product (calculated: 327.16326, observed: 327.1696).

2.7. Oxidation to Form Cyclic **16**

Controlled oxidation of the trithiol was also attempted. The reasons for this were twofold. Firstly, the disulfide material would be more stable than the trithiol which would be expected to oxidise during handling. Secondly, it would provide another material to test. The planned reaction is shown in Figure 2.9.

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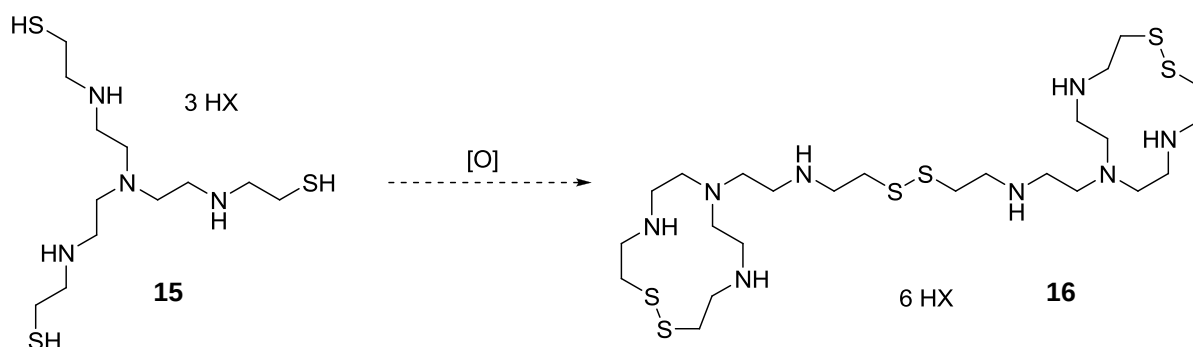


Figure 2.9 – Planned oxidation of trithiol **15 to form a double cyclic **16**.**

The first method used was the same as the one used for the preparation of the monocyclic disulfides:¹²⁰ namely, oxidation with DMSO in concentrated hydrochloric acid. A solution of crude trithiol as TFA salt was washed with ether and dissolved at 1 mgml⁻¹ in conc. HCl with DMSO. A solid formed that was insoluble in water, HCl and MeOH. The similarity of the material shown in Figure 2.9 to the previously synthesised cyclic disulfides suggested that it would be soluble in water suggesting something else had been formed. The solid was soluble in an aqueous solution of mercaptoethanol suggesting that it was a higher molecular weight oxidation product. The same result was obtained at 0.5 mgml⁻¹ – a far lower concentration than cyclic disulfide **7** which was formed at concentrations as high as 15 mgml⁻¹. A proposed mechanism for the formation of polymer at even low concentrations is shown in Figure 2.10 and Figure 2.11.

Figure 2.10 shows the ideal reaction. At high dilution, one would expect two of the thiols in the trithiol (A in the scheme) to form a cyclic with one thiol remaining (B). It was then envisaged that these “spare thiols” would pair off to form stable dimers (C).

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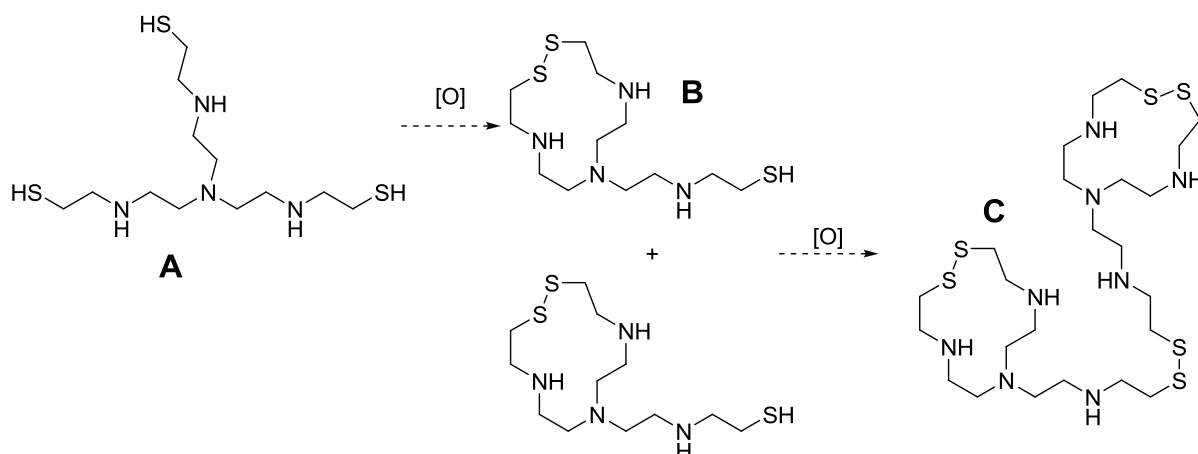


Figure 2.10 – Proposed two stage oxidation to form double cyclic.

However, a more complete analysis of the situation shows that as B is consumed it will eventually get to the point where a molecule of B is more likely to meet a molecule of C than it is to meet another molecule of B. When this happens, a trimer (D, Figure 2.11) will form possessing a free thiol. Though this would be reversible, it would only be a matter a time before a molecule of D reacted with a molecule of C, particularly if the concentration of C was higher than the concentration of B. This could go on until all of B and eventually C was consumed and so on leading to the formation of polymer (Figure 2.11). This makes the idea of forming dimers using this method totally redundant as this mechanism would be true for all dilutions.

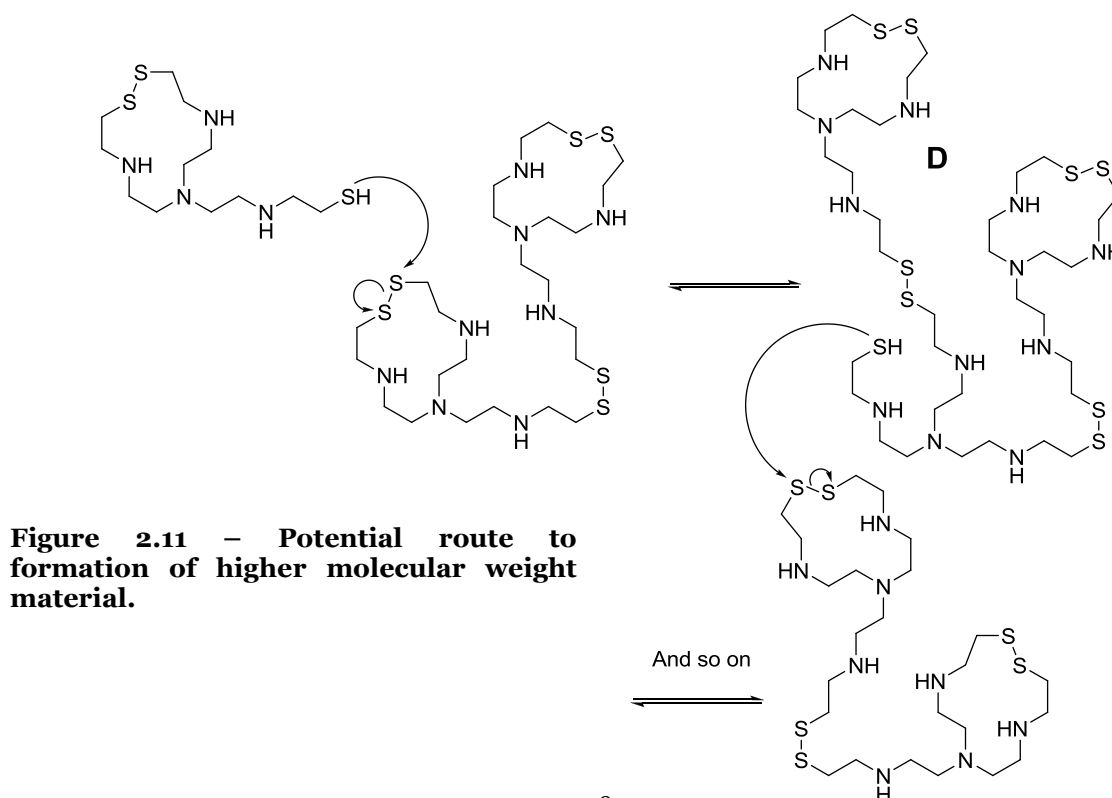


Figure 2.11 – Potential route to formation of higher molecular weight material.

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2.8. Controlled oxidation of trithiol

It was believed that the failure of the oxidation with DMSO to form the desired double cyclic disulfide was due to it being rapid enough to allow the formation of polymer. A more passive method of oxidation was required so it was decided to investigate the behaviour of the material when simply left in D₂O in an NMR tube. A 1 mgml⁻¹ solution in D₂O was prepared and proton NMR was periodically run. A method was devised to easily measure the relative proportions of disulfide and thiol in the sample.

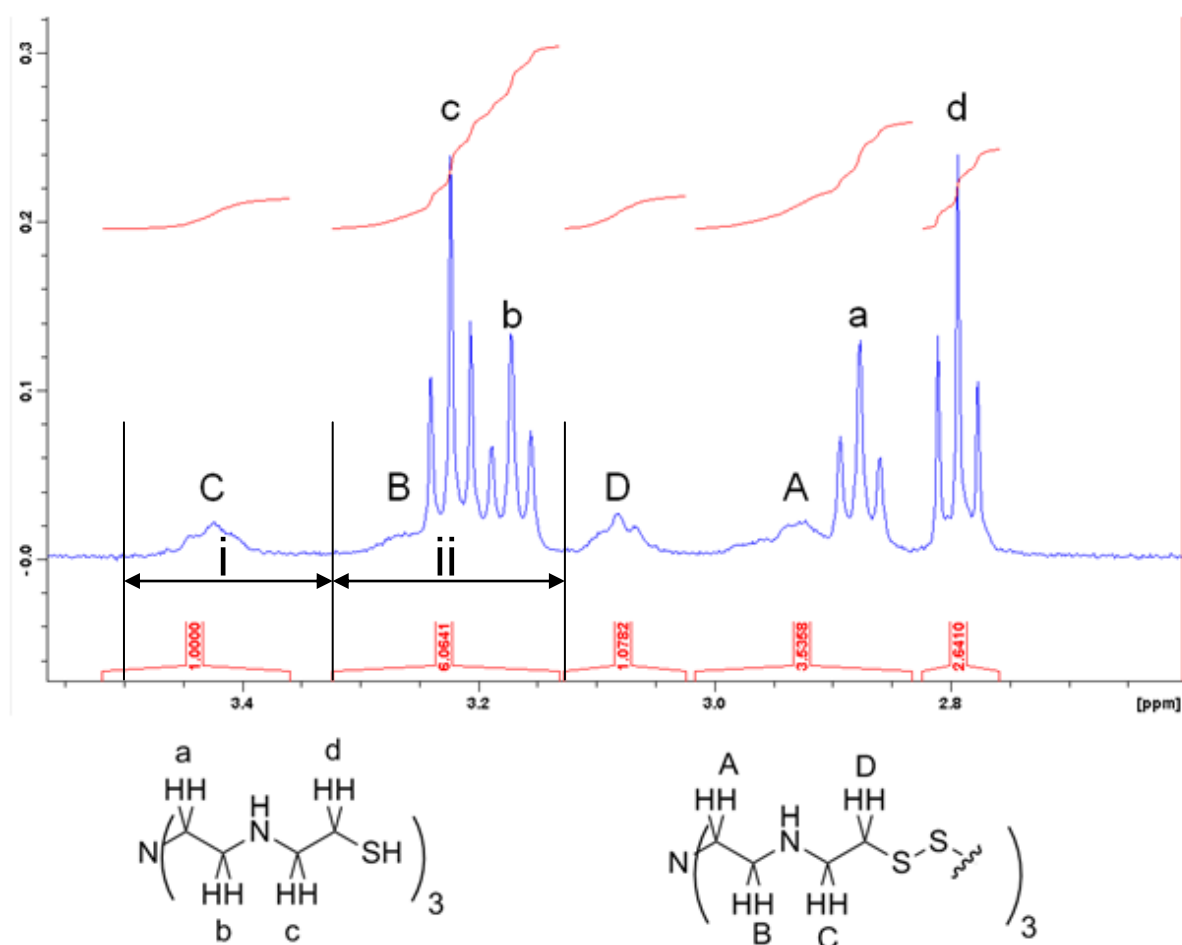


Figure 2.12 – ¹H NMR of partially oxidised brancher with assignment. In this case degree of oxidation was calculated to be 30.3%.

As shown in Figure 2.12, it is possible to differentiate between the spectra of the thiol and disulfide. This makes working out the degree of oxidation a simple case of comparing the integral of a resonance from the thiol with one from the disulfide. The

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simplest solution would have been to compare “d” with “D” although this was not possible because the “D” integral could be poorly defined. Degree of oxidation was therefore calculated by using the integrals of the regions $\delta = 3.50 - 3.32$ ppm (“i”) and $\delta = 3.32 - 3.12$ ppm (“ii”). Integral “i” was set to 1 and the following formula was used:

$$\text{Oxidation (\%)} = \frac{1}{1 + \frac{\text{ii}-1}{2}} * 100$$

As shown in Figure 2.12, region “ii” contains three proton environments: “B” from the disulfide and “b” and “c” from the thiol. To calculate “c”, C (set to one) is subtracted from region “ii” to give the value of b + c. Since b = c, division by two gives c. C (region “i”) is set to 1. The percentage oxidation is equal to C over C + c multiplied by 100. With this method in hand, the graph shown in Figure 2.13 was produced. Interestingly, oxidation was rapid over the first 24 hours followed by a change in rate. This was attributed to the availability of oxygen though there was also a possibility that the shape of the graph was due to the two stage process in forming the final material.

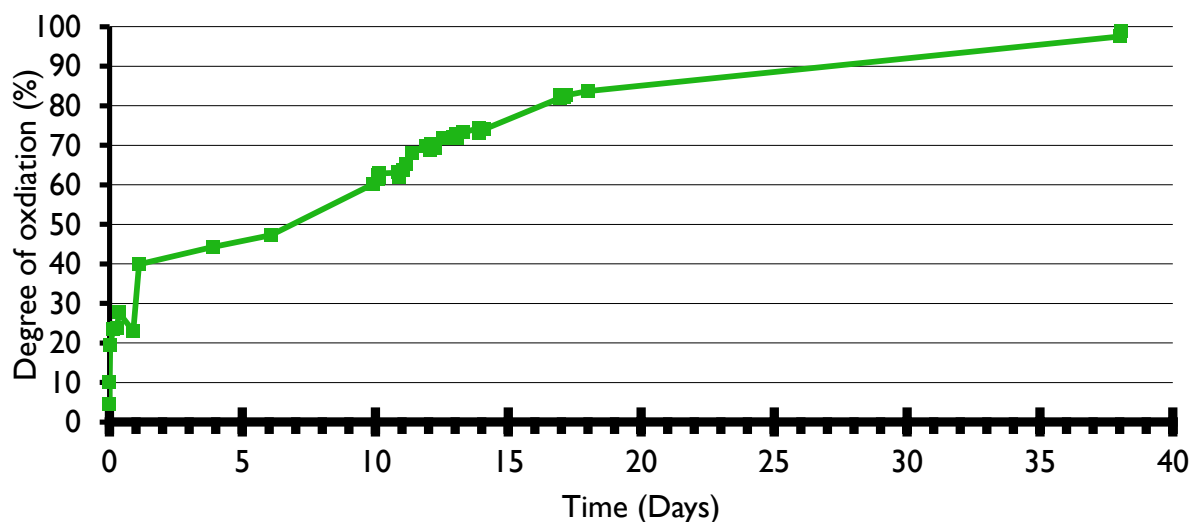


Figure 2.13 – Degree of oxidation of brancher in D₂O at 1 mgml⁻¹ over 40 days.

An investigation into the effect on oxidation of the amount of oxygen in the solvent was carried out (Figure 2.14). One sample was prepared in D₂O that had been

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degassed by bubbling nitrogen through. Another was prepared in the same D₂O after it had been “regassed” by bubbling air through. The shape of the graphs is consistent with previous experiments in that there is an initial period of rapid growth followed by a gradually slowing period of increase in the degree of oxidation.

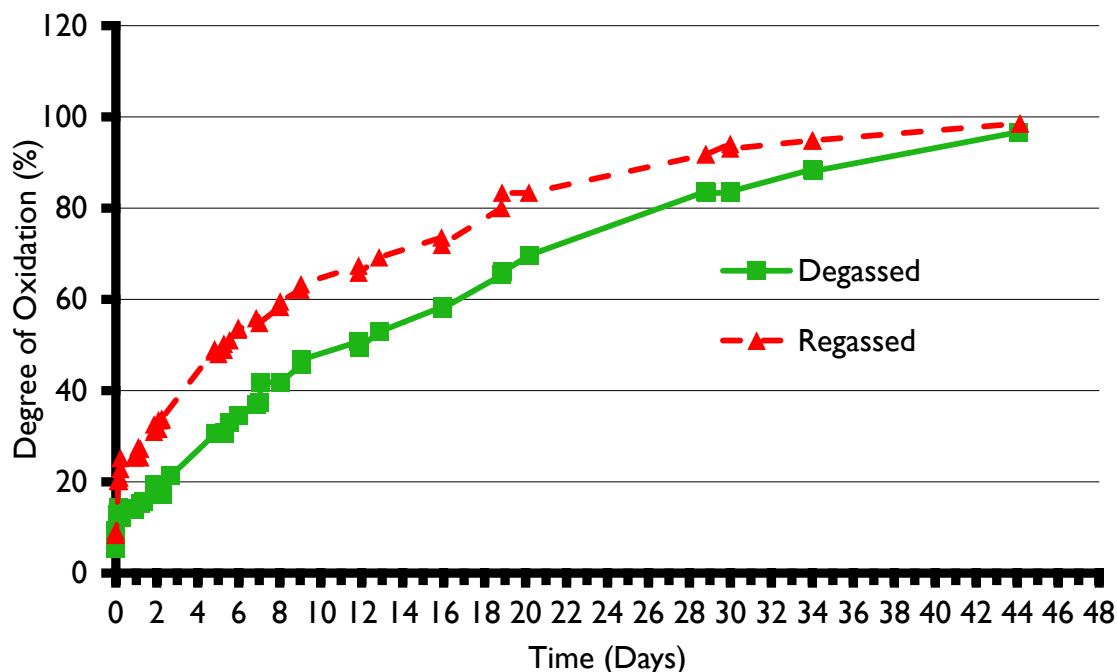


Figure 2.14 – Degree of oxidation of brancher in D₂O at 1 mgml⁻¹ over 16 days. Sample “Degassed” was prepared in D₂O that been degassed by bubbling N₂ through for 1 hr. Sample “Regassed” was made in the same D₂O after it had been regassed by bubbling air through for 15 mins.

Mass spectrometry (ESI) was performed on the materials produced using this method to elucidate whether the double cyclic disulfide had been produced. The most prominent peak was at $m/z = 647.2924$ and attributed to the dimer (calc. $m/z = 647.2796$). The other prominent peak (aside from those of the dimer and cations) was at $m/z = 324.1455$ consistent with the fragment resulting from cleavage of the central disulfide (calc. $m/z = 324.1476$). No higher molecular weight ions were observed providing compelling evidence for the formation of the double cyclic. The only suggestion that the picture may not entirely be complete is the imperfect integration in the proton NMR of one of the peaks (A in Figure 2.12). As shown in Figure 2.15, while the other three peaks integrate to 12, the peak attributed to the methylene protons alpha to the central tertiary amine only integrates to 11.2.

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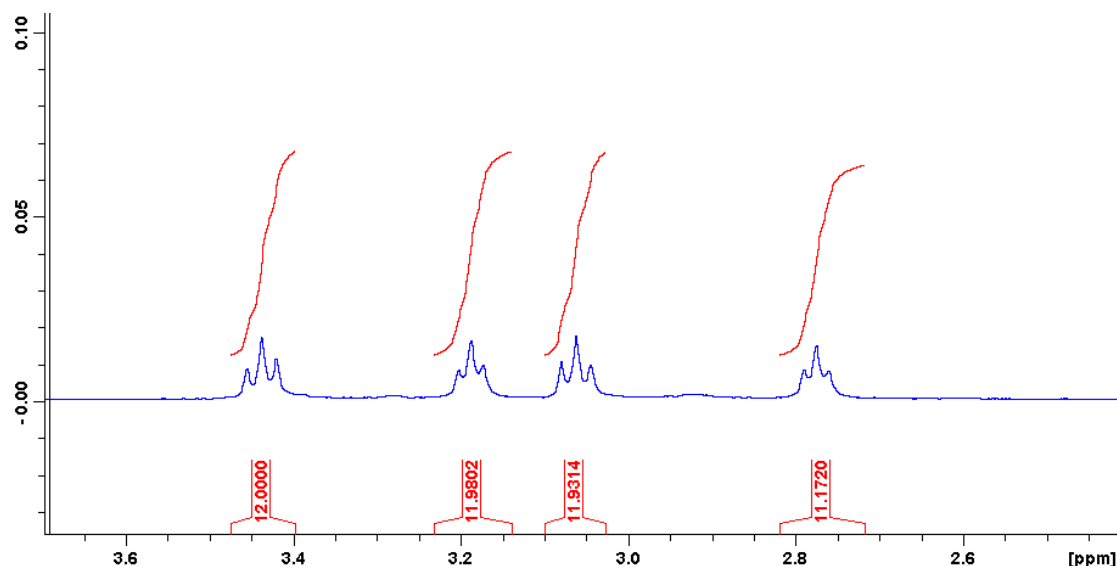


Figure 2.15 – Section of the NMR of the oxidised brancher. Assignment: 2.78 ppm = $\text{NCH}_2\text{CH}_2\text{NH}$, 3.06 ppm = $\text{CH}_2\text{CH}_2\text{SS}$, 3.19 ppm = $\text{NCH}_2\text{CH}_2\text{NH}$, 3.44 = $\text{CH}_2\text{CH}_2\text{SH}$

It can also be seen that there is some broadness around the baseline between $\delta = 2.8$ and 3.0 ppm so perhaps the lost integration is here. That said, the low concentrations involved make it difficult to draw any conclusions. It is possible that perhaps the oxidation behaviour is not a simple process and this is certainly an area that needs further investigation.

An experiment to confirm whether the period of initial fast oxidation was simply due to consumption of the oxygen in the solvent (followed by passive, slow dissolution of atmospheric oxygen into the solvent) was carried out. After a solution of brancher was prepared at 1 mgml^{-1} concentration in D_2O it was allowed to oxidise as normal. Once the oxidation had levelled out (at around 20%, as usual), air was bubbled through the sample for 5 mins. As shown in Figure 2.16, sharp increase in oxidation was observed further confirming that the initial rapid oxidation is due to the amount of oxygen dissolved in the solvent.

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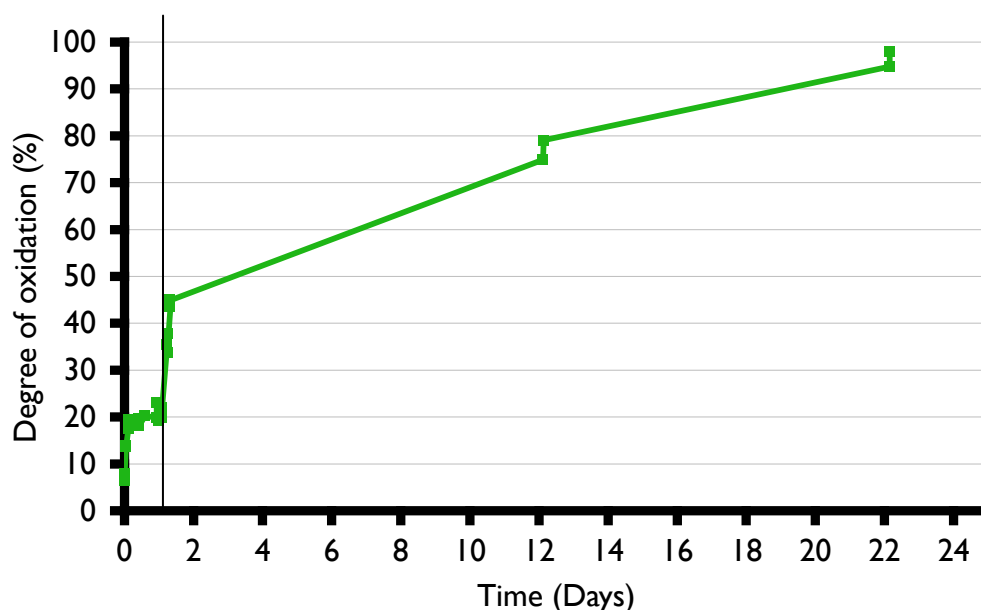


Figure 2.16 – Degree of oxidation of branched trithiol in D₂O at 1 mgml⁻¹ over 22 days. At the time indicated by the vertical line, air was bubbled through the sample for 5 mins using a hand bellows.

2.9. Attempted alkylation of tris(2-aminoethyl)amine using ethylene sulfide

Due to the initial failings of the reductive amination chemistry to produce the branching unit it was decided to attempt to use ethylene sulfide to alkylate the primary amine groups on tris(2-aminoethyl)amine with ethylenethiol groups. This method would also be very amenable to use on different amines. A previous attempt to alkylate spermidine in this way - using toluene as the solvent - based on the work of Wong and Dilworth¹²⁴ failed due to lack of pH control (Figure 2.17). A paper by Peng *et al.*¹²⁵ however, described the thiolation of branched PEI using propylene sulfide. They conducted the reaction in methanol at pH 7.2 to avoid the poly(ethylene sulphide)-generating side reaction caused by attack of the thiolate anion on the ring (Figure 2.17).

The reaction involved dissolving the amine in water and regulating the pH to 7.2 using hydrochloric acid. The water was then evaporated to give the hydrochloride salt of the amine which was then re-dissolved in methanol and reacted with the

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ethylene sulfide. It was found that the salt of the amine was insoluble in methanol and there was no evidence by NMR that any reaction had taken place when reacting it as a dispersion. The chloride counter-ions were exchanged for tetraphenylborate counter-ions and this salt became soluble in methanol.

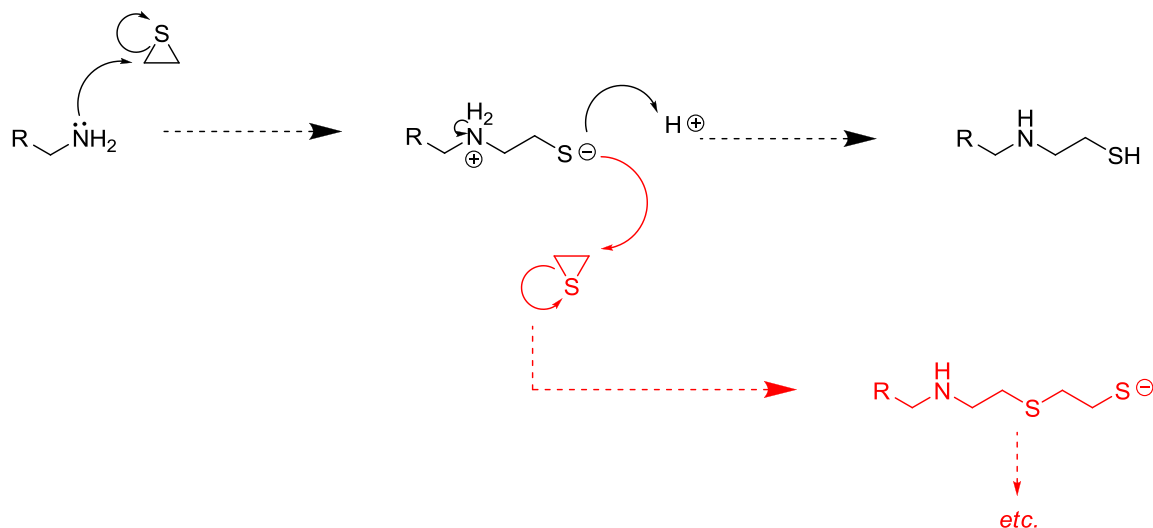


Figure 2.17 – Proposed mechanism of alkylation of primary amine with ethylene sulfide to give thiol.

Initially, the reaction was carried out on NMR scale using degassed solvent. Unfortunately, instead of the anticipated four, equally-sized triplets a more complex splitting pattern was observed (Figure 2.18). Due to the relatively similar chemical shift (all between $\delta = 2.2$ and 2.6 ppm) of the methylene protons involved in the molecule it was decided to attempt to follow the reaction at several different pH values to allow further interpretation of the spectrum.

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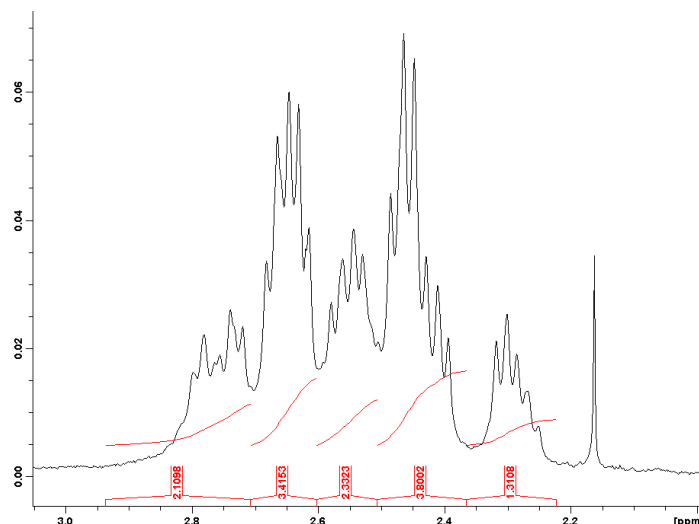


Figure 2.18 – Complex splitting pattern shown by NMR of attempted synthesis of trithiol 11.

Initially a spectrum was recorded at pH 2 and showed only two triplets due to the methylenes of the amine and a singlet at around $\delta = 2$ ppm due to the methylenes of the ethylene sulfide ring. As the pH was increased, two more triplets appeared which fitted in with the formation of an ethylene group between the sulfur and nitrogen atoms. When the experiment was conducted at pH 8, a spectrum was obtained which showed four clear triplets and two smaller triplets with coupling patterns consistent with the formation of the product (Figure 2.19).

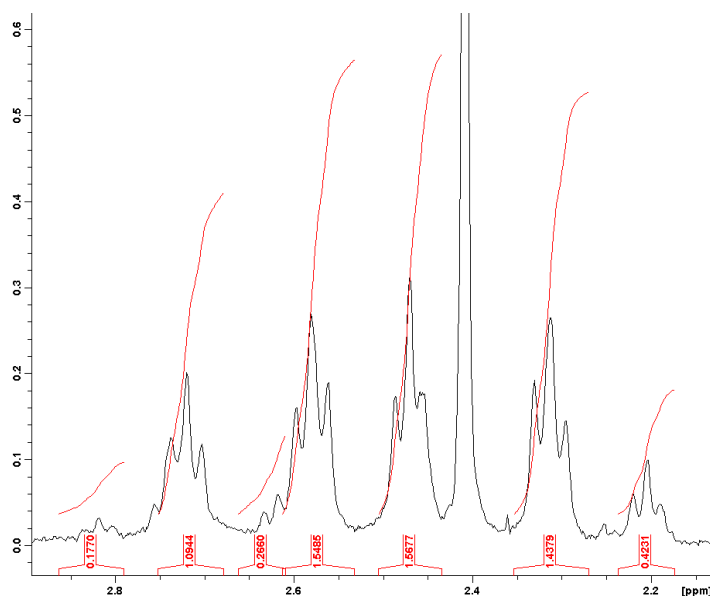


Figure 2.19 – Section of the NMR showing resonances originally believed to be due to formation of trithiol 15. The four prominent triplets are consistent with the four methylene environments.

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These were initially attributed to the protons α - to the amines and protons α - to either a thiol or disulfide. However, it was found that this pattern of resonances also appeared in the spectrum before ethylene sulfide was added showing that the pattern of resonances must simply be due to sufficiently slow proton exchange leading to an observable difference between the environments of methylene protons α - to protonated or unprotonated amines.

Because the aromatic protons in the tetraphenylborate were visible in the NMR spectrum it was possible to compare the integrations of the aromatic portion of the spectrum with the amine region and thereby deduce the degree of protonation. The pH 7.2 salts were found to have almost three counterions per molecule of tren. Electrostatic repulsion makes the central, tertiary amine extremely difficult to protonate if one of the primary amines is protonated. Therefore the only possibility at this protonation level is for the primary amines to be protonated resulting in lack of nucleophilicity. However, at pH 8 the degree of protonation was calculated to be 1.9 protonated amines per molecule of tren and there was still no evidence of reaction suggesting that this explanation is insufficient. However, pH 8 salts would often result in the polymer forming side reaction (Figure 2.17) indicating that this was the lower limit of pH that this reaction would take place. This would be expected bearing in mind the pKa of the thiol group which, based on the pKa of cysteamine, would be expected to be around 8⁹⁵ suggesting a significant amount of thiolate anion would exist at or above pH 8. The pH “window” where the reaction is practical is therefore extremely limited.

It is possible that if the reaction is failing for reasons related to pKa, the experiment would work using an oligoamine that would still have unprotonated amines at pH 7.2 like the branched PEI used by Peng *et al.*¹²⁵ A good candidate would be triethyltetramine which could lead to a product which would also fit the design criteria for a branching unit (Figure 2.20).

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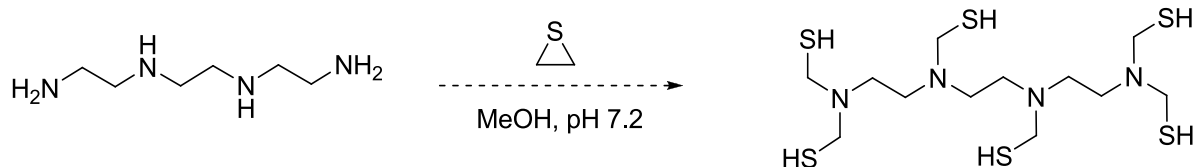


Figure 2.20 – Possible thiolation of triethyltetramine using ethylene sulfide.

2.10. Attempted alkylation of tosylated tris(2-aminoethyl)amine with ethylene sulfide

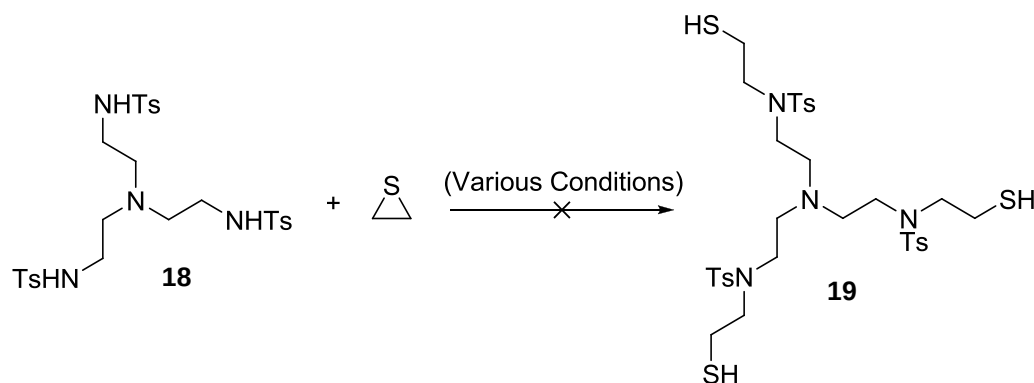


Figure 2.21 – Attempted reaction between tosylated amine 18 and ethylene sulfide

An alternative method of using ethylene sulfide to alkylate an amine was also considered (Figure 2.21). It was thought that tosylation of tren would avoid the problems associated with protonation of the amine, sulfonamides being acidic, not basic. Literature precedent exists for the ring-opening of both epoxides¹²⁶ and *N*-tosylaziridine using tosylamines¹²⁷. In the former case, base is used (with the more basic alkoxide rendering polymerisation a trivial concern) which would not be appropriate here. The aziridines are opened by refluxing in benzene without base.¹²⁷ Initially the reaction was attempted in methanol both with and without potassium carbonate at room temperature. The reaction with carbonate generated large amounts of white solid immediately (consistent with the polymer generated in previous ethylene sulfide experiments discussed in Section 2.9). The reaction without base generated a much smaller amount of insoluble solid though no other reaction was observable by TLC after 6 days. The reaction was repeated in DMF both with and without base. Once again the reaction with base generated solid precipitate very rapidly. The reaction without base was left heating for several days with no reaction observed by TLC after 1 week.

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2.11. Attempted NMR scale alkylation of tris(2-aminoethyl)amine using Traut's reagent

Traut's Reagent is an alternative name for 2-iminothiolane, commonly used to introduce thiol side chains in proteins *via* reaction with primary amine side chains such as lysine.¹²⁸ Typically, when this reagent is used with a protein or polymer¹²⁹ it is to introduce only a sub-equimolar amount of disulfide bonds whereas here, the intention was to react it with all three primary amines in a molecule of tris(2-aminoethyl)amine (Figure 2.22). Whilst it has been used in small molecule reactions, the free thiols generated are reacted *in situ* with maleamides or other thiol-reactive functional groups.^{130–132} The literature reactions where the free thiol was preserved on protein¹²⁸ or polymer¹²⁹ side-chains were carried out in aqueous buffer at a pH between 7 and 8.

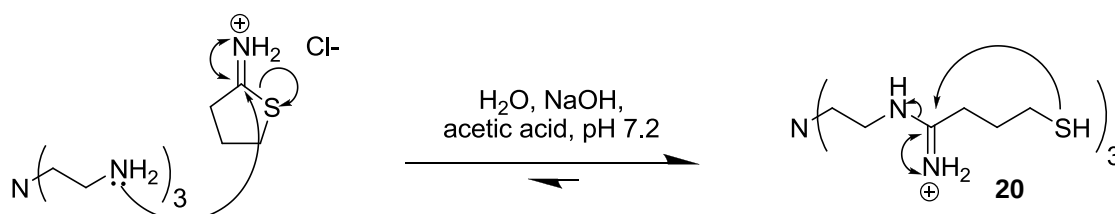


Figure 2.22 – A proposed mechanism of reaction between Tren and Traut's Reagent.

Initial spectra of the crude products from these reactions were complex and so it was decided to degas the water thoroughly with nitrogen to avoid the possibility of oxidation of the thiols to disulfides. Spectra of crude reaction products produced in this way were clearer but still difficult to assign. It was decided to acidify all samples with HCl and evaporate to dryness before running NMRs to prevent any oxidation, allow removal any residual acetic acid which obscured a key region of the ¹H NMR spectrum and to avoid a potential side reaction¹³³ (Figure 2.25) that would give substituted 2-iminothiolane products. This was first attempted during an experiment to investigate the rate of reaction as full conversion of all amines had not been reported in the literature. NMR aliquots of the reaction were taken every 15 minutes and whilst it was found the reaction proceeded slowly, the acidification of NMR aliquots led to less complex spectra that allowed interpretation.

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The question of assignment of resonances was solved using COSY which allowed identification of both starting materials and product (Figure 2.23). One of the clearest indications that a reaction had taken place were the protons initially α - to the sulfur in 2-iminothiolane shifting upfield by around 0.6 ppm to $\delta = 2.5$ ppm, characteristic of protons α - to an aliphatic thiol. Interestingly, after 72 h the reaction appeared to have proceeded further and now the product resonances were more prominent than the starting material resonances (Figure 2.24).

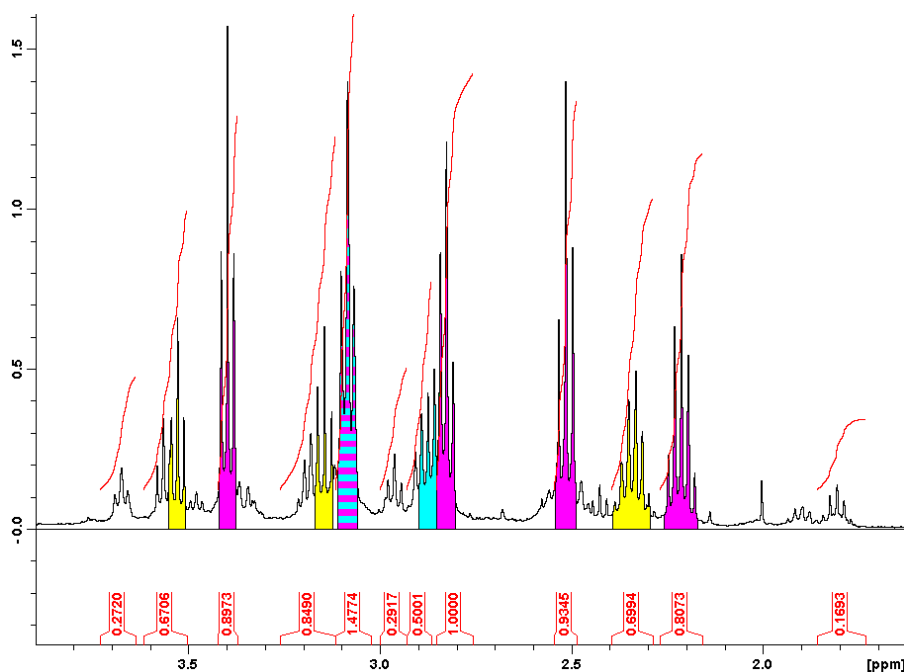


Figure 2.23 – A crude spectrum believed to show formation of the desired compound (magenta), Traut's Reagent (yellow) and the starting amine (cyan). Uncoloured resonances represent unidentified by-products.

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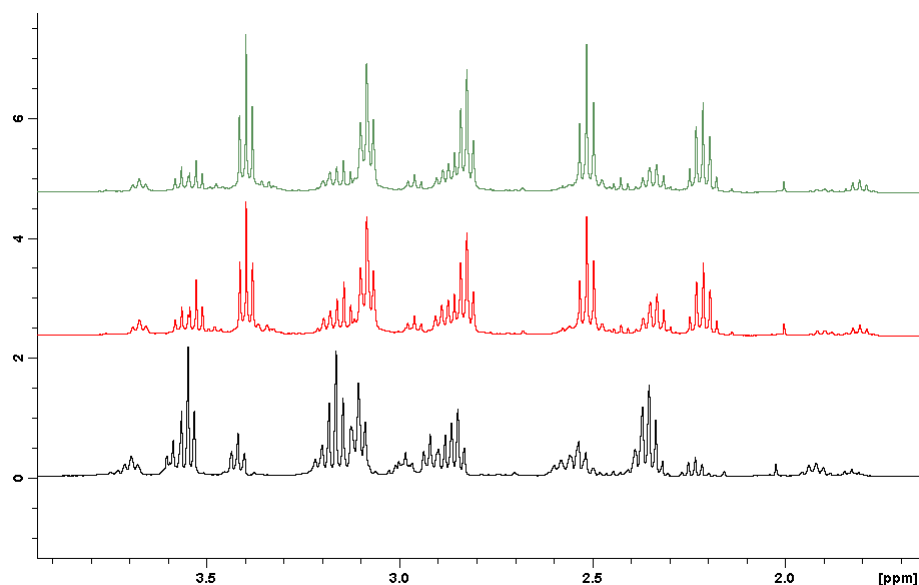


Figure 2.24 – A comparison of ^1H NMR spectra of a sample of crude compound 17. NMRs show the material immediately after acidification (black), after 72 hrs (red) and after 100 hrs (green).

It was eventually concluded after failed attempts at conducting the reaction at pH 2, that the appearance of reaction progress may in fact have been due to the hydrolysis of the Traut's Reagent. This could in-fact suggest that the product that was forming was the hydrolysis product of Traut's reagent, 4-mercapto-butyrimidic acid, the formation of which would cause similar changes in the chemical shifts of the methylene protons of Traut's reagent to reaction with the amine. However, this explanation would require an alternative explanation for the change in the amine resonances. As no NMR characterisation data for 4-mercapto-butyrimidic acid exists, it is difficult to say for certain if this is true though a peak was observed in the mass spectrum of the crude material at $m/z = 119.0646$ characteristic of 4-mercapto-butyrimidic acid (calc. $m/z = 119.045$). However, mass spectrometry evidence for substitution, albeit incomplete, was also obtained and showed peaks corresponding to mono- and di- substituted amines. There was also another peak observed in the mass spectrum that also helped shed some light on the nature of the unidentified impurities in the NMR: namely, a peak was observed at $m/z = 231.1669$ that corresponded to the degradation product mentioned in the literature that was the original reason that NMR samples were acidified (Figure 2.25).

2. Preparation of Novel Compounds for siRNA Delivery Systems

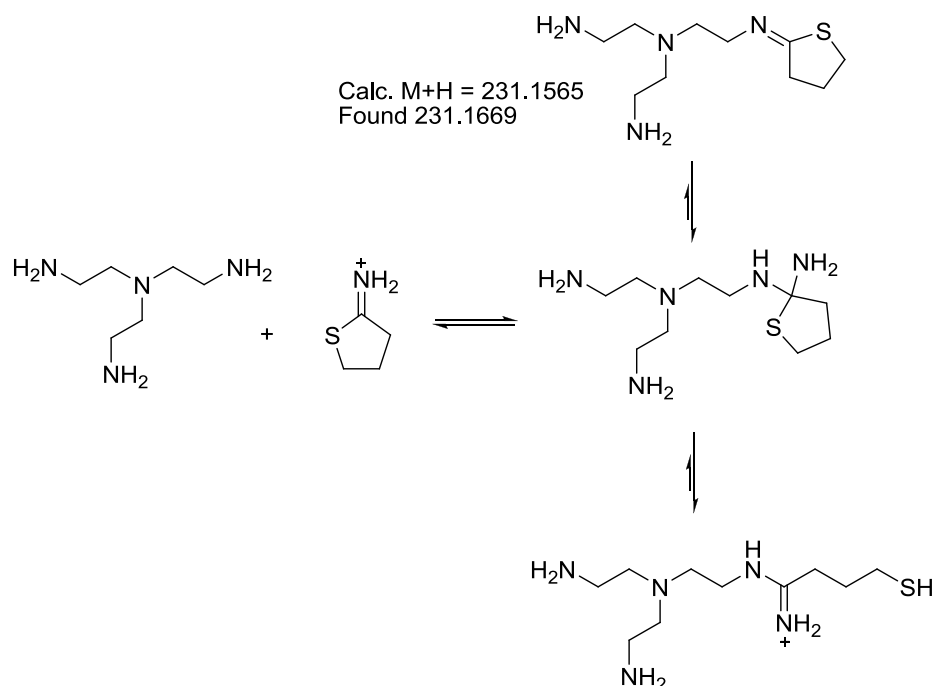


Figure 2.25 – Scheme showing possible degradation pathway.¹⁰² Calculated M+H value corresponds to a peak found in the mass spectrum of the crude material at m/z = 231.1669.

As previously mentioned, Traut's Reagent is predominantly used to add thiol functionalities to protein chains. In this instance, purification is simple dialysis. This is not appropriate in this case and clearly the reaction that is taking place is producing at least two by-products. Reverse-phase chromatography could be a possibility to separate the materials and will be investigated. One major problem that may make the reaction impractical is that a 50-fold excess of Traut's reagent may be necessary to guarantee full substitution.¹³⁴ The reaction could be attempted in organic solvents to avoid formation of the hydrolysis by-product though this would first require the freebase to be made or for ion-exchange of the chloride for an organic soluble counter-ion as the hydrochloride salt form would likely be insoluble. This reaction could be complicated by attack of the thiolate as a nucleophile which was encountered when ethylene sulfide was used in a ring-opening reaction in toluene (Section 2.3).

2. Preparation of Novel Compounds for siRNA Delivery Systems

2.12. Attempted tosylation of 2-(hydroxymethyl)-1,3-propanediol

A synthetic route to a branched trithiol not derived from tris(2-aminoethyl)amine was investigated as a possible means of developing a strategy that would allow a greater variety of amines to be prepared. Rather than being limited to adding $\text{CH}_2\text{CH}_2\text{SH}$ groups onto amines, this route would allow the addition of an $\text{NHCH}_2\text{CH}_2\text{SH}$ moiety onto any carbon bearing a good leaving group. A route involving a central carbon with leaving groups attached was identified as a possibility (Figure 2.26).

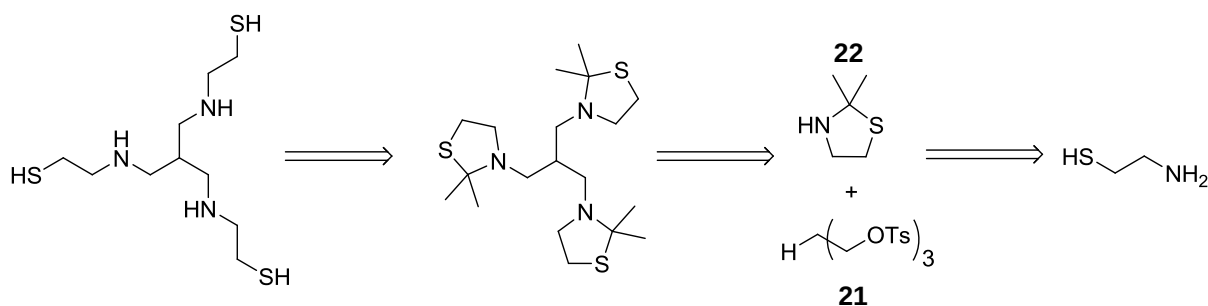


Figure 2.26 – Retrosynthetic analysis showing the potential usefulness of a protected cysteamine 22.

It was decided to attempt to activate 2-(hydroxymethyl)-1,3-propanediol with tosyl chloride for use as the core to which the amines would be attached. However, the tosylation reaction was terminated when it transpired that the alcohol was only sparingly soluble in DCM and the increased solubility imparted by the tosyl groups would not be enough to help the reaction to completion. By NMR, only 16% of the alcohol was tri substituted and it was decided that as a result, a column would not be worthwhile. 2-(Hydroxymethyl)-1,3-propanediol was poorly soluble in all polar aprotic solvents bar DMF (polar protic solvents would be inappropriate due to possibility of them reacting as nucleophiles) and there was also a risk of elimination so it was decided to use commercially available pentaerythritol tetrabromide instead. This would give a “four-armed” brancher and with minimal risk of elimination. While sterically challenging there was literature precedent for the substitution of all four bromines with secondary amines.¹³⁵

2. Preparation of Novel Compounds for siRNA Delivery Systems

2.13. Synthesis of 2,2-dimethyl-thiazolidine **22**

The success of the above mentioned scheme hinged on the successful synthesis of the protected cysteamine **22** (Figure 2.27). The reaction is analogous to the formation of an acetal with acetone and ethylene glycol.

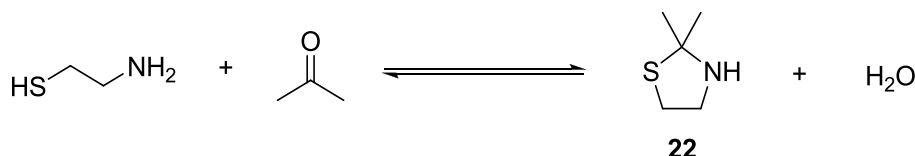


Figure 2.27 – Equilibrium reaction between cysteamine and acetone.

Literature precedent for the formation of this compound involved the refluxing of cysteamine in water and acetone¹³⁶ but it was thought that an example from the literature involving the protection of the amino acid cysteine¹³⁷ by refluxing in acetone would be a better starting point due to the more favourable equilibrium. This reaction was attempted and the desired product was obtained with a yield of 23%. A large amount of insoluble solid remained and this was believed to be cysteamine suggesting the reaction had not gone to completion. It was decided to repeat the experiment with the addition of MgSO₄ to force the equilibrium though this gave similar yields to before. The next attempt at this reaction was based on the analogous reaction between acetone and 2-hydroxyethylamine of which there were more examples in the literature.¹³⁸ The reaction was conducted at room temperature (though increased temperature was required to dissolve cysteamine) in 50:50 acetone/DCM (v/v) with MgSO₄. This gave material in a yield of 34% with ¹H NMR consistent with that which had been previously reported.¹³⁹

2. Preparation of Novel Compounds for siRNA Delivery Systems

2.14. Attempted tetrasubstitution of pentaerythritol tetrabromide with protected cysteamine **22**

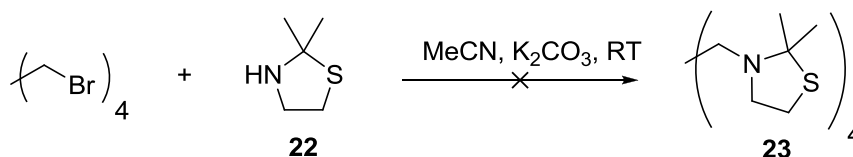


Figure 2.28 – Attempted tetra-substitution of pentaerythritol tetrabromide with **22.**

Based on a literature procedure,¹³⁵ 3.3 equivalents of compound **22** were reacted with one equivalent of pentaerythritol tetrabromide in acetonitrile with base (Figure 2.28). No reaction was observed by NMR even when NaI was used. Since the amine was completely unreactive, it is likely that steric factors were an issue.

2.15. Attempted tetrasubstitution of pentaerythritol tetrabromide nosylated amine

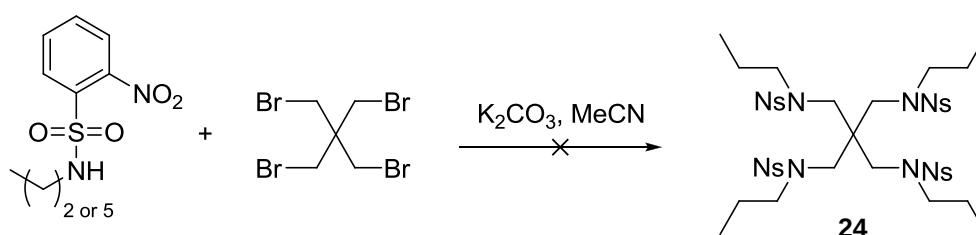


Figure 2.29 – Attempted reaction between nosylated amine and tetrabromide

It was thought that the most likely reason for the failure of the previous experiment was the low nucleophilicity of the secondary amine. Since the conjugation of amine-bearing “arms” to a functionality-lacking core was attractive – due to its potential for synthetic flexibility – alternative strategies were considered. The idea was to react a terminal sulphonamide with pentaerythritol tetrabromide (Figure 2.30). Ultimately, it was hoped that this would provide a procedure whereby several compounds containing different oligoamine motifs could be prepared.

2. Preparation of Novel Compounds for siRNA Delivery Systems

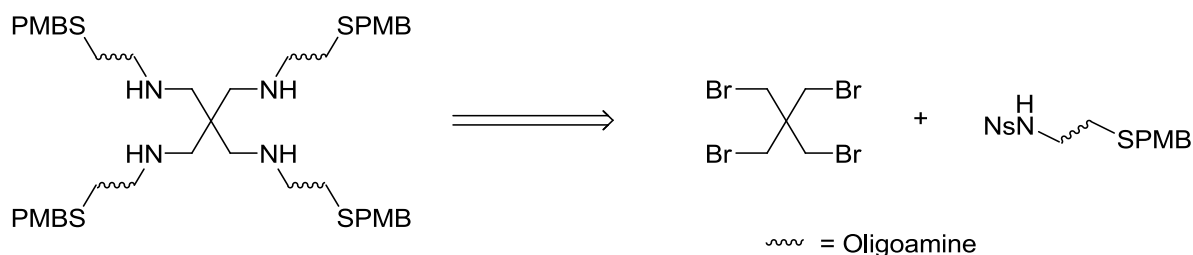


Figure 2.30 – Retrosynthetic analysis showing how protected oligoamines could be attached to a four-armed core.

A test reaction was carried out using nosylated propyl-1-amine (

Figure 2.29). After refluxing in acetonitrile for 48 h in the presence of potassium carbonate no reaction was observed by either TLC or aliquot NMR. The experiment was repeated (using nosylated hexyl-1-amine) using harsher reaction conditions in an attempt to drive the reaction to completion. DMF was used as solvent allowing the reaction to be refluxed at 153 °C and a catalytic amount of NaI was also used. Characterisation showed that a reaction had taken place: the reaction mixture changed colour, the aliquot NMR became complex and a new faster running, orange spot was observable on TLC. Basic work-up and flash column chromatography on the crude material led to the isolation of an orange oil. However, the NMR did not indicate substitution had taken place due to the absence of a resonance for the methylene protons alpha to the quaternary carbon. Unexpectedly, there was an upfield shift among the aromatic protons suggesting the presence of an electron donating substituent. It was eventually deduced that SO₂ had eliminated from the nosyl-amine to give a mono-alkylated 2-nitro aniline. The NMR was consistent with the literature for the proposed product¹⁴⁰ and ESI-MS showed the expected ion. There is precedent for this reaction occurring when nosylated amines are heated too strongly in the presence of base.¹⁴¹ The suggested mechanism, analogous to the nosyl deprotection mechanism is shown in Figure 2.31.

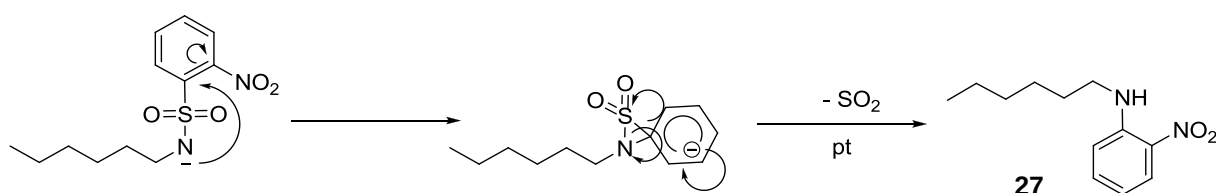


Figure 2.31 – Proposed mechanism¹⁴¹ for the formation of the nitroaniline byproduct 27.

2. Preparation of Novel Compounds for siRNA Delivery Systems

The literature suggests this reaction only normally occurs as a side reaction competing with the desired reaction. In this instance, the desired reaction would appear to be poor competition as no substitution was observed. The experiment was repeated at 90 °C and the yield of the aniline **27** was recorded as 77%; no substitution product was identified.

2.16. Attempted hexa-alkylation of Tris(2-aminoethyl)amine

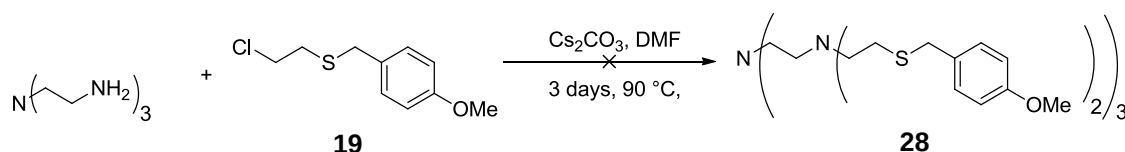


Figure 2.32 – Attempted hexaalkylation of tris(2-aminoethyl)amine

Synthesis of a hexathiol was also planned via reaction of the chloride **12** with unprotected tris(2-aminoethyl)amine (tren) followed by removal of the PMB groups. This would give a different branching unit and use two fewer synthetic steps than the synthesis of the trithiol. A TLC in 100 % EtOAc of the product - obtained after 3 days of reaction and basic aqueous work-up - showed three spots of $R_f = 0$, 0.68 and 0.87. It was believed that the product would likely stick to silica under these conditions and the spot at $R_f = 0$ also turned blue when treated with ninhydrin indicating the presence of primary amine. Flash column chromatography was carried out on the crude sample. 36% (429 mg) of the material was revealed to be the alkene elimination product of the chloride. This was a sufficiently large percentage of the chloride to suggest that full substitution could not have occurred. The other fraction – comprising 3.6% of the crude was consistent with the alcohol analogue of the chloride **12**. The remainder of the material put through the column was unrecovered even after several column lengths of 20% MeOH/DCM were used. Mass spectrometry of the crude material showed no evidence for product formation. NMR of the crude was complex in the region of methylenes adjacent to amines, suggesting some reaction may have taken place; however, the spectrum was dominated by the alkene elimination product prohibiting full analysis. A possible explanation for this is that the amine was not sufficiently reactive to allow substitution to compete with

2. Preparation of Novel Compounds for siRNA Delivery Systems

elimination. Use of a stronger base such as DBU was considered but not pursued due to success in other areas.

2.17. Summary

In conclusion, a synthetic route to the target trithiol compound was developed. The oxidation behaviour of the material was also investigated and an oxidised trithiol was prepared. Both of these compounds were appropriate to be tested for their siRNA delivery efficiency and combined with and compared to the original cyclic disulfide as described in Section 1.11. Alternative synthetic routes were attempted and the possibility of preparing alternative thiol bearing structures was also investigated. Unfortunately, none of these pathways led to useful compounds.

3. Formation and Characterisation of siRNA Nanoparticles

3. Formation and Characterisation of Particles

3.1. Initial siRNA Complexation Studies

As described in Section 1.11, it was envisaged that a combination of branched and linear compounds would be used in combination to afford an siRNA vector. As a starting point, it was decided to investigate the interactions between cyclic TetN343 (7) and siRNA.

The first step in ascertaining if cyclic TetN343 (7) was able to complex siRNA in a useful manner was to carry out a gel electrophoresis experiment as this had strongly demonstrated effective binding of pDNA in the earlier work.¹²⁰ As shown in Figure 3.1, no band of nucleic acid is visible at an N/P ratio of five or greater. Though a similar experiment using pDNA had previously shown movement of the nucleic acid to be completely retarded at N/P 2.5, this result still indicated the possibility of useful particle formation.



Figure 3.1 – A UV photograph of a polyacrylamide gel showing retardation of siRNA by TetN343 at an N/P ratio of 5 and above.

For a more qualitative examination of siRNA binding a propidium iodide exclusion assay was used. This experiment relies on the propensity of propidium iodide to intercalate inbetween the base pairs of siRNA and the fact that it only fluoresces strongly when intercalated (Figure 3.2). Should all the available siRNA be bound by a cation, no fluorescence should be visible. Comparison of results to the fluorescence of free siRNA ($N/P = 0$) allows the degree of complexation to be obtained as a percentage.

3. Formation and Characterisation of Particles

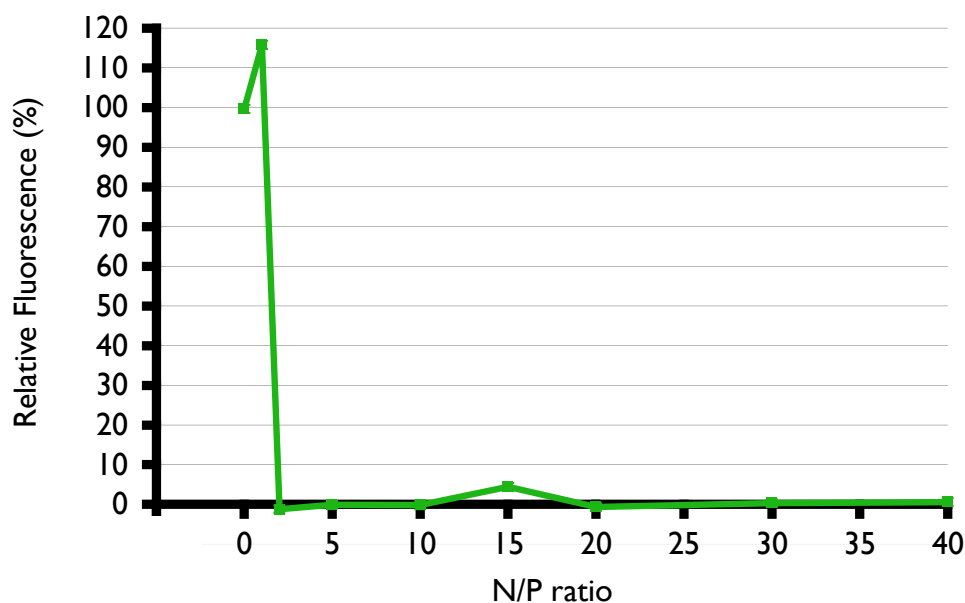


Figure 3.2 – A graph showing the proportion of siRNA encapsulated by TetN343 at different N/P ratios as indicated by a propidium iodide exclusion assay. Data show mean and standard deviation of three measurements.

As shown in Figure 3.2, full encapsulation takes place at an N/P ratio of two or greater. The difference between this value and the results from the gel suggests that though the siRNA may be fully encapsulated at N/P 2, the complex is not very strong and falls apart under electrophoresis conditions.

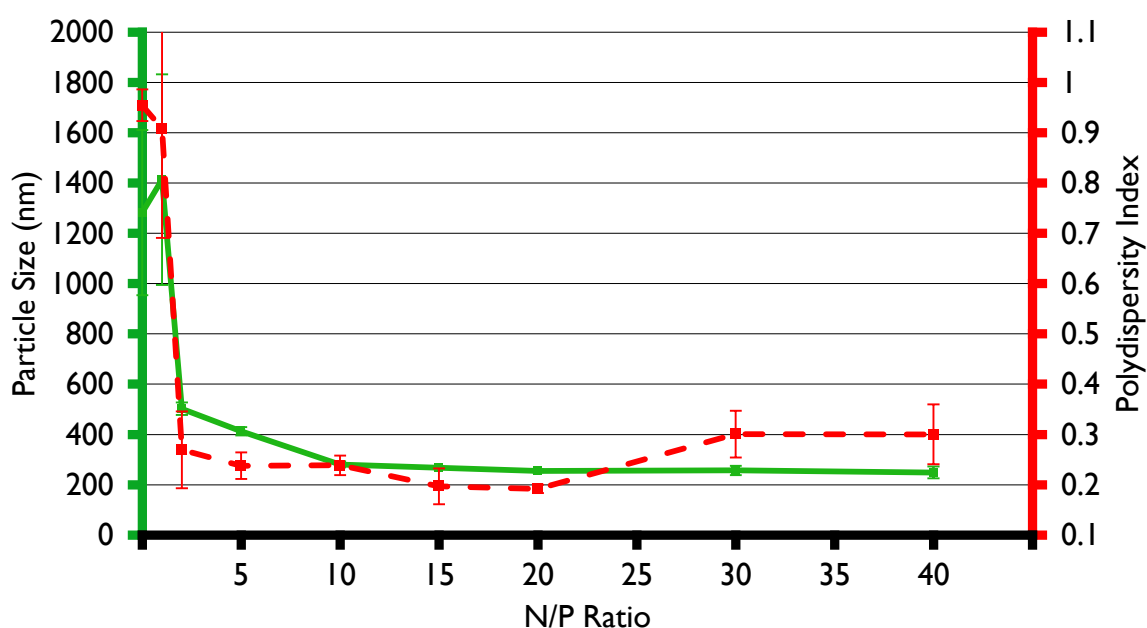


Figure 3.3 – Size and polydispersity of particles formed between siRNA and cyclic TetN343 at a range of N/P ratios. Data show mean and standard deviation of three measurements.

3. Formation and Characterisation of Particles

The final experiment to be conducted as part of the initial particle formation experiment was particle sizing. A similar experiment conducted with pDNA led to the formation of particles of 100 – 150 nm but due to the shorter length of siRNA and the evidence for weaker binding observed in the electrophoresis experiment, it was expected that the particles formed with siRNA would be larger (as discussed in Section 1.4). This was indeed the case and the data showed particle formation between 250 and 280 nm at and above an N/P ratio of ten. An N/P ratio of five gave somewhat larger particles (412 nm) suggesting that there was insufficient cationic charge for stable particle formation. An N/P ratio of one – in common with free siRNA – displayed size and polydispersity values of high standard deviation suggesting no particle formation and corroborating the previous experiments.

From these three experiments it was concluded that cyclic TetN343 was able to form complexes with siRNA albeit larger than the ones formed with pDNA. An N/P ratio of ten was regarded as the minimum required to obtain robust, small particles and this would be treated as the starting point for the following formulation experiments.

3.2. Optimisation of Formulation Conditions

3.2.1. Particle formation over time

Once it had been established that siRNA complexes could be formed, it was decided to investigate the effects of the formulation protocol on particle size. The first element of particle preparation to be considered was the time taken for incubation. Figure 3.4 shows the change in particle size after the cation is added to the siRNA ($t=0$ represents the solution prior to the addition of cation). Particle size slowly increases over time but not by a significant amount. It was thought that by adding the cation gradually, particle formation could be controlled. The blue and orange lines in Figure 3.4, representing size and PI respectively show that adding cation over time (specifically, in six portions, the data point at time 0 contains no polymer, the data point at time 70 contains polymer to N/P 10) leads to larger particle sizes than adding simply adding all the polymer in one portion. It must therefore be the case

3. Formation and Characterisation of Particles

that the large particles formed at low N/P ratios early in the experiment do not provide a good “foundation” for further portions of cation to bind.

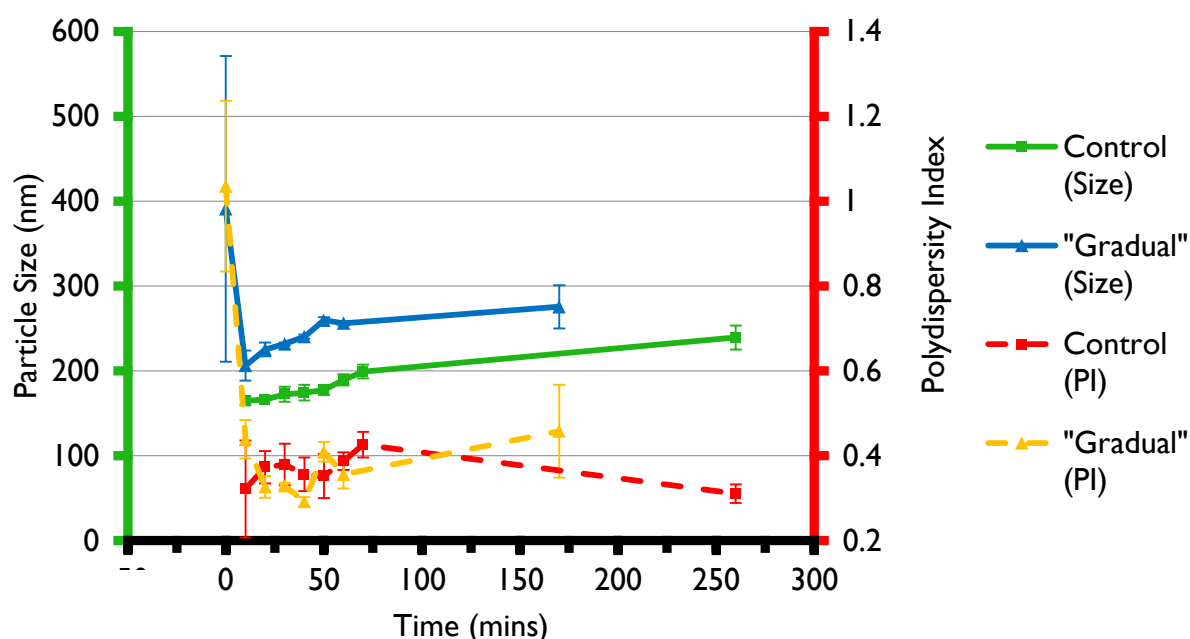


Figure 3.4 – Size and polydispersity of particles formed between siRNA and cyclic TetN343 at an N/P ratio of 10 over time. Green square/red square: Particles formed by simple mixing of cation and anion followed by vortexing. Blue triangle/orange triangle: Particle formation over time with polymer added in 6 portions over 1 hr from t=10 mins. Data show mean and standard deviation of three measurements.

3.2.2. Effect of the order of mixing on the cationic and anionic components

In every example of polyplex formation found in the literature, cation is added to anion. The reason for this has not been studied in detail and no comparisons with opposite addition order exist for either siRNA or pDNA. Because many siRNA polyplex formation protocols are simply unmodified pDNA protocols, and because of the proposed mechanistic differences between our transfection agents and PEI-like materials an “anion to cation” procedure was investigated. Two sets of three samples of TetN343 – siRNA complexes were prepared at N/P 10. One set of polyplexes was prepared by adding cation to anion and the other by doing the opposite. Both samples were then vortexed for five seconds and left at room temperature for one hour.

3. Formation and Characterisation of Particles

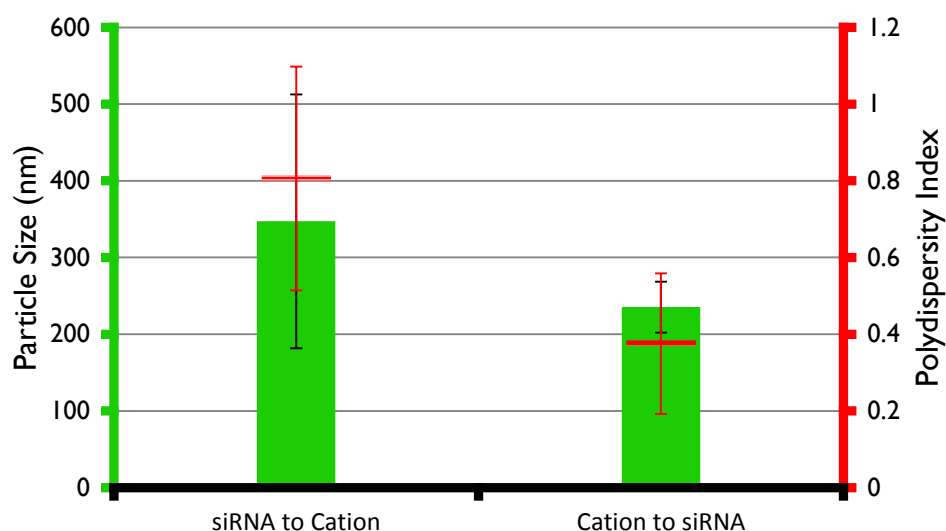


Figure 3.5 – Size and polydispersity of particles formed between siRNA and cyclic TetN343 at an N/P ratio of 10. In one instance siRNA solution was added to cation solution and in the other the standard cation to siRNA procedure was used. Green bars indicate particle size, red horizontal lines indicate polydispersity index. Data show mean and standard deviation of three measurements.

As shown in Figure 3.5, the addition order makes a profound difference; the particles prepared by adding siRNA to TetN343 are shown to be over 900 nm in diameter. In fact, particle sizing data showing great size along side high polydispersity have been found to be characteristic of lack of particle formation (*cf.* N/P ratio 1 in Figure 3.3) suggesting addition order is vital to the formation of polyplexes.

3.2.3. The effect of pH on particle size

The next condition to be investigated was pH. The majority of polyplex formation in the literature is carried out between pH 7.0 and pH 7.4, *i.e.* slightly below or at physiological pH. All of the experiments conducted so far had been carried out in 4 mM HEPES at pH 7.0 so samples at a range of pH values around this were prepared. For conventional PEI-like polymeric vectors the impact of pH on polyplex formation is limited to its effect on amine protonation with high pH resulting in unprotonated, neutral amines incapable of forming complexes. For our system however, pH also has the potential to affect the proposed disulfide rearrangement through the

3. Formation and Characterisation of Particles

deprotonation of the thiol groups that it is believed initiate the polymerisation (see Section 1.11).

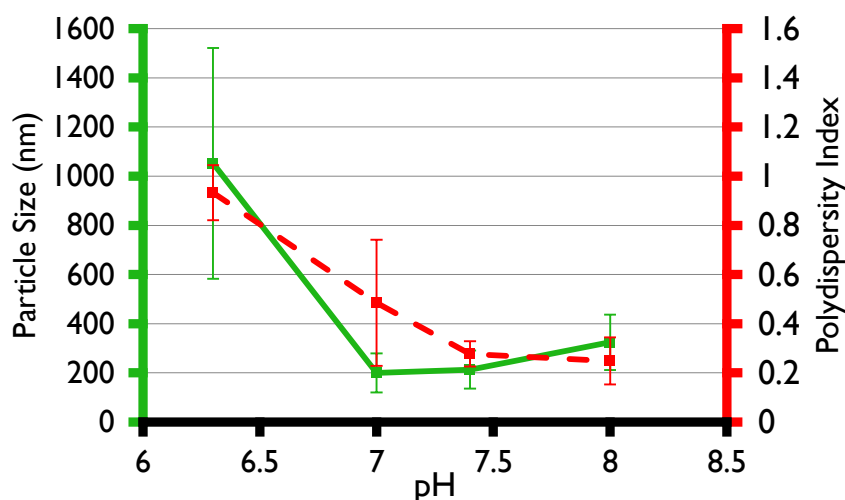


Figure 3.6 – Size and polydispersity of particles formed between siRNA and cyclic TetN343 at N/P 10 at a range of pH values. Data show mean and standard deviation of three measurements of three samples.

Figure 3.6 shows the results of the pH dependency experiment. The difference between size at pH 7.0 and pH 7.4 is trivial with the pH 7.4 shown to be a negligible 12 nm larger. The higher PI of the pH 7.0 particles can be discounted as this was caused by one anomalously high reading not seen in other experiments at this pH. The trend with increasing pH was an upwards one and at pH 8.0 the particles were over 300 nm. This was entirely in line with expectation given the decreased protonation of the amines at this pH. The most interesting data point proved to be the pH 6.3 sample. It would have been expected to produce particles at least as small as those at pH 7.0 and yet the data would suggest no particle formation at all. Figure 1.34 shows the mechanism of disulfide exchange and it is likely that at a pH of 6.3, this is suppressed due to lack of thiolate. If the cyclic disulfides are unable to polymerise the situation is more akin to polyplex formation using spermine alone. Indeed, it is likely to be worse than spermine due to restriction of the charged amines by the cyclic structure (see Section 3.3.4 for discussion of this). Based on this experiment there was not a great deal to choose between pH 7.0 and 7.4 so it was decided to keep buffer pH at 7.0 due the extra strength this would impart to the polyplexes.

3. Formation and Characterisation of Particles

3.3. Particle Formation Using Linear TetN343

3.3.1. Initial particle sizing

Perhaps the most surprising result was obtained when observing the formation of particles over time using linear dithiol. A steady increase in size from 400 nm to over 1000 nm was observed (Figure 3.7). The previous work had identified that the dithiol did form larger particles than the cyclic disulfide and this was attributed to polymerisation in this instance being a kinetic process rather than a thermodynamic one for the disulfides.

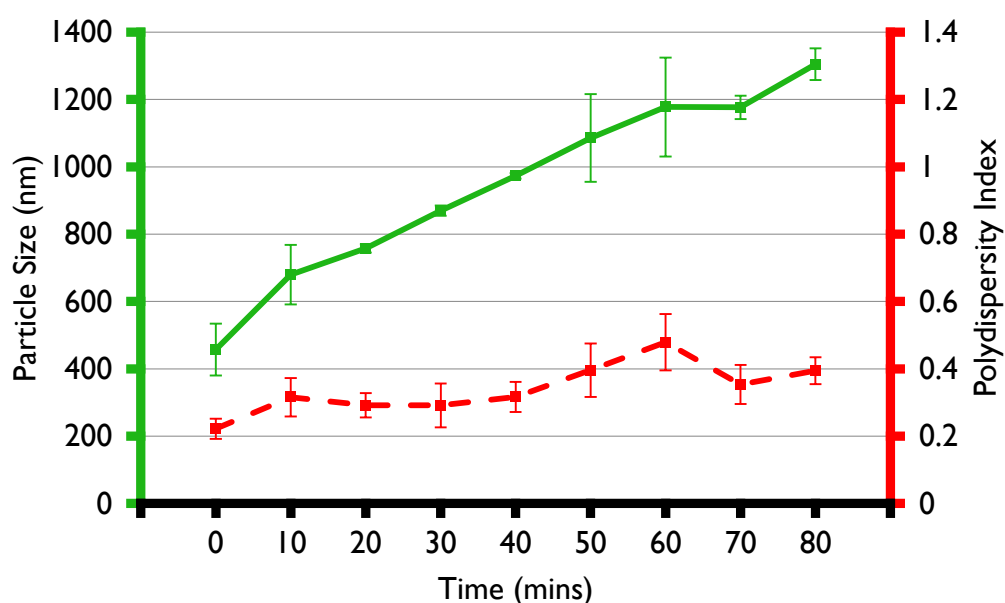


Figure 3.7 – Size and polydispersity of particles formed between siRNA and linear TetN343 at N/P ratio 10 over time. Data show mean and standard deviation of three measurements.

It was thought that the aggregation behaviour that was suggested by the data could be attributed to inter-particle disulfide bond formation. For the cyclic, particle size remains relatively constant from ten minutes after mixing however in this case, there are few thiols present. Most polymerisation events that takes place must, therefore, be the result of disulfide rearrangement and this is unlikely to occur inter-particle. Particles formed with dithiol on the other hand could be rich in surface thiol groups and perhaps this makes inter-particle disulfide bond formation more likely and leads to the large aggregates.

3. Formation and Characterisation of Particles

3.3.2. Reduction of particles formed with linear TetN343

If it were true that the large aggregates formed when using dithiols were due to inter-particle disulfide bond formation, the presence of ME would reduce the disulfide bonds and prevent particle formation. Varying amounts of ME would also perhaps allow a degree of control over particle size. An experiment was devised where particles would be formed for an hour and then exposed to an increasing amount of ME. The amount of ME was initially 0.001 eq and ultimately 0.5 eq (See Table 2 for further details). It was therefore expected that introduction of a reducing agent would cause a change in the particle size since the newly formed disulfide bonds would be cleaved. However, as shown in Figure 3.8 the trend matches the size increase in particles when no ME is added.

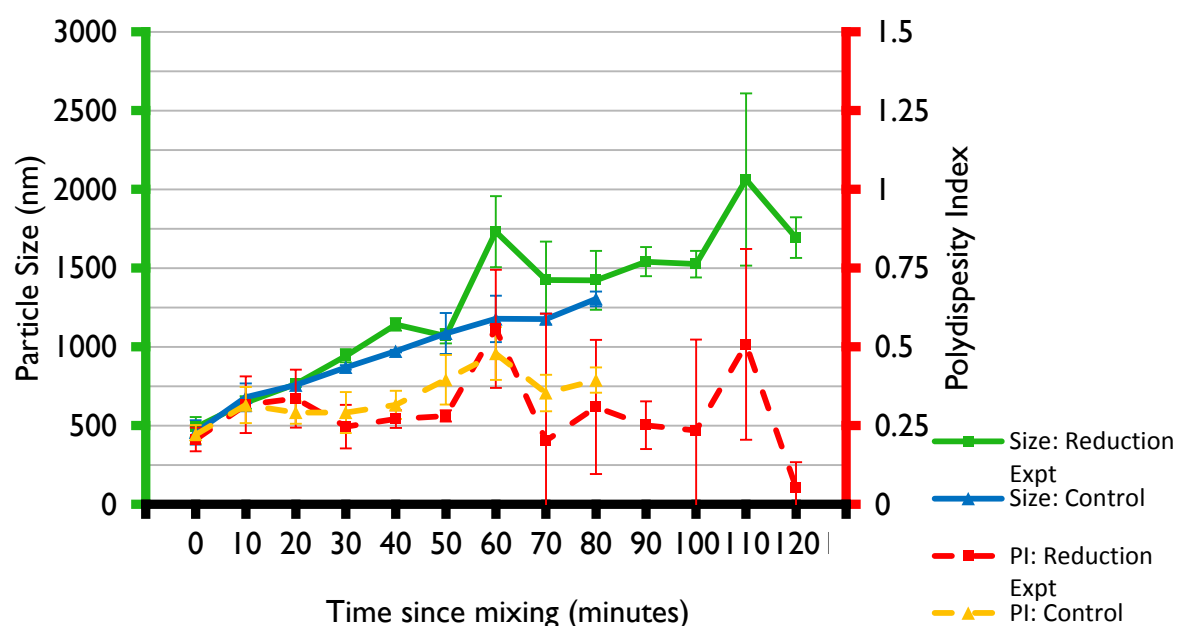


Figure 3.8 – Size and polydispersity of particles formed between siRNA and linear TetN343 at N/P ratio 10 over time. Green square/red square: At t=60 mins 0.001 eq of mercaptoethanol was added. An additional portion was added every 10 mins until at 110 mins 0.5 eq had been added (See Table 2, Section 6.3.3.9). Blue triangle/orange triangle: Size and polydispersity of particles formed between siRNA and linear TetN343 at N/P ratio 10 over time (as Figure 3.7). Data show mean and standard deviation of three measurements.

3. Formation and Characterisation of Particles

At this stage it was considered important to find out whether enough mercaptoethanol was being added and it was in fact possible for reduction to take place. An experiment analogous to the linear TetN343 reduction experiment was carried out using cyclic TetN343. Since this did contain disulfide bonds, the ME was expected to have an effect. As shown in Figure 3.9, particle size begins to increase as soon as ME is added. This demonstrates the amounts and mode of addition of the ME was appropriate to reduce disulfide bonds and that the lack of impact observed on the particles formed with linear TetN343 was probably due to the lack of disulfide bonds in the particle.

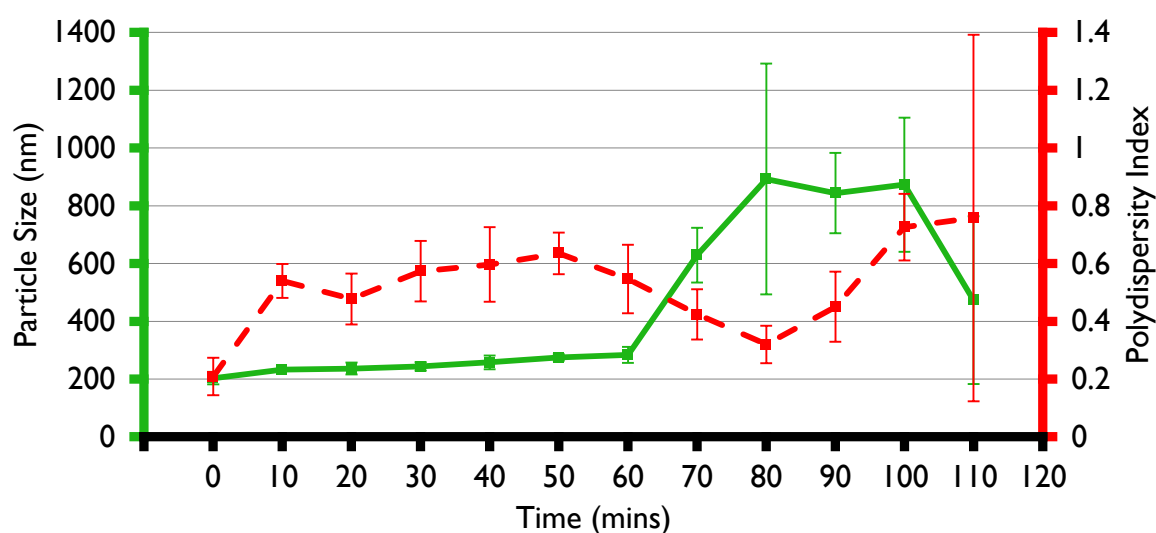


Figure 3.9 – Size and polydispersity of particles formed between siRNA and cyclic TetN343 at N/P ratio 10 over time. At t=60 mins 0.001 eq of mercaptoethanol was added. An additional portion was added every 10 mins until at 110 mins 0.5 eq had been added (See Table 2, Chapter 6). Data show mean and standard deviation of three measurements.

3.3.3. Propidium iodide exclusions assays on particles formed with linear TetN343

The principle disadvantages of using PCS to study the particles are that it provides very little information regarding the stability of the particles formed and that it doesn't describe lack of particle formation very well. Propidium iodide exclusion assays however, can show both of these things. Figure 3.10 shows that whilst cyclic TetN343 was able to encapsulate siRNA at low N/P ratios, linear TetN343 appeared to reach a limit of encapsulation at around 20% of the total

3. Formation and Characterisation of Particles

fluorescence of free siRNA. This suggested that the particles formed with linear TetN343 were not only large but that they also failed to contain the siRNA. This could be because they are diffuse or the siRNA is not bound strongly.

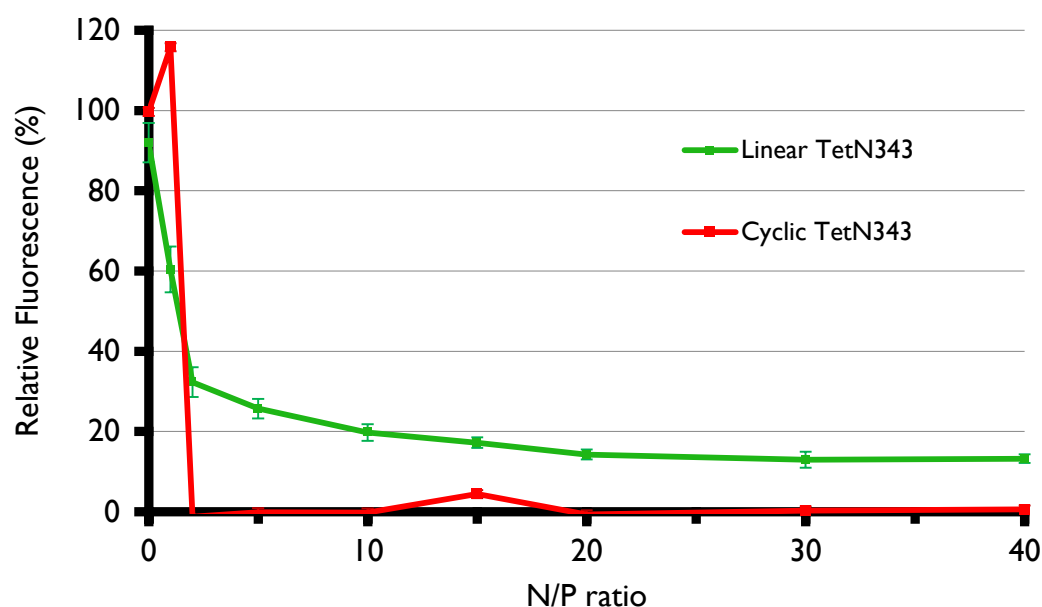


Figure 3.10 – Comparison of the encapsulation efficiency of linear and cyclic TetN343 at a range of N/P ratios as demonstrated by a propidium iodide exclusion assay. Percentage values are calculated relative to the fluorescence observed for siRNA in the presence of no cation. Data show mean and standard deviation of six measurements.

Since PCS suggested growth of the polyplexes formed with linear TetN343 over the course of one hour, it was also decided to investigate the encapsulation efficiency with respect to time. The lack of effect when the particles were reduced also suggested that the mechanism of particle formation was not entirely understood. This was done by mixing TetN343 and siRNA at 10 minute intervals then reading all samples at the same time such that at the time of reading, each polyplex had been forming for different lengths of time. It had been anticipated that as the particles aggregated, the siRNA would become more inaccessible to the propidium iodide. however shows that encapsulation stays constant over the same timeframe that the size increased in the PCS experiment (Figure 3.11). The graph also shows that while the linear material binds to siRNA strongly at even the shortest incubation time, the cyclic requires more than ten minutes to effectively bind the siRNA. This is perhaps indicative of the polymerisation of the cyclic being slower than the process of particle formation undergone by the linear.

3. Formation and Characterisation of Particles

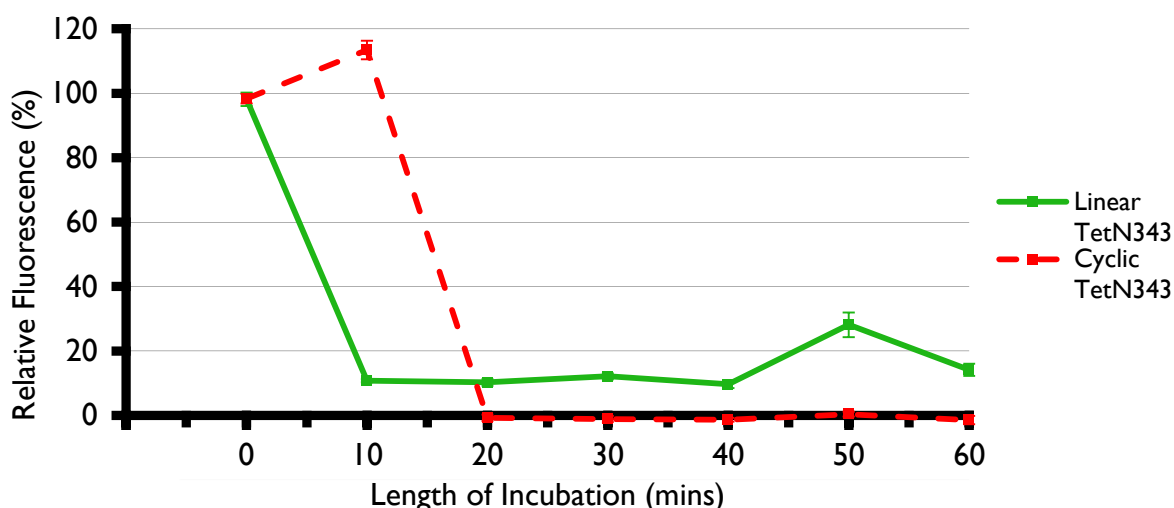


Figure 3.11 – Comparison of encapsulation efficiency of linear and cyclic TetN343 as a function of incubation time as demonstrated by a propidium iodide exclusion assay. Percentage values are calculated relative to the fluorescence observed for siRNA in the presence of no cation. t = 0 indicates no cation added. Data show mean and standard deviation of six measurements.

The lack of full encapsulation was puzzling. An experiment was carried out using combinations of linear and cyclic to see if full encapsulation could be achieved with linear present at all. As shown in Figure 3.12, it could, though a 50:50 mixture was not as effective as might be expected given that it had enough cyclic present (effectively N/P 5) to fully encapsulate.

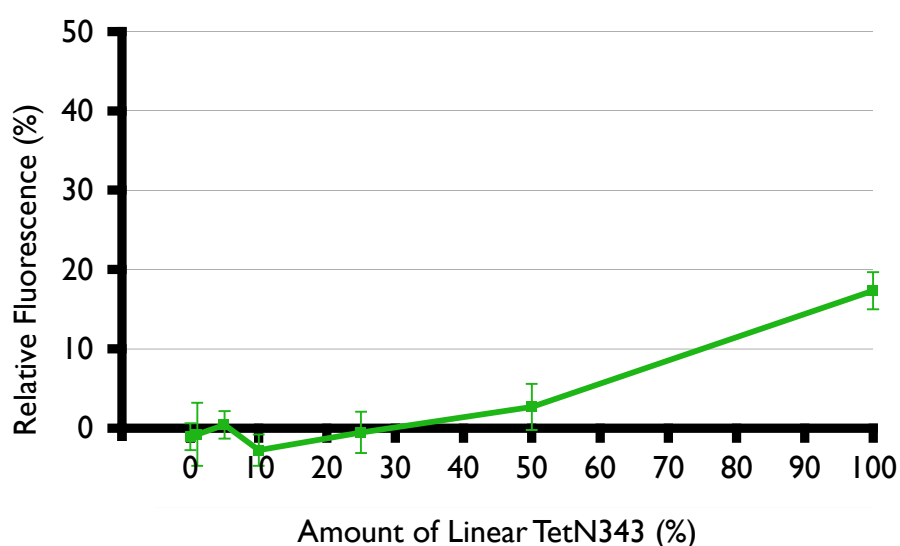


Figure 3.12 – Encapsulation efficiency of combinations of linear and cyclic TetN343 as demonstrated by a propidium iodide exclusion assay. Percentage values are calculated relative to the fluorescence observed for siRNA in the presence of no cation. Data show mean and standard deviation of six measurements.

3. Formation and Characterisation of Particles

This suggested that not only did the linear dithiol form inferior particles to those formed by the cyclic, but that it also had a disruptive effect on the formation of particles by the cyclic disulfide. As shown in linear dithiol could 99% encapsulate siRNA at an N/P ratio of 400 and fully encapsulate at an N/P ratio of 1000 – both values far greater than is typically used in polyplex formation or transfection.

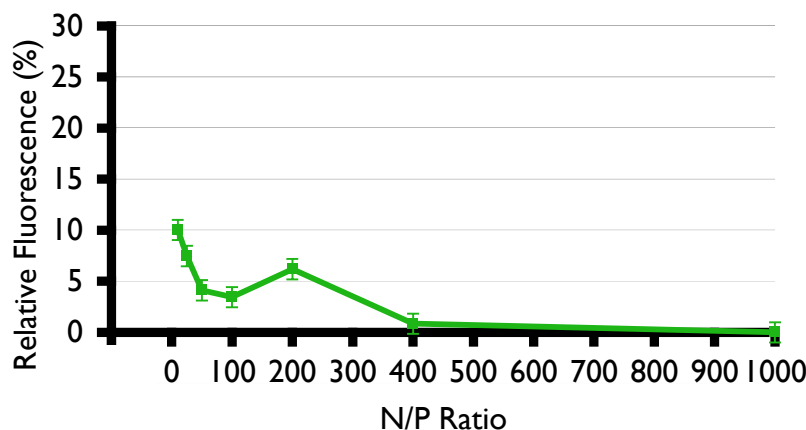


Figure 3.13 – Encapsulation efficiency of linear TetN343 at high N/P ratios as demonstrated by a propidium iodide exclusion assay. Percentage values are calculated relative to the fluorescence observed for siRNA in the presence of no cation. Data show mean and standard deviation of three measurements.

Until this point, it had been assumed that – like the cyclic disulfide, though possibly with a different mechanism – the linear dithiol template-polymerised to form a high molecular weight polycation. However, the final two encapsulation experiments seem to dispute this. The encapsulation data – particularly at high N/P ratios – show no behaviour that wouldn't be expected from any material that could compete with propidium iodide for binding. Upon adding PrI to the mixture of dithiol and siRNA, some of the siRNA remains bound to cation and some of the cation is competed away by the dye. This also explains the disruptive effect observed in Figure 3.12. The cyclic and linear will have similar binding affinities due to their shared spermine motif (indeed, the linear may be a better binder, see Figure 3.16) and should bind in similar proportions. However, if only adjacent cyclic molecules rearrange to form higher M_w species it can be envisaged that any dithiol present in the helix will interrupt this. Given the higher binding affinity of the more multivalent oligomers/polymers these will not be expected to be displaced; the monomeric dithiols on the other hand would be. This process is represented in Figure 3.14.

3. Formation and Characterisation of Particles

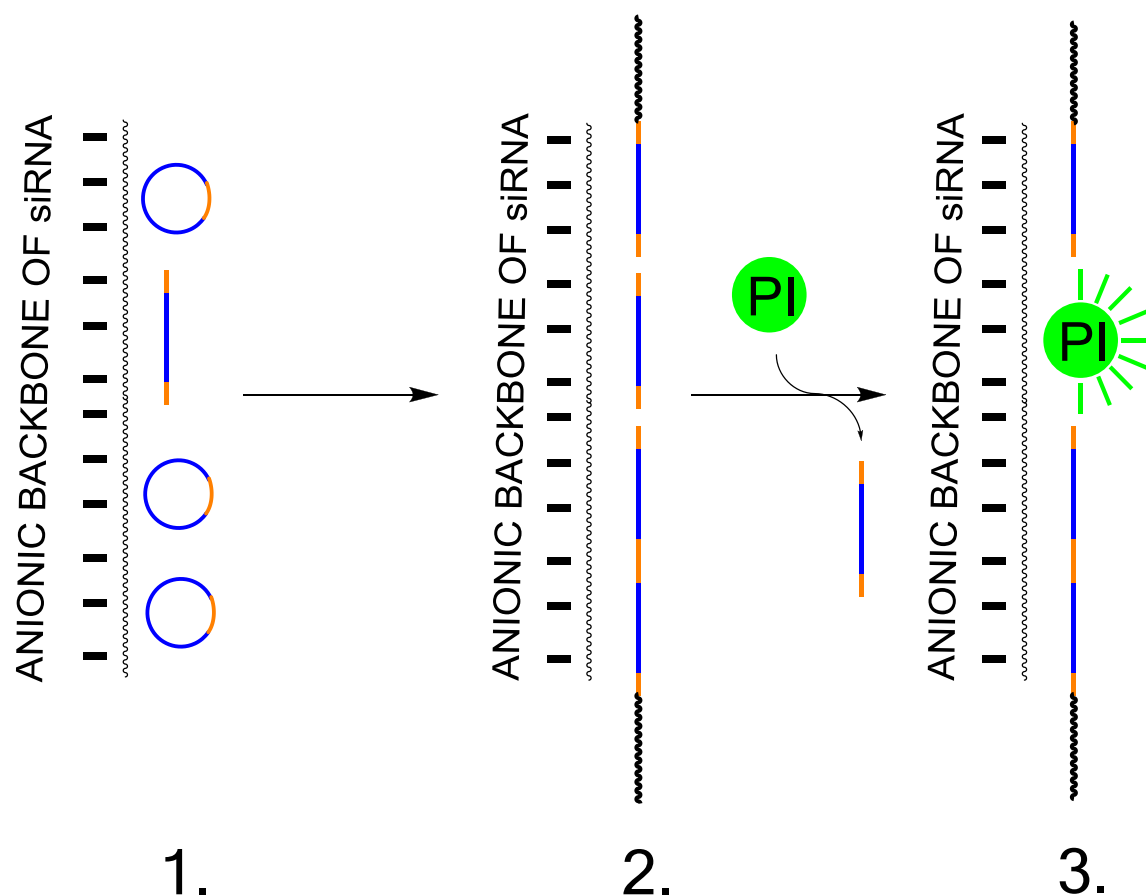


Figure 3.14 – Scheme showing the disruption of polymerisation by linear TetN343 through its inability to polymerise and subsequent displacement from the helix of siRNA. 1) All polycations bind to siRNA backbone. 2) Adjacent cyclic disulfides rearrange to form higher MW species. 3) Dithiols are not part of a higher MW species and are displaced by propidium iodide causing fluorescence to be observed.

For further proof of the non-polymerisation of the linear dithiol, an encapsulation assay was carried out using spermine hydrochloride. If the linear dithiol was not polymerising, its encapsulation behaviour should mirror the unpolymerisable spermine. As shown in Figure 3.15, spermine behaved in a manner reminiscent of the linear TetN343 suggesting that the thiol groups were not taking part in the formation of complexes between linear TetN343 and siRNA.

3. Formation and Characterisation of Particles

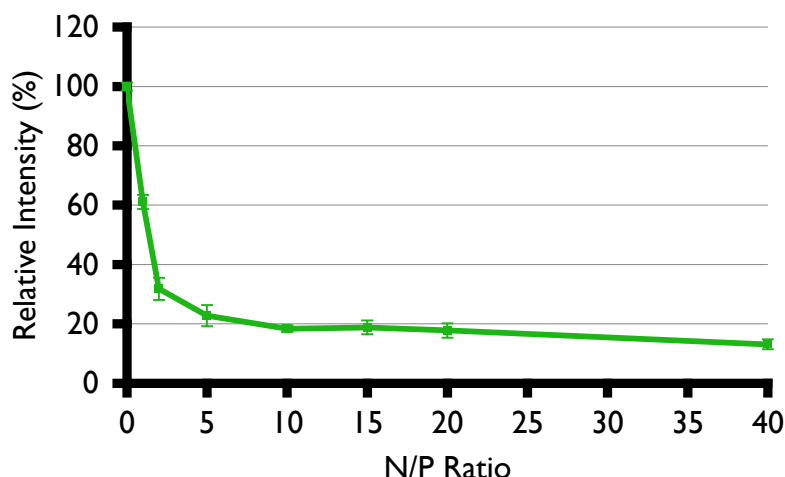


Figure 3.15 – Encapsulation efficiency of spermine tetrahydrochloride at a range of N/P ratios as demonstrated by a propidium iodide exclusion assay. Percentage values are calculated relative to the fluorescence observed for siRNA in the presence of no cation. Data show mean and standard deviation of three measurements.

3.3.4. Consideration of the Polymerisation Mechanism of Cyclic Disulfides

The current theory to explain the polymerisation of the linear and cyclic TetN343 (See Section 1.11) is insufficient to explain why no polymerisation would take place with linear TetN343 in the presence of siRNA. The shorter length of siRNA means that the template for polymerisation is also shorter (only 3 - 4 dithiols could be accommodated in the helix compared to hundreds in pDNA). While this could explain a lack of all polymerisation, it does not appear to prevent polymerisation of the cyclic disulfides. This suggests that there may be a driving force behind polymerisation that is unique to the cyclic.

Ring strain in a single, isolated cyclic disulfide is not likely to be very high given that it contains 40 atoms and indeed, forms in the first place. However, the spermine motif of the cyclic disulfide would appear to be unable to fully bind electrostatically to the siRNA backbone due to the restricted conformations it can assume (See Figure 3.16). In this instance, forming a linear polymer chain would allow stronger binding. Linear TetN343 is already fully capable of assuming the most favourable conformation to bind and would therefore lack this incentive to polymerise.

3. Formation and Characterisation of Particles

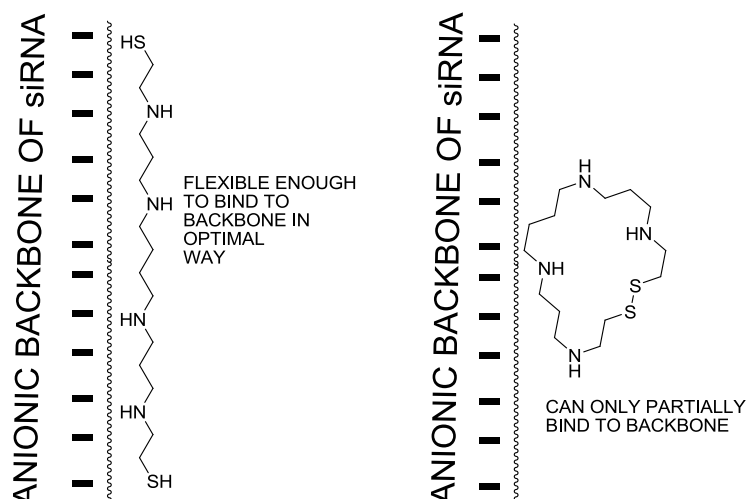


Figure 3.16 – Diagram showing the potential for the disulfide bond to restrict the binding ability of the spermine motif in the cyclic version of TetN343.

Of the original cyclic disulfides produced in the earlier work (Figure 1.33),¹²⁰ only one of them would not gain any additional binding by ring-opening: the ethylene diamine based cyclic DiN2 (Figure 3.17). This material had previously shown poor pDNA particle formation (3154 nm at N/P 12.5) which, considering that TriN23 – which differed only by three methylene groups and an amine – formed small particles (128 nm at N/P 12.5) suggested the effect might not entirely be due to the amine number and spacing.

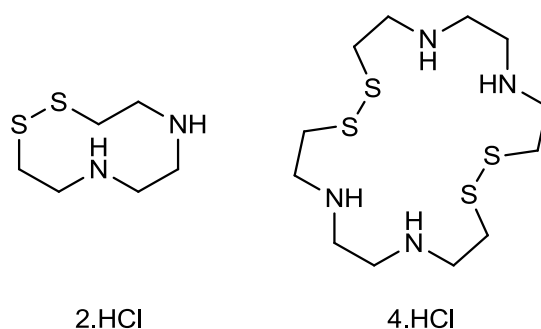


Figure 3.17 - Ethylene diamine based cyclic DiN2 and the dimeric byproduct that is believed to have been formed.

Unfortunately, attempts to prepare the material (Section 6.2.30 - 6.2.33) led to formation of a significant quantity (46% by NMR) of what was believed to be the dimer despite also attempting the cyclisation at more than an order of magnitude lower concentration. Since the dimer would be expected to gain binding strength

3. Formation and Characterisation of Particles

through ring-opening it was not considered worthwhile testing the mixture. Indeed, if it does become possible to prepare a sample of the monocyclic species it would allow a very interesting experiment to take place: namely, comparison of the relative binding strengths of the monocyclic and the dicyclic mixture. Were the dimer mixture to form more stable, smaller particles it would provide compelling evidence for the driving force of the ring-opening. Without this, it could be argued that the large polyplexes formed by DiN2 in the previous work was simply due to it containing fewer amine groups.

An NMR experiment was carried out to investigate the oxidation of the linear TetN343 at pH 7 without template. Aside from the lack of siRNA there were two main differences between the NMR experiment and the encapsulation experiments. Firstly, concentration was around one-hundred times greater due to the requirements of NMR. In principle, this would increase the amount of polymerisation. Second, instead of aqueous buffer the experiment was carried out in D₂O with pH controlled with DCl and NaOD. Despite these differences, the experiment would give some idea as to the propensity of the thiols to oxidise. As shown in Figure 3.18, no change was observed over the initial 136 minutes and only minimal decrease in the CH₂SH triplet ($\delta = 2.7$ ppm) was observed after 6 days. The extra complexity in the spectrum is believed to be due to pH change since as can be seen from the spectra at the top, the region $\delta = 1.5 - 2.0$ ppm is the same in both linear and cyclic. This experiment showed that oxidation is not favoured at pH 7 and is unlikely to take place in timeframes relevant to the particle sizing experiments.

3. Formation and Characterisation of Particles

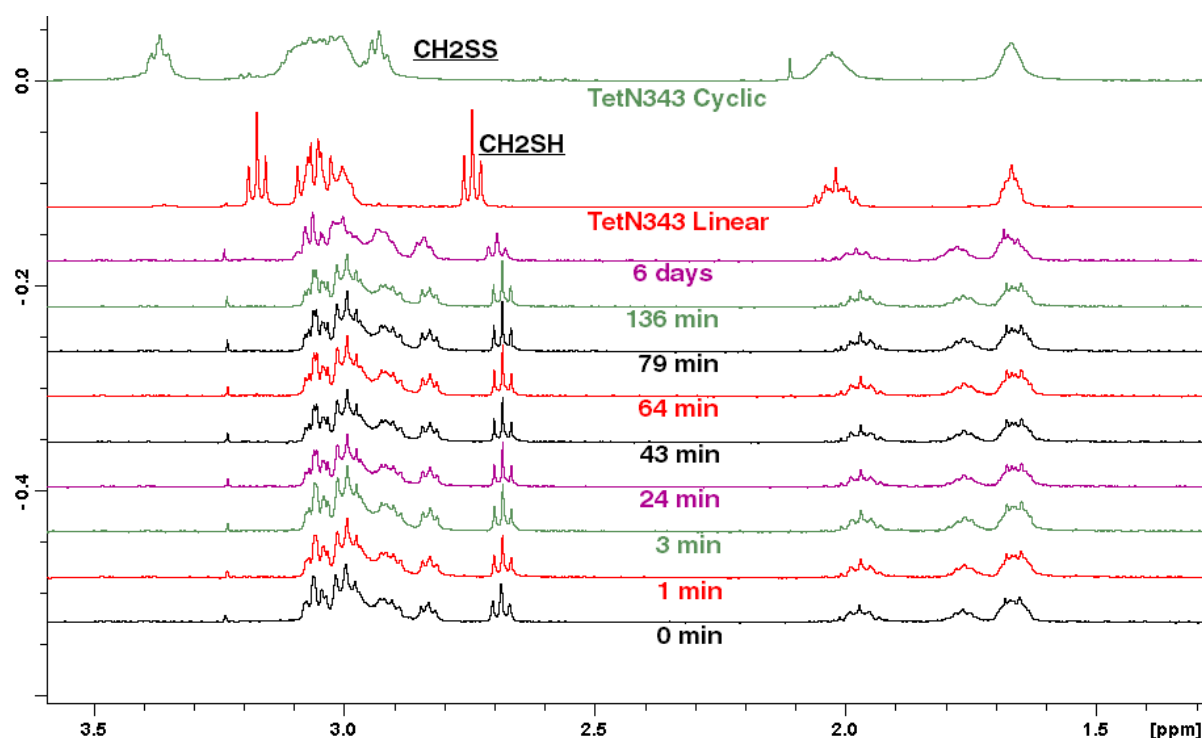


Figure 3.18 – NMR spectra of cyclic TetN343, linear TetN343 (both in D₂O) and linear TetN343 in pH7 D₂O at various points in time.

It was thought that perhaps the likelihood of polymerisation of the linear material could be increased by raising the pH of the buffer solution (so far, pH 7.0 has been used). Increased polymerisation would be expected to result in a stronger binding to the siRNA as with the cyclic. However, in a propidium iodide encapsulation assay the opposite effect was observed as shown in Figure 3.19. This is most probably because the increase in pH also serves to reduce the protonation level of the amine resulting in weaker competition with the propidium iodide dye. This would also explain why the sample at pH 6.3 gave the lowest fluorescence. For this reason, it was decided that pH control would not be appropriate for encouraging polymerisation. It was considered that having pH high initially to encourage polymerisation followed by a pH drop to increase binding would be useful. However, since the mechanism of polyplex formation is believed to rely on templating it was not considered prudent to reduce the binding ability of the cation.

3. Formation and Characterisation of Particles

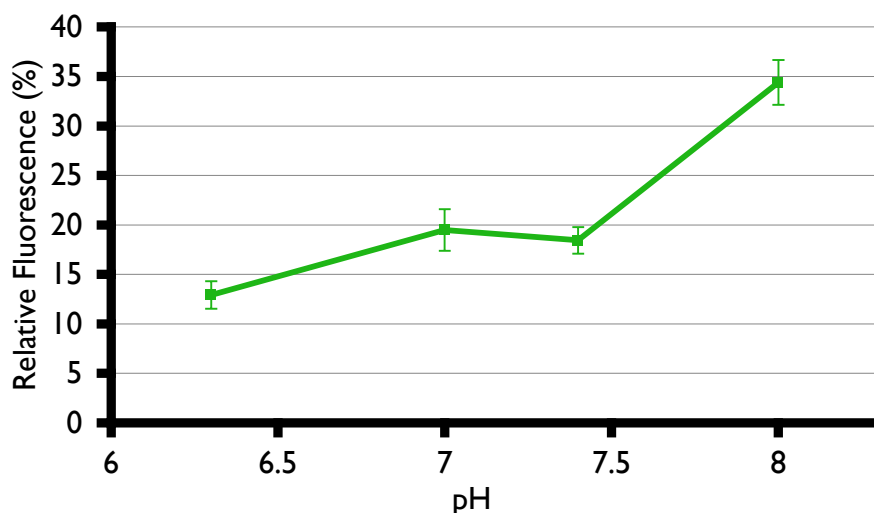


Figure 3.19 – Encapsulation efficiency of linear TetN343 at N/P 10 at a range of pH values as demonstrated by a propidium iodide exclusion assay. Percentage values are calculated relative to the fluorescence observed for siRNA in the presence of no cation. Data show mean and standard deviation of six measurements of three repeats.

For comparison, the same experiment was carried out using the cyclic TetN343 (Full encapsulation was observed at all pH values which considering the particle sizing experiment shown in Figure 3.6 is an interesting result. The large particles formed at pH 6.3 were initially attributed to lack of particle formation due to lack of thiolates to act as initiators. If this was indeed the case, one would expect the encapsulation efficiency of the unpolymerised cyclic to be similar to – if not worse than – the encapsulation efficiency of linear dithiol. The total encapsulation indicated by this data suggests that the particles formed at pH 6.3 also consist of polymerised cyclic and that lack of polymerisation is not sufficient to explain the large particles observed previously. The pH 8 data-point is also interesting because it shows that even at a pH where the linear dithiol can only encapsulate 65% of the siRNA the cyclic disulfide is able to achieve full encapsulation. This further underlines the difference in the mechanism of particle formation of these two materials.

3. Formation and Characterisation of Particles

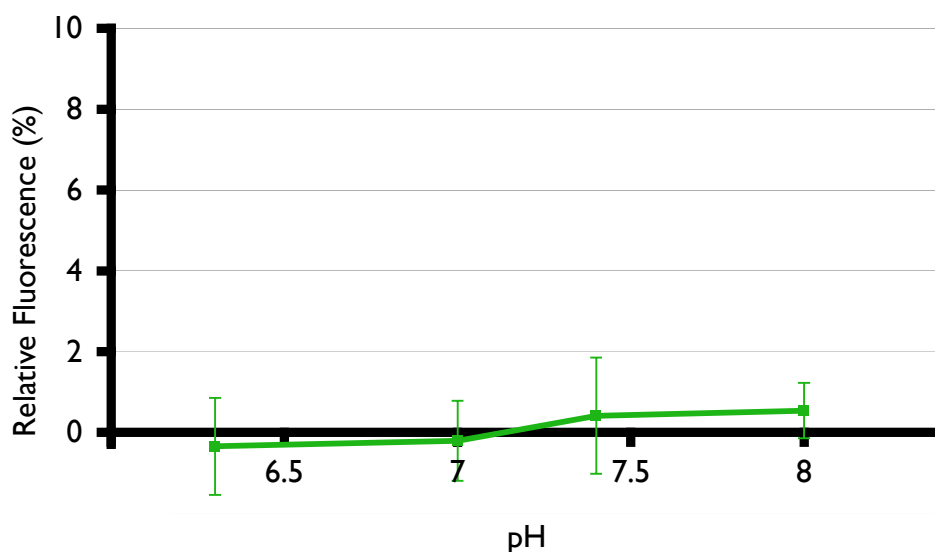


Figure 3.20 – Encapsulation efficiency of cyclic TetN343 at N/P 10 at a range of pH values as demonstrated by a propidium iodide exclusion assay. Percentage values are calculated relative to the fluorescence observed for siRNA in the presence of no cation. Data show mean and standard deviation of three measurements of three repeats.

3.3.5. Comparison with the pDNA encapsulation efficiency of linear TetN343

So far it was unknown whether the non-polymerisation of the linear dithiol was only an issue for siRNA or whether it was also the case for pDNA. One possible reason for the non-polymerisation was that siRNA didn't provide a sufficiently long template – only four dithiols could fit along the groove of an siRNA helix – for any useful polymer to form. A simple propidium iodide experiment was therefore tried with pDNA (Figure 3.21) and can be considered a direct analogue of the graph shown in Figure 3.10. The graph shows that the linear is not capable of fully encapsulating pDNA either. The overall encapsulation is higher than for siRNA which plateaued at around 80% encapsulated at N/P 40. At first glance, this would suggest that binding is stronger with pDNA suggesting polymerisation is more facile. This could however be due to differences in binding affinity as interactions between the dye and siRNA/pDNA are likely to be different. The datapoint for the cyclic at N/P 5 was unexpected. The previous work showed via gel electrophoresis that polyplex formation was stable by this N/P ratio so the lack of full encapsulation is more likely an experimental error.

3. Formation and Characterisation of Particles

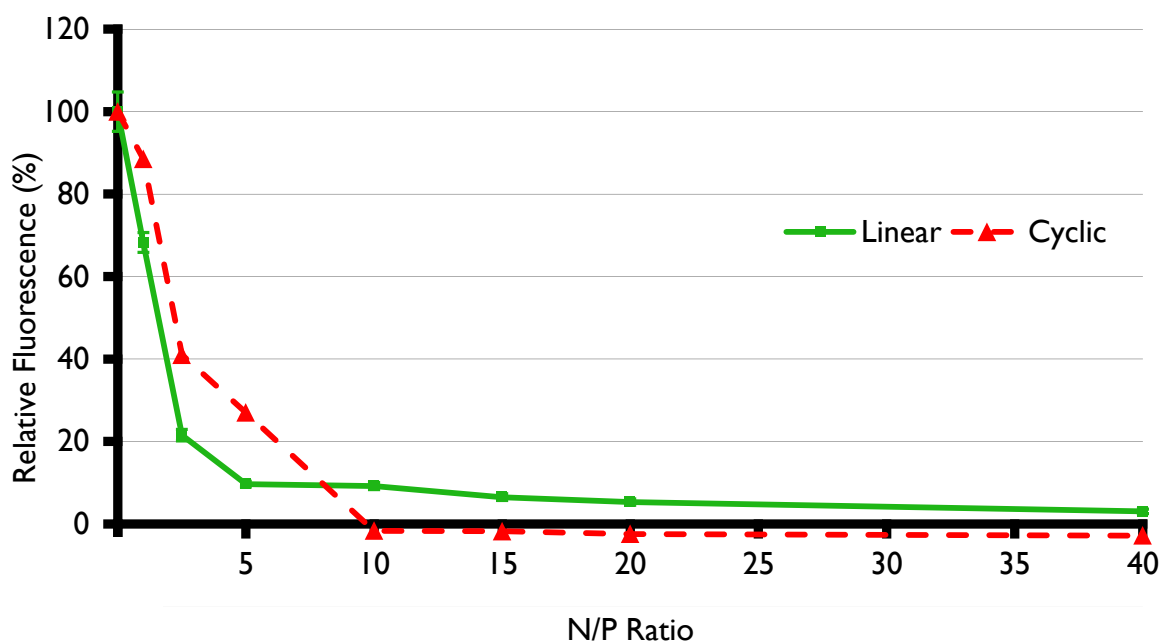


Figure 3.21 – pDNA encapsulation efficiency of cyclic or linear TetN₃₄₃ at a range of N/P ratios as demonstrated by a propidium iodide exclusion assay. Percentage values are calculated relative to the fluorescence observed for pDNA in the presence of no cation. Data show mean and standard deviation of six measurements.

3.4. Formation of Particles Using Branched Materials

3.4.1. Anticipated problems regarding the use of trithiol

The apparent difficulty in encapsulation of siRNA when dithiol was used in conjunction with disulfide suggested that using a tren-based trithiol in conjunction with the cyclic disulfide would also be problematic. A series of experiments similar to the ones described in Section 3.3.3 were conducted. In all discussion of data relating to combinations of spermine and tren monomers, the percentage represents the percentage of the total protonable nitrogens from the branched trithiol.

3. Formation and Characterisation of Particles

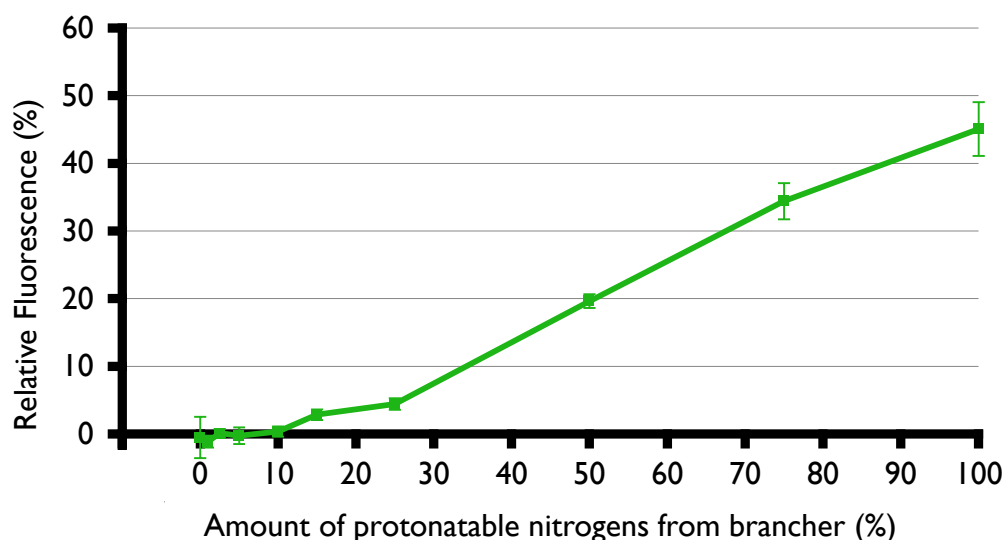


Figure 3.22 – Encapsulation efficiency of different combinations of cyclic TetN343 and branched trithiol at N/P 10 as demonstrated by a propidium iodide exclusion assay. Percentage values are calculated relative to the fluorescence observed for siRNA in the presence of no cation. Data show mean and standard deviation of six measurements.

As would be expected – considering the results with the linear materials – increasing the proportion of trithiol led to a decrease in encapsulation efficiency once more than 10% of the protonatable amines were being contributed by the trithiol (Figure 3.22). Due to the lower binding affinity of the trithiol, the disruptive effect is also more pronounced than for the dithiol. This is reflected in both the lower proportion of trithiol that can be used before incomplete encapsulation is observed (10 vs 25% for linear dithiol) and the less efficient encapsulation of brancher on its own (40 vs. 20% encapsulation using linear dithiol). The data also show that the binding strength of the trithiol to siRNA is sufficient to disrupt encapsulation by the cyclic since if no binding by the trithiol was taking place, one would expect full encapsulation at 50 and 75 % trithiol based purely on the encapsulation efficiency of the cyclic by itself at N/P 5 and 2.5. It is assumed that a similar disruptive effect to that seen with the linear/cyclic combinations could be taking place where the branched trithiol reversibly binds to some siRNA and is later displaced by the dye (See Figure 3.14). To confirm this it was decided to see if the presence of branched trithiol would disrupt full encapsulation of siRNA with TetN343. The encapsulation of TetN343 at different N/P ratios was compared to the encapsulation of TetN343 at the same N/P ratios in solutions that also contained an equivalent N/P ratio of branched trithiol (ie. the N/P 10 sample contained total N/P 20; 10 each from TetN343 and branched trithiol).

3. Formation and Characterisation of Particles

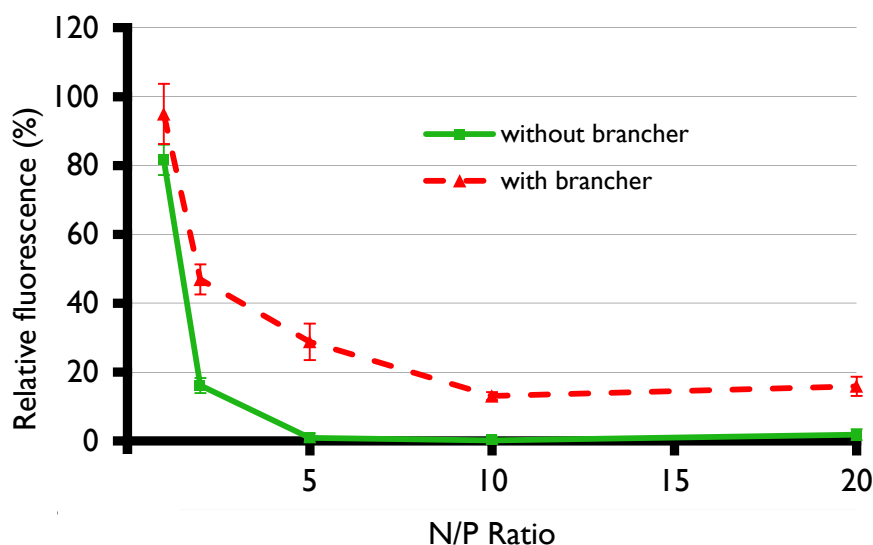


Figure 3.23 – Encapsulation efficiency of cyclic TetN343 at a range of N/P ratios in the absence (green) and presence (red) of an equivalent amount of branched trithiol. Percentage values are calculated relative to the fluorescence observed for siRNA in the presence of no cation. Data show mean and standard deviation of six measurements.

As shown in Figure 3.23, full encapsulation by cyclic TetN343 is possible at N/P 5 and higher. However, this is not the case in the presence of branched trithiol. This proves that the branched trithiol binds, doesn't polymerise and is subsequently displaced by the propidium iodide.

An experiment into the effect of pH was carried out to see if any polymerisation could be encouraged (similar to the experiments discussed in Section 3.3.4, Figure 3.19). The experiment was carried out at 50% branched trihiol since this offered the possibility of change both upwards and downwards. A slight upward trend was observed in fluorescence as for the dithiol experiment. As before, if polymerisation is being encouraged by higher pH, it is inobservable do to the reduced binding of the amines caused by the reduced protonation of the amines.

3. Formation and Characterisation of Particles

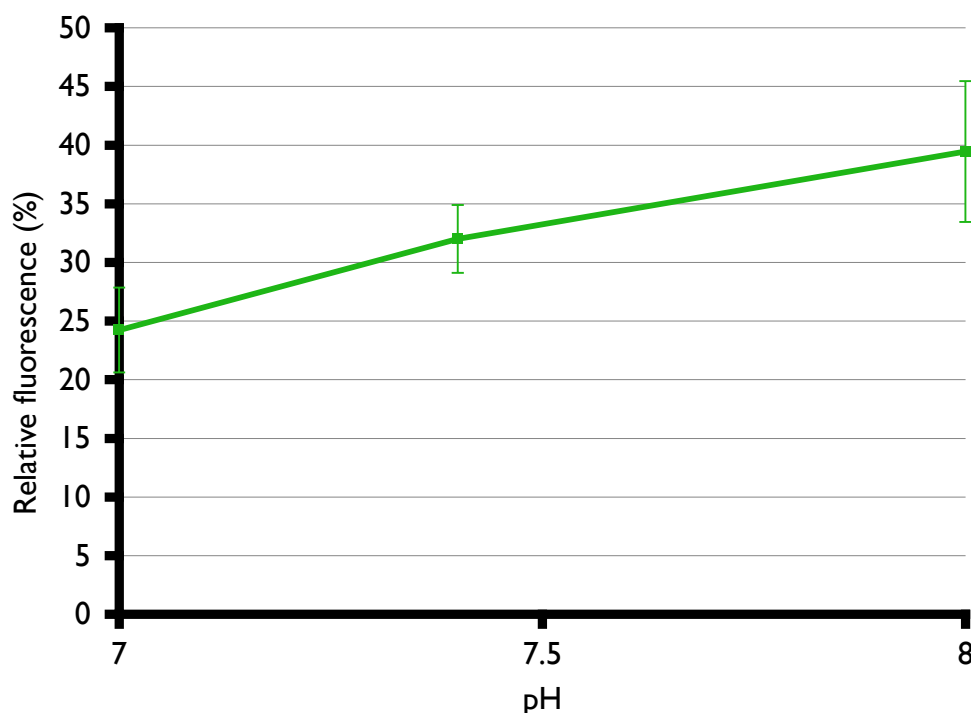


Figure 3.24 – Encapsulation efficiency of a 50% combination of cyclic TetN343 and branched trithiol at N/P 10 at a range of pH values as demonstrated by a propidium iodide exclusion assay. Percentage values are calculated relative to the fluorescence observed for siRNA in the presence of no cation. Data show mean and standard deviation of three measurements of three repeats.

3.4.2. Photon correlation spectroscopy of particles formed using branched trithiol

The encapsulation assays carried out using combinations of trithiol and cyclic disulfide demonstrated that the maximum amount of trithiol that could be used while still obtaining full encapsulation was 10%. PCS measurements were therefore carried out on particles formed using 0-10% brancher and showed particle size increasing and polydispersity decreasing as the proportion of brancher was raised (Figure 3.25). The polydispersity change is the less interesting due to the abnormally high polydispersity observed for the particles formed using only cyclic disulfide. Indeed, the value only decreases to the same value as was typically seen whenever this formulation had been prepared before. The increase in size as the proportion of trithiol is increased is beyond doubt though and does suggest that the trithiol is substantially altering the particle formation process.

3. Formation and Characterisation of Particles

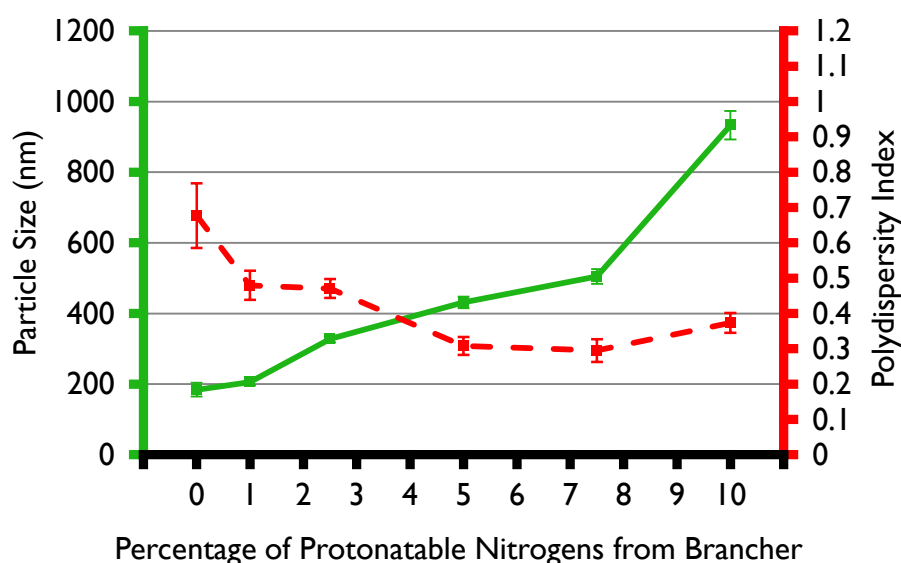


Figure 3.25 – Particle size using TetN343 and a percentage of brancher (calculated that a certain percentage of protonatable N's come from brancher). Carried out using 1.5 mM siRNA solutions at N/P 10. Freshly prepared brancher solutions added to TetN343 and buffer. Cation solutions were then added to siRNA solution, vortexed for 5s and left for 1 hr at RT. Data show mean and standard deviation of three measurements.

The use of two different types of cations with the potential to interact raised the question of how the two components were to be mixed. For the experiment shown in Figure 3.25, the cyclic TetN343 was dissolved in buffer (in the manner used for the standard single component system) followed by the addition of the brancher immediately before mixing with the siRNA. It was thought that perhaps the concentration of the two types of cations was too low for them to interact prior to the addition of siRNA so the formulation was altered slightly. In the modified procedure, fifteen minutes of incubation time was allowed between the two cations as a mixture of 1 mgml⁻¹ solutions before dilution in buffer. As shown in Figure 3.26, the trends observed were broadly similar (i.e. increase in size from ~200 to ~900 nm) though an increase in particle size is also observed at 1% trithiol; in the previous experiment, 1% had affected almost no change at all. A possible explanation for this is that when incubated together at high concentration there is a strong chance that a disulfide exchange between a molecule of trithiol and a molecule of cyclic disulfide could take place and ring open a portion of the cyclic disulfides (Figure 3.27) thus removing the possible driving force discussed in Section 3.3.4.

3. Formation and Characterisation of Particles

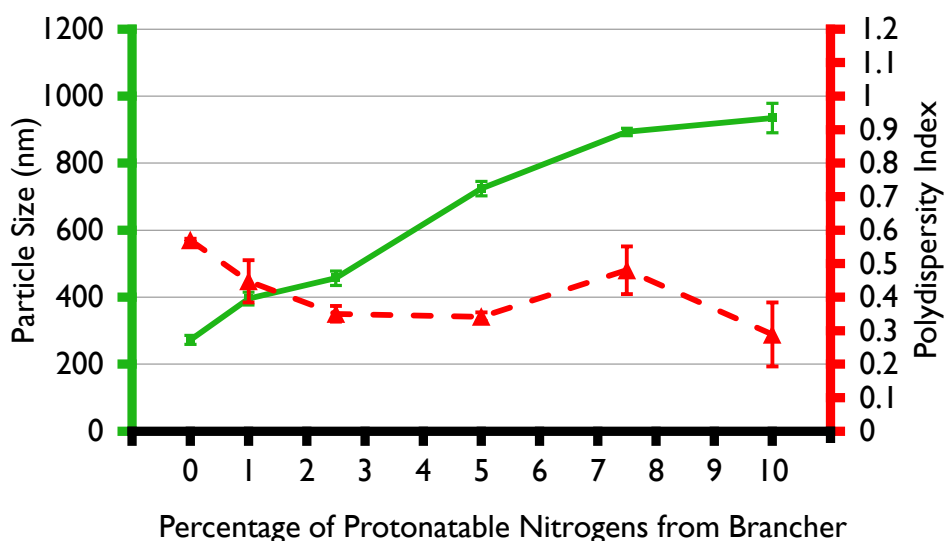


Figure 3.26 – Particle size using TetN343 and a percentage of brancher (calculated that a certain percentage of protonatable N's come from brancher). Carried out using 1.5 mM siRNA solutions at N/P 10. Freshly prepared brancher solutions added to TetN343 solutions, incubated for 15 minutes and then added to siRNA solution, vortexed for 5s and left for 1 hr at RT. Data show mean and standard deviation of three measurements.

Overall, the fact that when using both methods particle size increased suggested that the brancher was definitely having an impact on polyplex formation. The cause of this effect could be attributed to many things: the reaction of thiols with disulfides, the presence of cationic moieties that interact with the siRNA but don't polymerise, different buffering behaviour of the amines or simply a change in the concentration of the cyclic (though in N/P terms the change is small – even with 10% of protonatable nitrogens coming from the branched trithiol, the effective N/P ratio of the cyclic is 9).

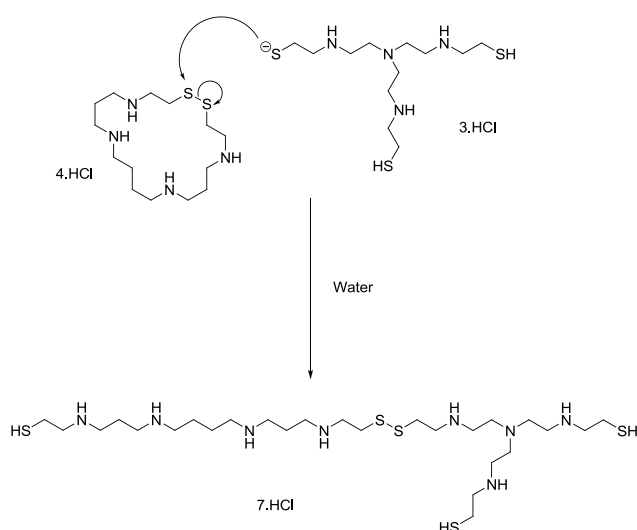


Figure 3.27 – Ring-opening of cyclic by trithiol. The resulting polyamine would not be expected to polymerise for the reasons give in Section 3.3.4

3. Formation and Characterisation of Particles

Many of these possibilities could be ruled out by preparing particles using tren (tris(2-aminoethyl)amine) trihydrochloride in place of brancher; if the same pattern of particle size increase was not observed when using simple tren, it would be reasonable to conclude that it was due to an impact on the polymerisation. Figure 3.28 shows the results obtained from just such an experiment alongside the data from Figure 3.25. Particle size does increase as the proportion of tren is increased but it is not the dramatic increase observed for the trithiol. It is also worth noting that the particles started off at the upper limits of particle size typically observed for the cyclic disulfide (~250 nm). The slight increase can be explained by considering the interference that would be caused by having non-polymerising cations in the sample (see Figure 3.14). This experiment strongly suggests that the increase in particle size after introducing the trithiol is due to the thiols, not the amine.

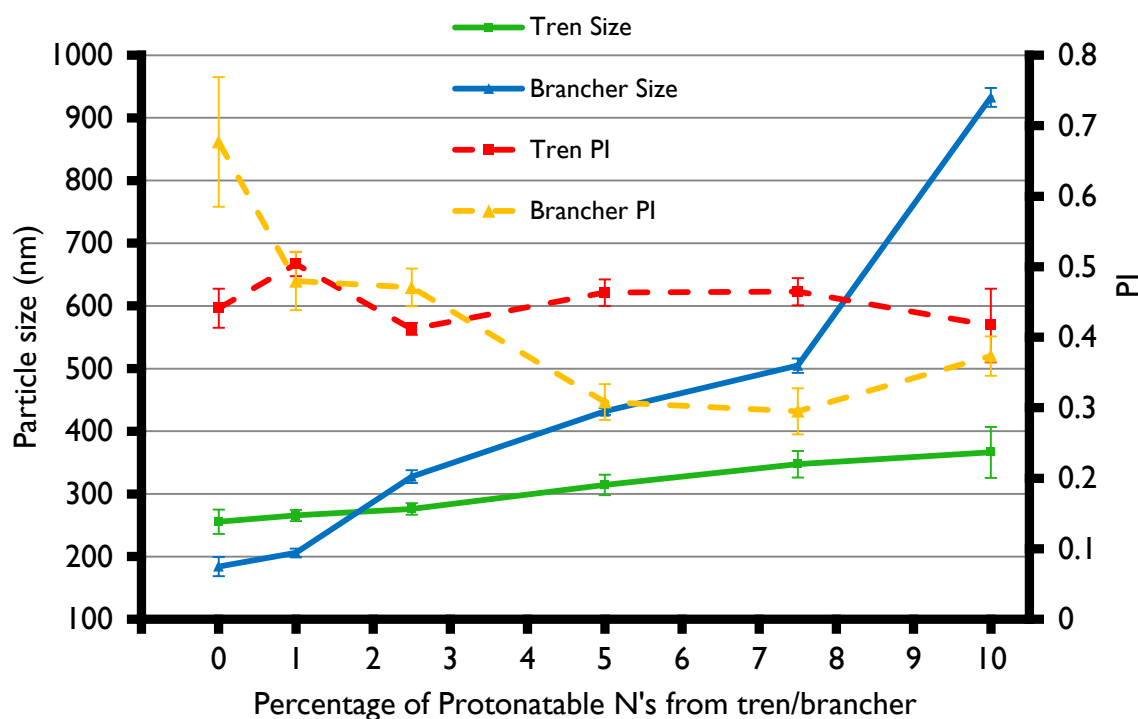


Figure 3.28 – Particle size using TetN343 and a percentage of tren (tris(2-aminoethyl)amine trihydrochloride) (calculated that a certain percentage of protonatable N's come from tren) . Carried out using 1.5 mM siRNA solutions at N/P 10. Freshly prepared tren solutions added to TetN343 (both in water) After 15 mins, pH 7 buffer was added. Data using brancher (from Figure 1) are shown for comparison. Data show mean and standard deviation of three measurements.

3. Formation and Characterisation of Particles

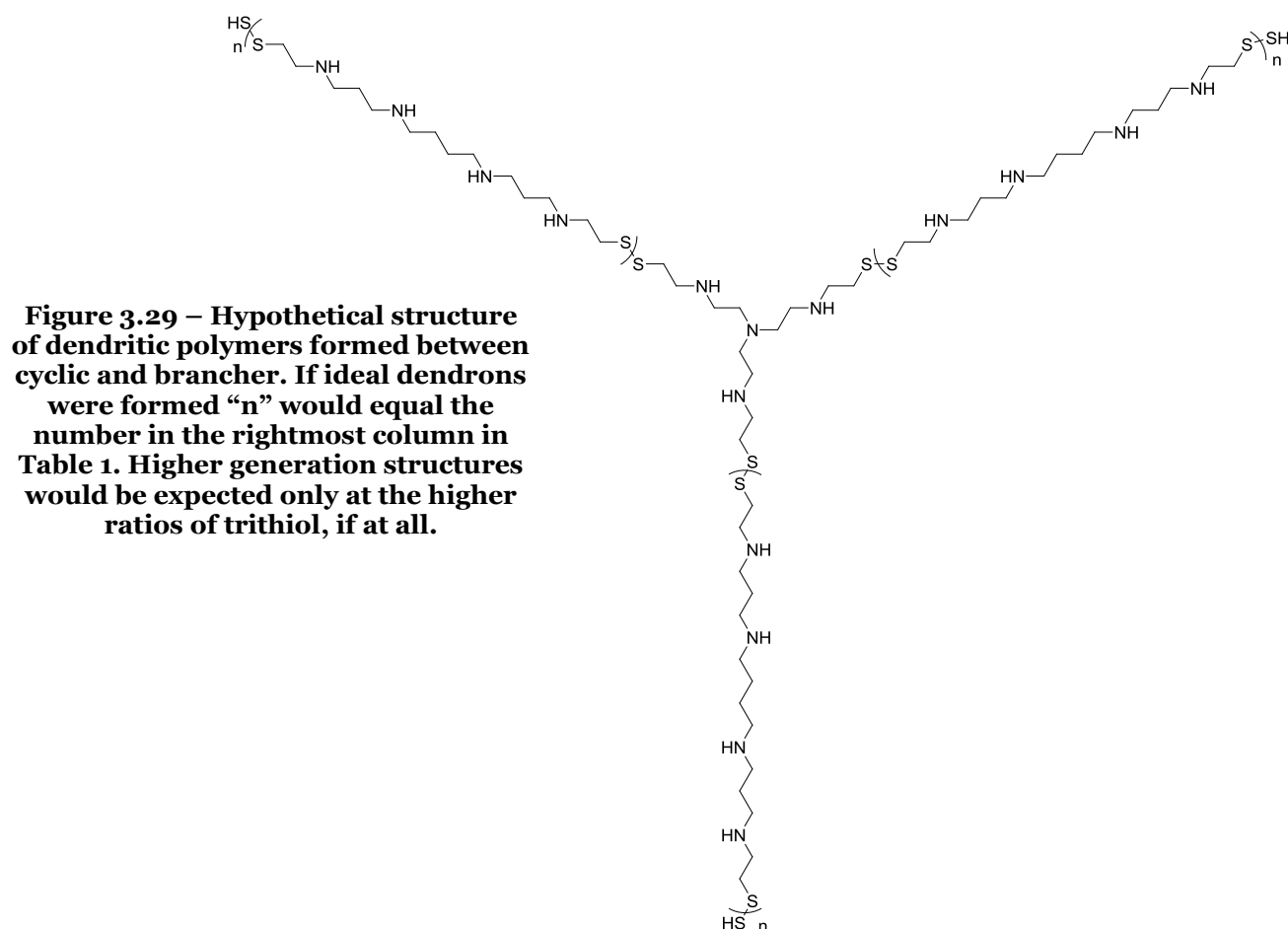
Despite the lack of a method of characterising the polymers away from the siRNA, it is possible to consider the type of structures that may be formed when trithiol molecules are introduced. To do this the molar ratios of the branched trithiol and cyclic disulfide become important. These can be seen in Table 1 which also shows the expected molecular weight per “arm” assuming a perfect 1G dendrimer is formed using one molecule of trithiol as the core. For this discussion it is assumed that all polymerisation is initiated by a trithiol given that they would be expected to be the most numerous thiol bearing species.

% of N from branched trithiol	% Branched trithiol (mol/mol)	Moles of cyclic disulfide per moles of branched trithiol	MW of cyclic per moles of branched trithiol (Da)	MW of cyclic disulfide per “arm” (Da)	Repeat units of cyclic disulfide per “arm”
0	0	N/A	N/A	N/A	N/A
1	1.35	74.25	34630.2	11543.4	24.75
2.5	3.42	29.25	13642.2	4547.4	9.75
5	7.02	14.25	6646.2	2215.4	4.75
7.5	10.81	9.25	4314.2	1438.1	3.08
10	14.81	6.75	3148.2	1049.4	2.25

Table 1 – A table showing molar ratios and derived data for hypothetical polymers formed between branched trithiol and cyclic disulfide.

As can be seen, 1% of protonable nitrogens from the brancher results in there being 74.25 moles of cyclic disulfide for each mole of branched trithiol. This accounts for 34.6 kDa of disulfide polymer possible per branching unit. Figure 3.29 shows an idealised example of the structure one would expect. It could be hypothesised that the introduction of thiol-bearing branching points into the system would introduce more order into the polymerisation mechanism with the thiols acting as the initiators. This would perhaps in turn lead to lower polydispersity. This increase in order could also explain increased particle size as it would also possibly restrict the system from reaching equilibrium.

3. Formation and Characterisation of Particles



One other experiment carried out using this system was a time course experiment observing 1% brancher, N/P 10 particles over 110 minutes (Figure 3.30). It was hypothesised that more time could allow more interaction between the thiols and the disulfides. As can be seen in the graph, particle size increased steadily in a manner reminiscent of the experiments using linear dithiol (Figure 3.7).

3. Formation and Characterisation of Particles

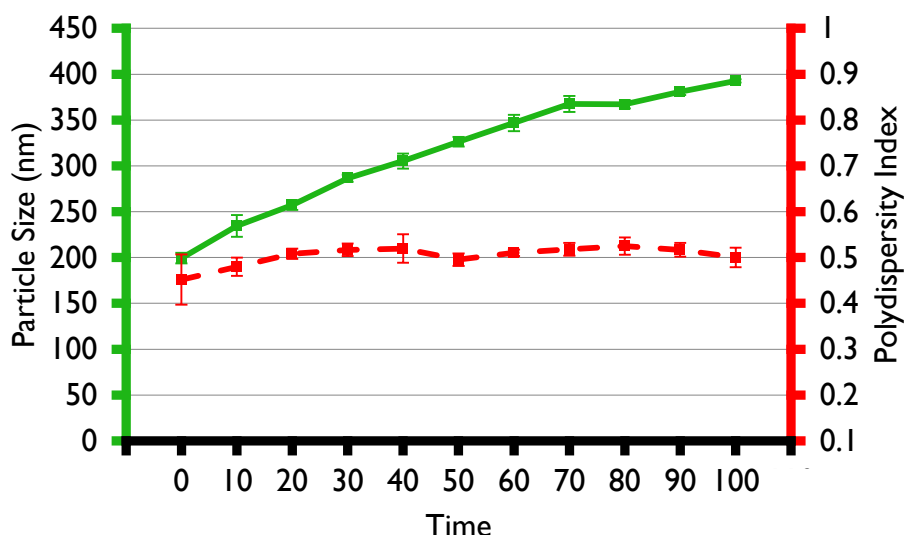


Figure 3.30 – Particle size at over 110 minutes using TetN343 and 1 % of brancher (calculated that 1 % of protonatable N's come from brancher). Carried out using 1.5 mM siRNA solutions at N/P 10. Freshly prepared brancher solutions added to TetN343 and buffer. Cation solution then added to siRNA solution, vortexed for 5s and then measured three times every ten minutes. Data show mean and standard deviation of three measurements.

3.5. Formation and Testing of a Disulfide-Containing, Branched Amine

3.5.1. Propidium iodide exclusion assays

The encapsulation data obtained so far had suggested that thiols did not oxidise to form disulfide polymers in the way envisaged when the work began. It was therefore decided to also investigate branched disulfides of the type shown in Figure 3.31. The preparation of these materials is discussed in depth in Section 2.7.

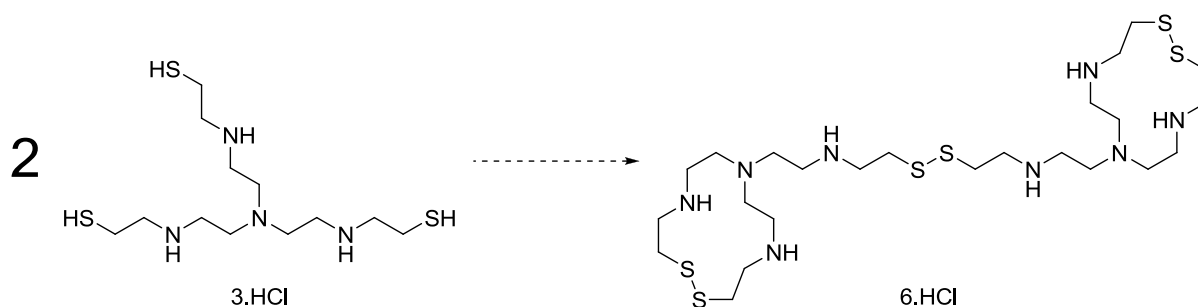


Figure 3.31 – Possible route to a “double cyclic” formed *via* the oxidation of two molecules of trithiol.

3. Formation and Characterisation of Particles

The encapsulation efficiencies of a solution of trithiol that had been left in aqueous solution for one week and one that had been freshly prepared (initially unoxidised but assumed to oxidise a small amount during mixing, sample preparation, etc) were compared. It was assumed that by leaving the trithiol in water it would slowly oxidise and that, at a low dilution of 1 mgml^{-1} there would be limited polymerisation. The oxidised trithiol was found to encapsulate more effectively than the non-oxidised material as shown in Figure 3.32.

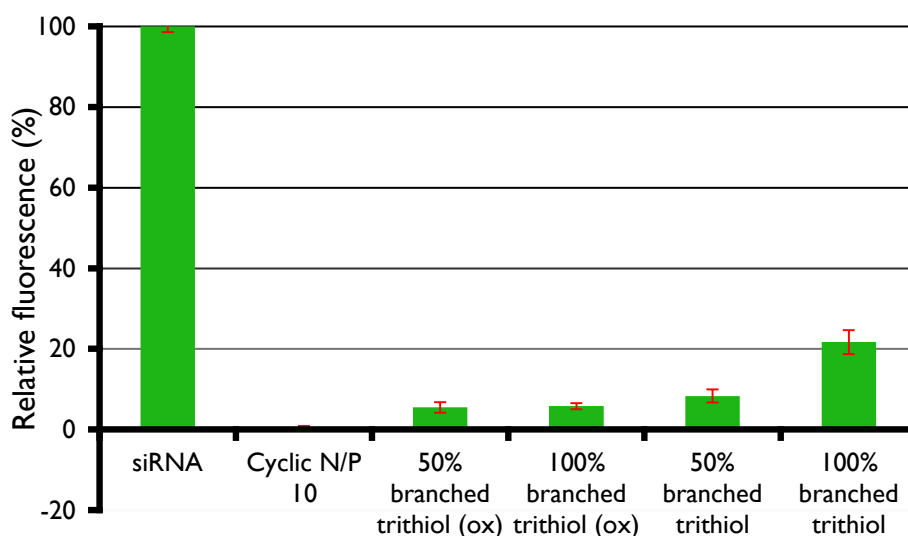


Figure 3.32 – Comparison of the encapsulation efficiency of fresh and partially (70%) oxidised branched trithiol (percentages refer to amount of branched trithiol relative to amount of TetN343). (Fluorescence relative to fluorescence of free siRNA). Data show mean and standard deviation of three measurements of three repeats.

NMR of the oxidised material after freeze-drying revealed that 70% of the thiol groups were oxidised in the sample that had been left (see Section 2.8 for a detailed discussion of how this was investigated). In a different experiment, another sample that was 80% oxidised (difference due to lack of precise control of degree of oxidation) was used as the sole cation across a range of N/P ratios. As shown in Figure 3.33, encapsulation was much improved over the values obtained using unoxidised trithiol and was in fact almost total at N/P ratio 20 and above.

3. Formation and Characterisation of Particles

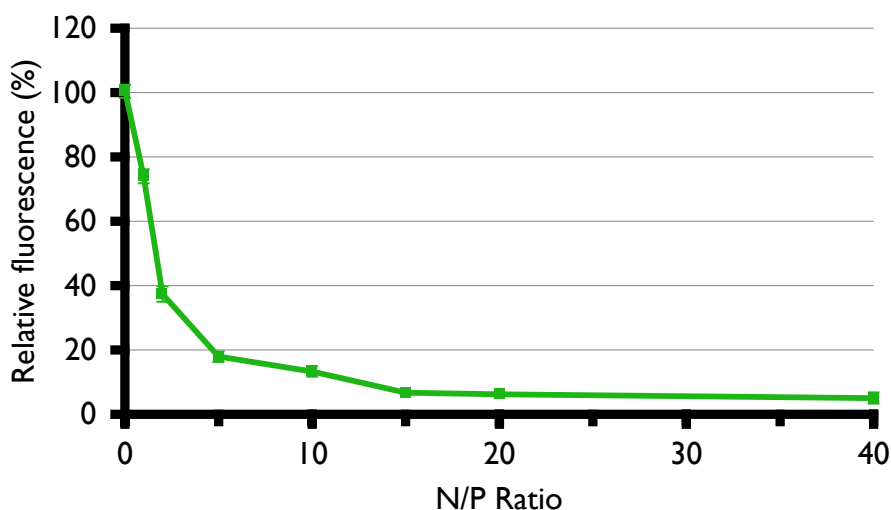


Figure 3.33 – Encapsulation efficiency of 80% oxidised trithiol at a range of N/P ratios as demonstrated by a propidium iodide exclusion assay. Percentage values are calculated relative to the fluorescence observed for siRNA in the presence of no cation. Data show mean and standard deviation of six measurements.

The degree of oxidation was potentially important so a comparison between branched trithiol solutions of different ages (and consequently different degrees of oxidation) was carried out. A range of brancher samples that had been in solution for some time were tested at N/P 10. As shown in Graph 7, the 99% oxidised (by NMR) sample affords full encapsulation at N/P 10. A range of samples of different oxidation levels were compared also compared. Figure 3.34 shows a compelling trend that increased oxidation increases binding efficiency. For the 99% oxidised sample, encapsulation is in fact complete.

There are two possibilities for this increased binding ability. Firstly, the oxidation could lead to the formation of higher MW species before the siRNA is even introduced. These would bind to siRNA more strongly due the multivalency effect and be more comparable with standard polymeric transfection agents. The second possibility is that, by pre-oxidising, formation of higher MW species through templating is more favourable as only disulfide exchange needs to take place, not oxidation. This is also in agreement with the results for the cyclic and linear spermine compounds.

3. Formation and Characterisation of Particles

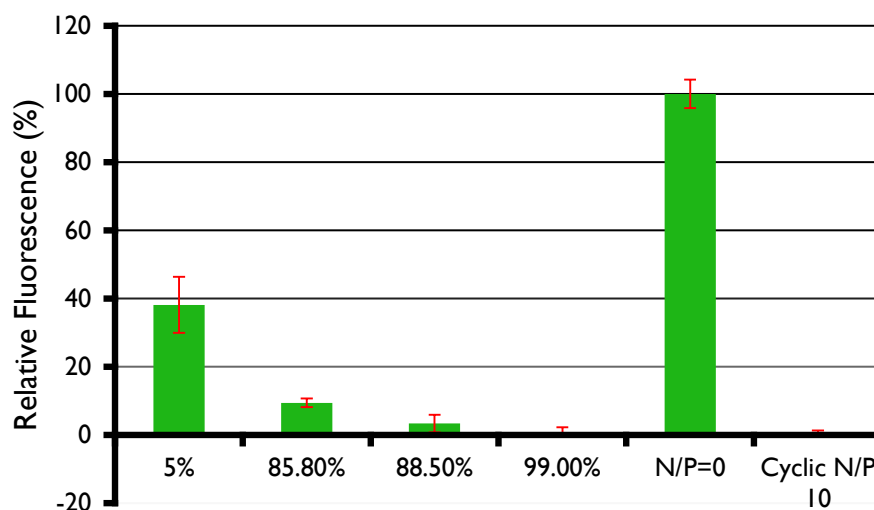


Figure 3.34 – Encapsulation efficiency of oxidised trithiol at N/P 10 at a range of degrees of oxidation as demonstrated by a propidium iodide exclusion assay. Percentage values are calculated relative to the fluorescence observed for siRNA in the presence of no cation. Data show mean and standard deviation of three measurements of six repeats.

Figure 3.35 shows the use of the 99% oxidised trithiol (**16**) in conjunction with cyclic TetN343 (**7**) (at N/P 10). Unlike the unoxidised trithiol, full encapsulation is possible up to 50% trithiol making this a more viable way to include more branching (the limit was 10% with fresh trithiol). Even above 50%, encapsulation is very high suggesting that these materials could have applications even without cyclic disulfide. Indeed, observation of full encapsulation with the 99% oxidised trithiol had been observed previously (Figure 3.3).

3. Formation and Characterisation of Particles

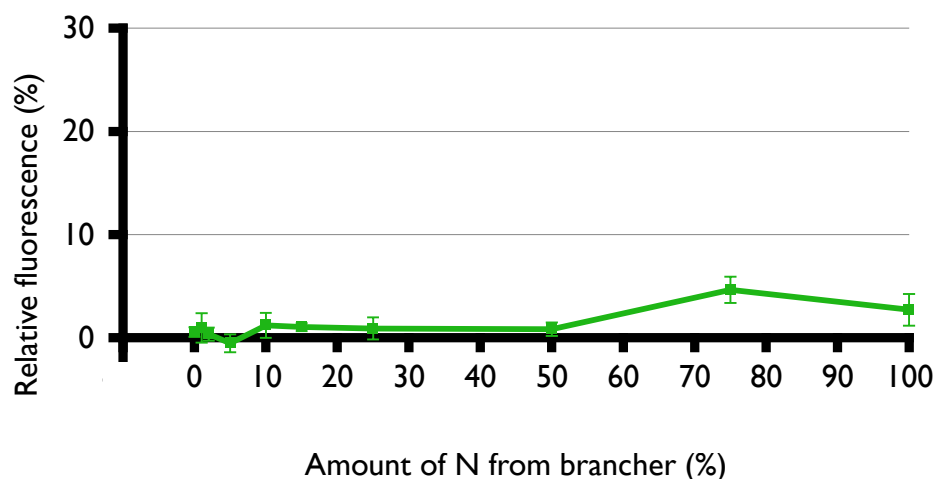


Figure 3.35 – Encapsulation efficiency of mixtures of brancher and cyclic TetN343. Percentage indicates the amount of protonable nitrogen due to brancher. (Fluorescence relative to fluorescence of free siRNA). Data show mean and standard deviation of three measurements.

The 99% oxidised branched disulfide was tested across N/P ratios (). The results showed encapsulation that was within one standard deviation of totality at N/P ratio 10 and above. This would also suggest that the branched disulfide would be appropriate for polyplex formation.

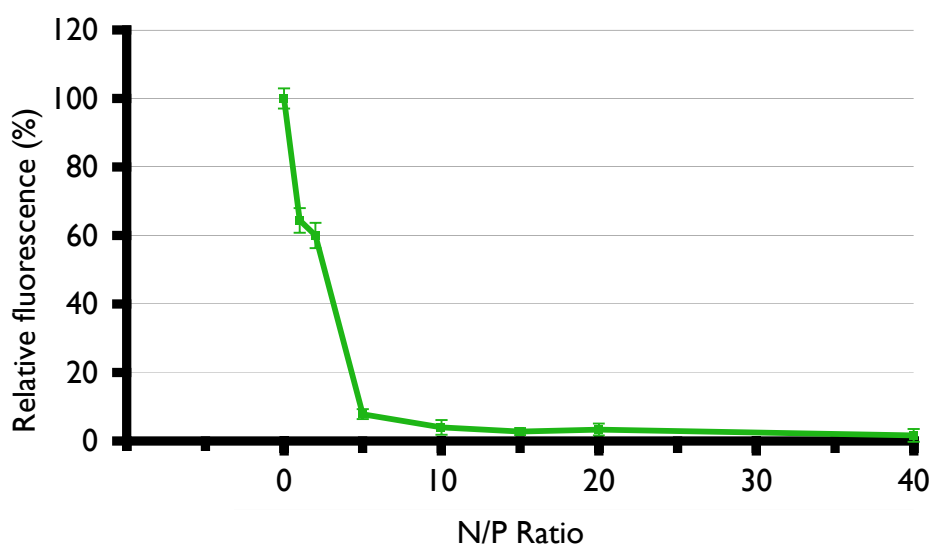


Figure 3.36 – Encapsulation efficiency of ~100% oxidised trithiol at a range of N/P ratios as demonstrated by a propidium iodide exclusion assay. Percentage values are calculated relative to the fluorescence observed for siRNA in the presence of no cation. Data show mean and standard deviation of three measurements.

3. Formation and Characterisation of Particles

3.5.2. Photon correlation spectroscopy of particles formed using oxidised brancher

Particles formed using the branched disulfide across N/P ratios showed sub-200 nm particles with low polydispersity (Figure 3.37). In addition, the existence of a minimum was observed (necessitating the additional data points compared to previous experiments) unlike the results from the cyclic disulfide that plateaued. The small size is further evidence of the formation of high quality particles with the branched disulfide.

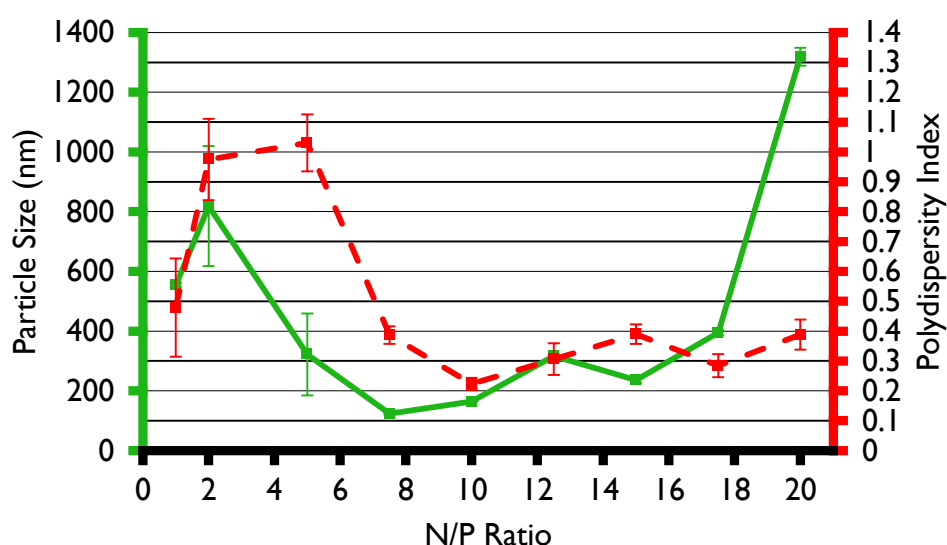


Figure 3.37 – Size and polydispersity of particles formed between siRNA and ~100% oxidised brancher at a range of N/P ratios. Data show mean and standard deviation of three measurements.

Figure 3.38 shows a time course using the branched disulfide. The particle size is lower than that observed in the previous experiments suggesting that the 200 nm obtained before may be the upper limit. Compared to the analogous experiment using cyclic disulfide (see Figure 3.4), it takes a while for polydispersity to come down but when it does, it is very steady. Particle size also shows improved stability compared to the cyclic disulfide.

3. Formation and Characterisation of Particles

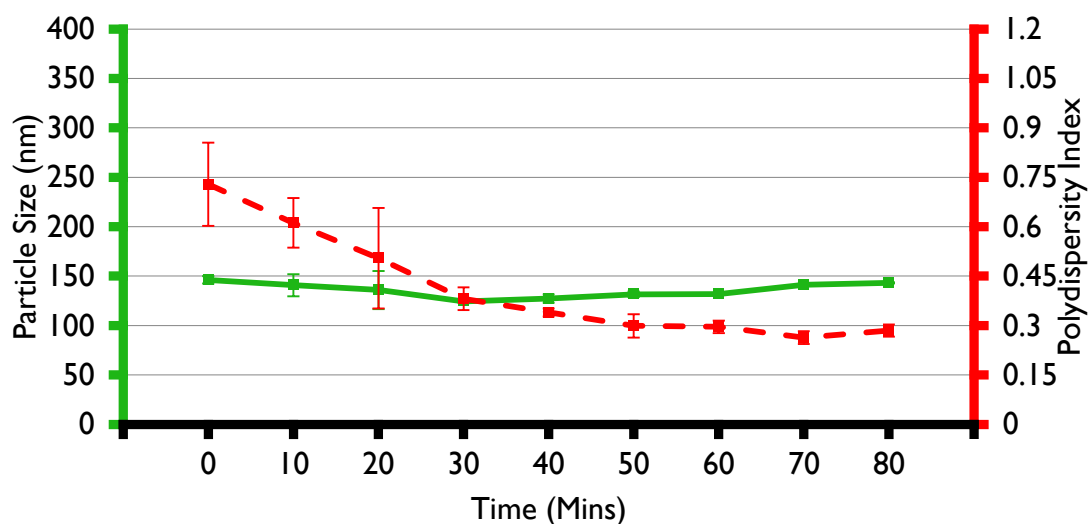


Figure 3.38 – Size and polydispersity of particles formed between siRNA and ~100% oxidised brancher at N/P 10 over 80 minutes. Data show mean and standard deviation of three measurements.

Due to the excellent stability of the material over one hour, an extended time course experiment was also run to investigate whether any significant change would eventually be observed in the manner of the cyclic disulfide particles. After 6 hours, size had increased though particles were still below 200 nm (Figure 3.39). This suggests that the time frame of aggregation is sufficiently long that it will not prove to be a problem in a delivery context.

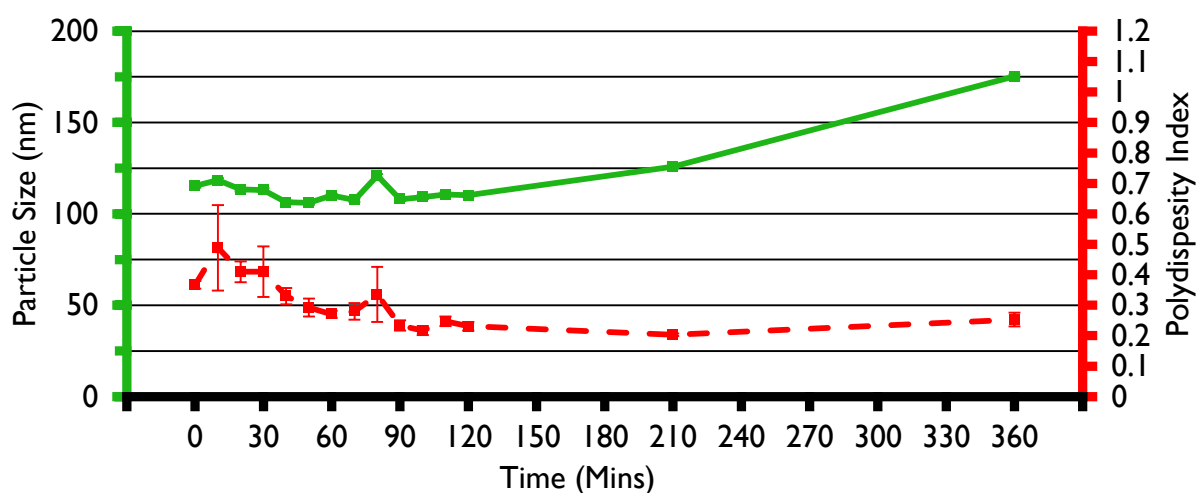


Figure 3.39 – Size and polydispersity of particles formed between siRNA and ~100% oxidised brancher at N/P 10 over 6 h. Data show mean and standard deviation of three measurements.

3. Formation and Characterisation of Particles

3.5.3. Using branched disulfide (**16**) in conjunction with cyclic TetN343

The results of an experiment using combinations of branched disulfide and cyclic disulfide is shown in Figure 3.40. This experiment is analogous to the one discussed in Figure 3.25 using trithiol and in common with that, the percentage shown is the percentage of protonatable nitrogens from the branched disulfide (**16**). Both the 0% and 100% branched disulfide (**16**) particles are consistent with previous experiments which makes the strange trend observed over the entire graph very interesting. The data show the branched disulfide (**16**) to make very little difference until the proportion reaches 75% where it causes a massive increase in size (though this is only one data point). It would have been expected that the size would gradually transition from the size of the particles formed with the linear polymer to the size for the branched.

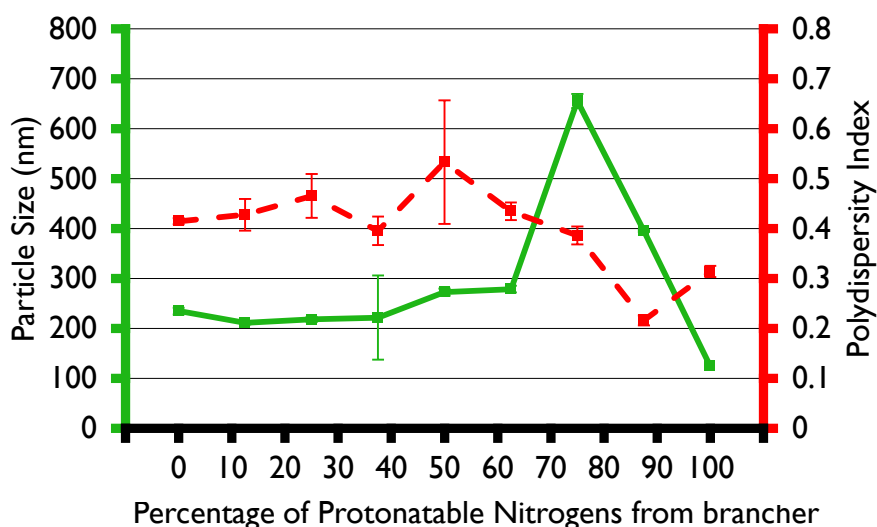


Figure 3.40 – Particle size using TetN343 and a percentage of oxidised brancher (calculated that a certain percentage of protonatable amines come from brancher at N/P 10. Freshly prepared brancher solutions added to TetN343 and buffer. Cation solutions were then added to siRNA solution, vortexed for 5s and left for 1 hr at RT. Data show mean and standard deviation of three measurements.

For further clarification, the experiment was reproduced using more samples in the interesting range. As shown in Figure 3.41, the trend is still present and size peaks at 80%. Above this value, particles return to having more in common with the 100%

3. Formation and Characterisation of Particles

branched disulfide particles that it should be noted, were measured to be larger on this run compared to other runs with the exact same material (175 nm vs. 150 nm).

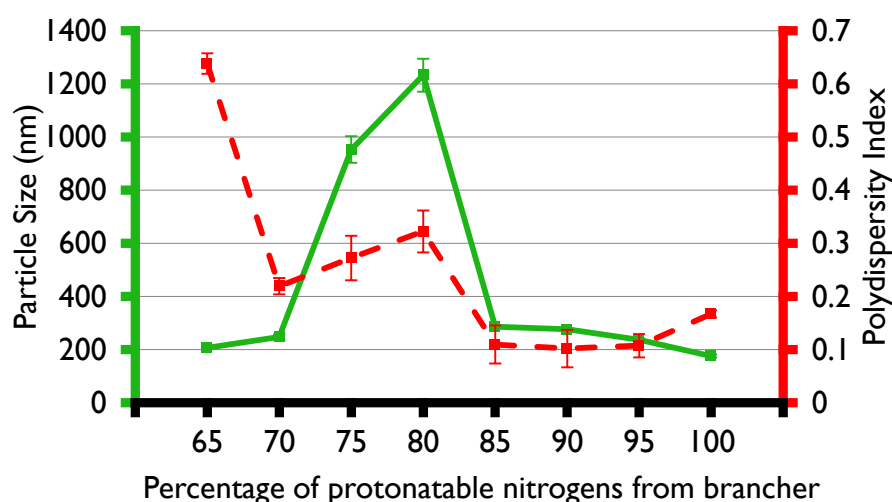


Figure 3.41 – Particle size using TetN343 and a percentage of oxidised brancher (calculated that a certain percentage of protonatable amines come from brancher at N/P 10. Freshly prepared brancher solutions added to TetN343 and buffer. Cation solutions were then added to siRNA solution, vortexed for 5s and left for 1 hr at RT. Data show mean and standard deviation of three measurements.

These results suggest that there is a certain proportion of branching points that produce a material unsuitable for siRNA complexation. It is therefore important to consider the results in terms of branched cations to linear ones; Figure 3.42 shows Figure 3.41 replotted in terms of this relationship. The peak is between four and six branching units per spermine. It is however unlikely that the dimeric branched disulfide will break up so one would expect pairs of brancher to occur in the chain.

3. Formation and Characterisation of Particles

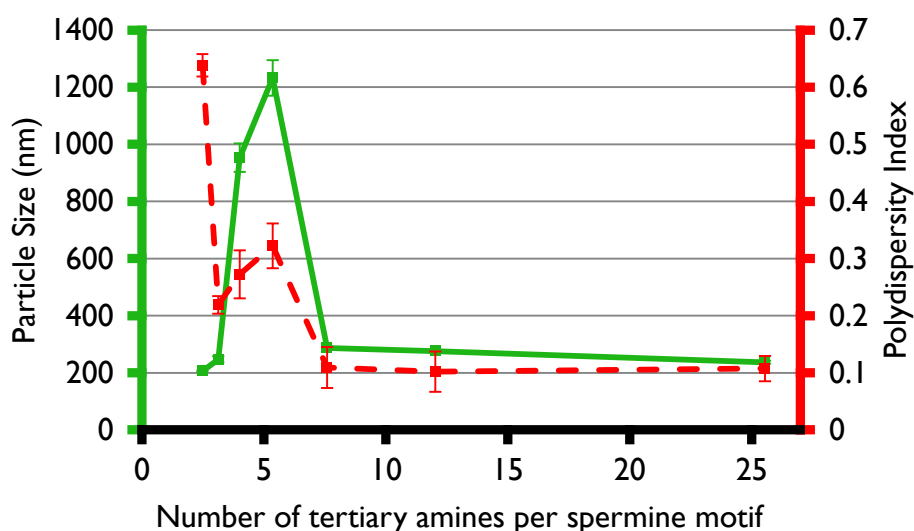


Figure 3.42 – Figure 3.41 replotted to show particle size in terms of the number of spermine motifs per tertiary amine. Data show mean and standard deviation of three measurements.

The reason that this particular combination causes such a dramatic increase in the size of the particles formed is unclear. Especially puzzling is the fact that the peak in particle size is so narrow: the difference between combinations that form the large particles and the small is as low as two tertiary amines. This underlines the need to develop a method of characterising the polymer away from the siRNA after particle formation. The investigation of the particles using propidium iodide assays would also be useful.

3.6. Particle Sizing in Biologically Relevant Media

The particles formed with the branched disulfide were also tested in media less conducive to particle formation. Higher molarity buffer would be expected to reduce protonation of the amines and reduce complexation strength. Figure 2.9 shows that particles formed in 20 mM buffer are indeed slightly larger – except at low N/P ratio where data are typically unreliable due to incomplete particle formation). The particles formed at N/P 7.5 and 10 were still within the desired size range at 184.0 and 227.5 nm respectively.

3. Formation and Characterisation of Particles

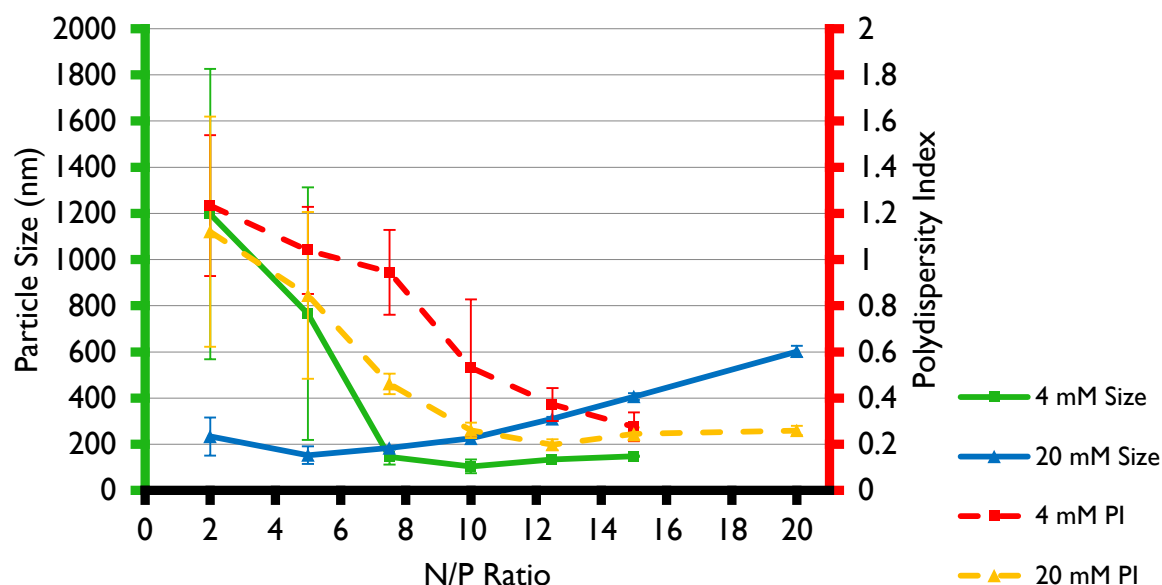


Figure 3.43 – Particle size using oxidised brancher at different N/P ratios in different buffers. Green/Red: pH 7, 4 mM HEPES; Blue/Yellow: pH 7 20 mM HEPES. Data show mean and standard deviation of three measurements.

Due to its greater biological relevance, experiments were carried out in Opti-MEM a medium with higher salt content and buffering strength that will be similar to the media used during transfection. Due to the greater ionic strength of the medium, it would be expected that polyplex formation would be less favourable. Also, as discussed in Section 1.4, the extra salts will remove one of the principle driving forces of the interaction between the cations and nucleic acid. While larger than those formed in HEPES, the particles formed using the oxidised brancher were still of an acceptable size and particles were stable between 200 and 300 nm for two hours (Figure 3.44). However, the particles formed with the cyclic disulfide were much larger (Figure 3.45). This suggests that the binding of the branched material to siRNA is significantly stronger than the linear polymer formed with the cyclic disulfides. Indeed, the rapid increase in size observed for the cyclic disulfide particles is suggestive of a lack of suitability for transfection.

3. Formation and Characterisation of Particles

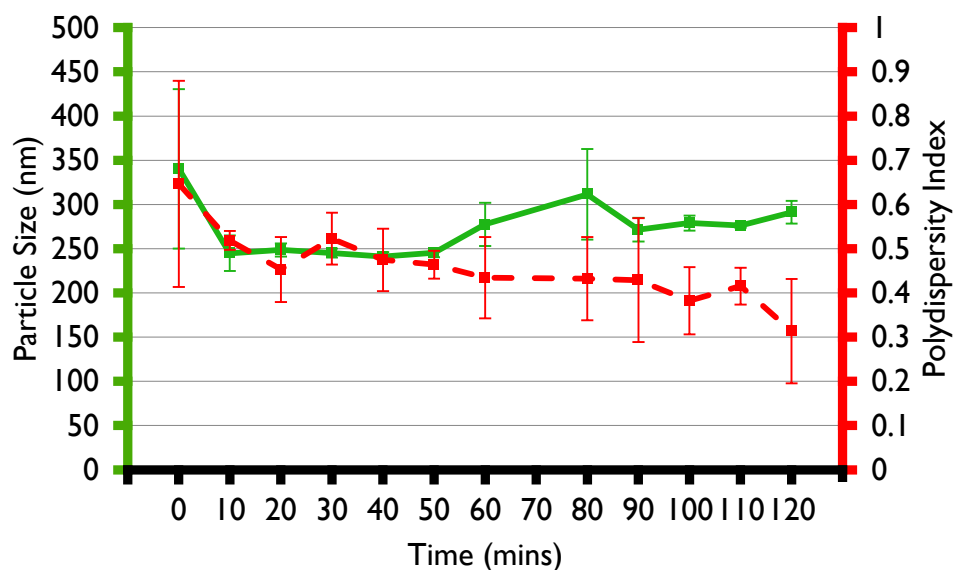


Figure 3.44 – Size and polydispersity of particles formed between siRNA and ~100% oxidised brancher at N/P 10 over 2 h in OptiMEM buffer. Data show mean and standard deviation of three measurements.

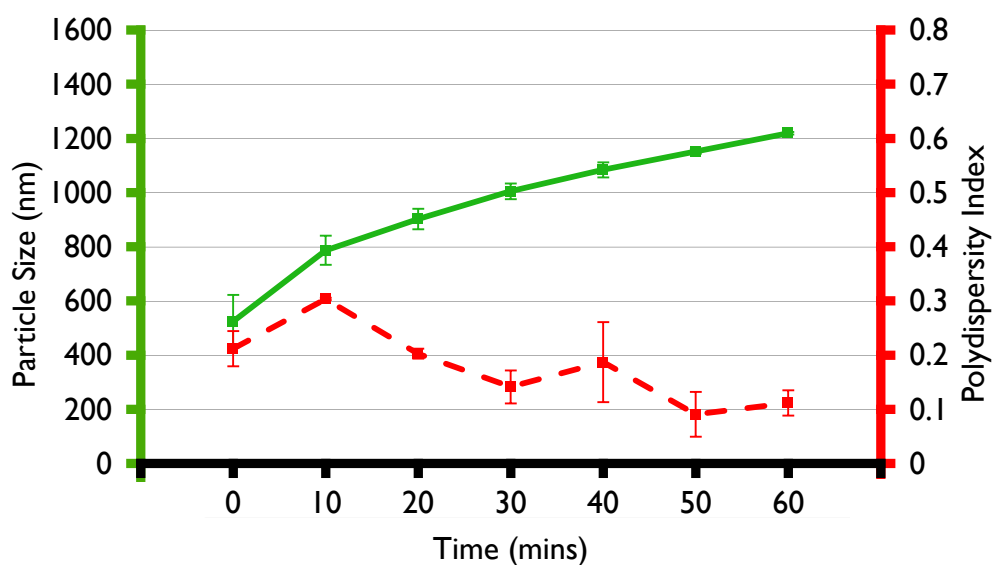


Figure 3.45 – Size and polydispersity of particles formed between siRNA and cyclic TetN343 at N/P 10 over 1 h in OptiMEM buffer. Data show mean and standard deviation of three measurements.

3.7. Summary

From the particle sizing and encapsulation experiments carried out it can be surmised that both the cyclic TetN343 and the cyclic brancher are suitable for siRNA delivery. The sizes of particles formed with these materials are of the appropriate size

3. Formation and Characterisation of Particles

and in the case of those formed with the branched material are temporally stable in biologically relevant media.

The particles formed with the combinations of material will also be useful for *in vitro* testing as they will potentially give an interesting insight into the effect of size on siRNA delivery. That is assuming that the difference in size is solely a result of the structure of the polymer formed.

4. *In Vitro* Testing of Nanoparticles

4. *In Vitro* Testing of Nanoparticles

4.1. Introduction

After appropriate conditions for the formation of particles had been found, *in vitro* studies could begin. In the previous work¹²⁰ the cyclic disulfides had been used to successfully deliver pDNA to both CHO-K1 (Chinese hamster ovary) cells and A549 (human lung carcinoma) cells; since A549 cells represent a more meaningful disease target these were selected for this work.

The GAPDH assay was selected as a means of measuring knockdown. GAPDH is a stably expressed “house-keeping” enzyme the amount of which can be quantified by adding a dye to the lysed cells and measuring fluorescence.¹⁴² Since the amount of GAPDH is proportional to the amount of cells, toxicity can also be judged by comparing the amount of GAPDH present in a sample treated with non-targeting siRNA with a sample that has not been transfected. Any GAPDH “knockdown” in this instance can be attributed to cell death caused by the vector itself. Whilst not as meaningful as dedicated cytotoxicity assays such as MTT¹⁴³ (offering a count of live cells) or LDH¹⁴⁴ (which measures membrane integrity) it would at least be able to give some insight into whether the materials maintained the biocompatibility they had previously displayed.¹²⁰

4.2. Determination of Optimum Number of Cells and Dose of siRNA

Once the cell line and assay had been identified the dose of siRNA and the number of cells to use in each experiment had to be identified. This was done by testing 5000 or 10000 cells per well of a 96-well plate using a varying dose of siRNA in particles formed with cyclic TetN343 (7) at N/P 20. Cells were grown in serum-containing media for 24 h after which time the media was changed for serum-free media and a solution of polyplexes in HEPES prepared exactly as in the PCS and PrI exclusion experiments. After a further 24 h the polyplexes and media were removed and replaced with serum-containing media and left for another 24 h. All polyplex solutions were tested with payloads of GAPDH targeted siRNA and scrambled siRNA.

4. *In Vitro* Testing of Nanoparticles

As shown in Figure 4.1, the levels of fluorescence recorded using 5000 cells were too low given the size of even the smallest error bars. 80 ng of siRNA per well was found to suggest siRNA knockdown and was chosen as the starting point for the *in vitro* assays.

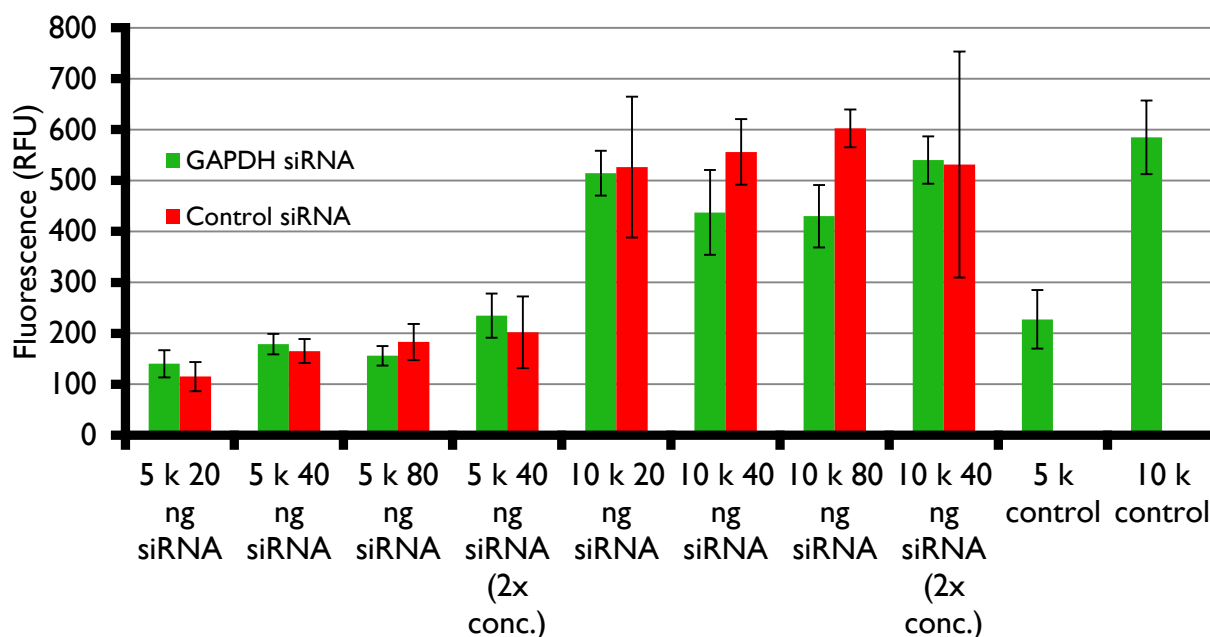


Figure 4.1 – A graph of the relative fluorescence due to the presence of GAPDH enzyme after treatment of the named number of cells with cyclic TetN343 (7) polyplexes containing the named amount of siRNA at N/P 20. 5 k and 10 k controls refer to wells treated with serum-free medium in place of polyplex solution. Data shows mean and standard deviation of three repeats.

4.3. Determination of Optimum DharmaFECT® Conditions

Another requirement was a reliable vector to use as a positive control. DharmaFECT® is a commercially available, lipidic siRNA transfection agent. An experiment was run to determine the optimum transfection conditions to use for our experiments as shown in Figure 4.2. Interestingly, although the manufacturer's instructions indicate that the lipoplexes should be prepared in serum-free media and placed on cells in the presence of serum-containing media we had greater success using our serum-free media/HEPES system. The results identified that 0.25 µl of DharmaFECT® per well was optimum using both methods of transfection. Not only

4. *In Vitro* Testing of Nanoparticles

was clearer transfection obtained by using the HEPES, using the same conditions as for the polycations would enable more meaningful comparisons to be made.

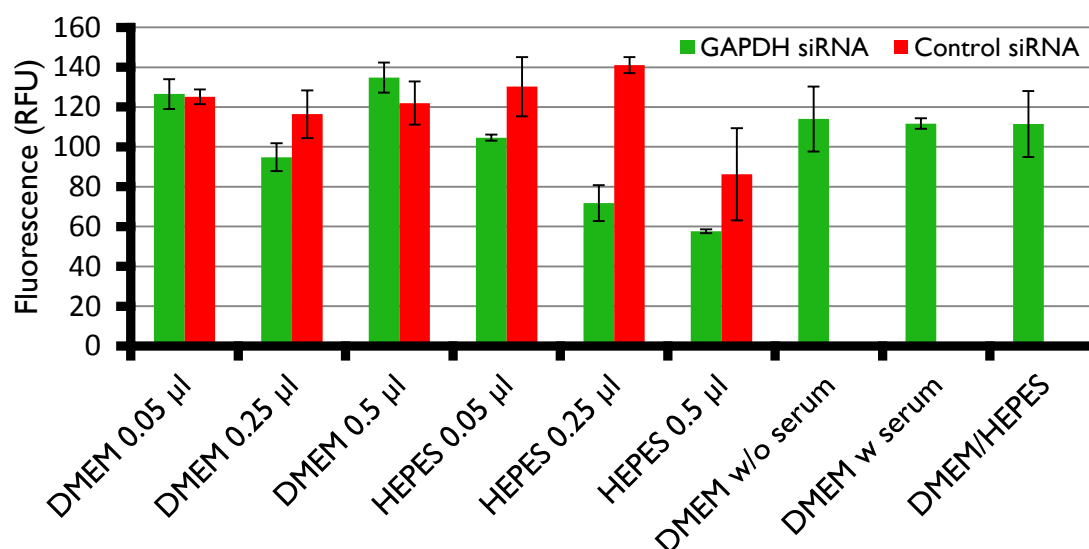


Figure 4.2 – A graph of the relative fluorescence due to the presence of GAPDH enzyme after treatment of 10 k cells with with DharmaFECT® lipoplexes. Each well was transfected with 80 ng of siRNA and the named amount of amount DharmaFECT®. “DMEM” data show lipoplexes formed in serum-free DMEM and added to wells containing serum-containing DMEM. “HEPES” data show lipoplexes formed in HEPES buffer and added to wells containing serum-free DMEM. Data shows mean and standard deviation of at least three repeats.

4.4. Transfection Efficiency of Cyclic TetN343 (7)

Figure 4.3 shows a study using cyclic TetN343 across a range of N/P ratios. It is important to note that the experiment was conducted across two plates with the N/P 0 through N/P 20 corresponding with the untreated cells labelled DMEM 1. The other samples correspond to DMEM 2. Although the experiments were performed in parallel the error bars are generally larger on the plate containing the controls and a lack of common samples between the two does not allow this to be definitively attributed to a particular cause. The principle conclusion that can be drawn from this dataset is that polyplexes prepared at an N/P ratio of 15 suggested siRNA knockdown of around 25%. N/P 10 also appeared to mediate some knockdown although the data for this are not especially compelling and the knockdown is less than 10%. jetPEI™ (used according to the manufacturer’s instructions and delivered as a solution in saline) proved to be the only toxic material although as this is the only material not delivered in HEPES buffer it is unclear if this could be the cause. It is extremely

4. *In Vitro* Testing of Nanoparticles

unlikely that saline would prove to be so toxic though. Not unsurprisingly, the low molecular weight cations 600 kDa bPEI and spermine displayed no transfection efficiency. The latter is particularly important as it demonstrates the difference in behaviour between the cyclic and similar low molecular weight cations. It is surprising that the 25 kDa bPEI did not mediate knockdown given that this has been observed Grayson *et al.*³² who used the same buffer (albeit at pH 7.2, 10 mM not pH 7.0, 4 mM) and similar numbers of cells (8000 per well). This suggests that our lack of success could be attributed to the different cell line used. Indeed, work by Creusat *et al.*¹⁴⁵ reports low effectiveness (~10% knockdown) using 25 kDa bPEI on A549 cells albeit using a different transfection procedure.

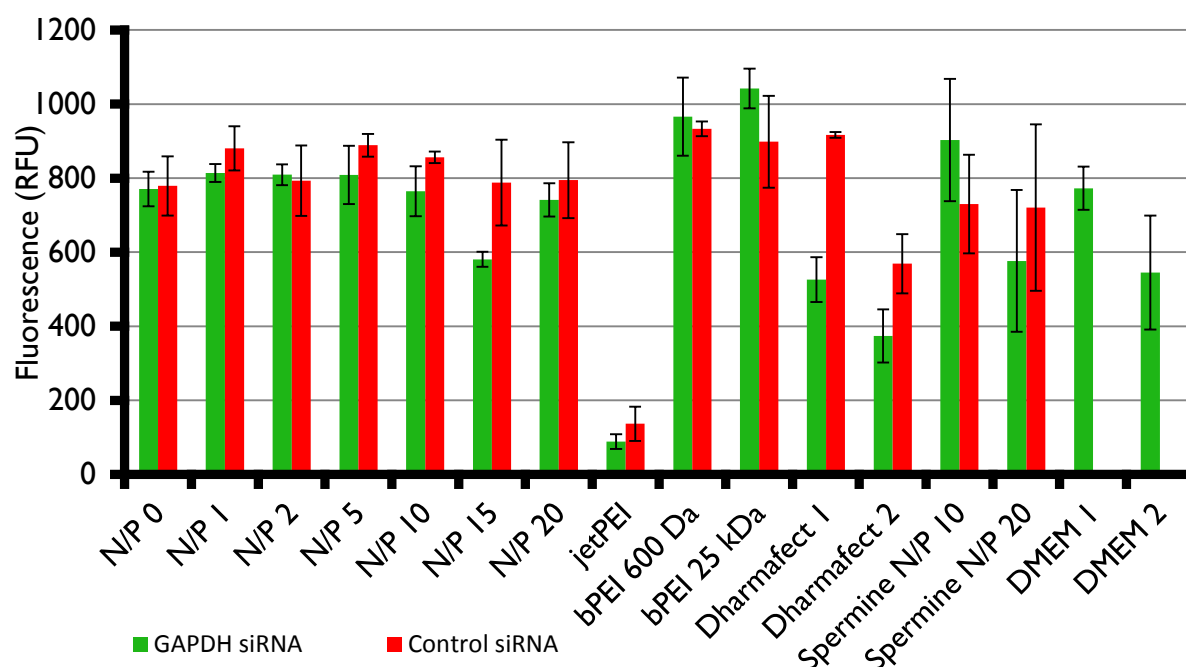


Figure 4.3 – A graph of the relative fluorescence due to the presence of GAPDH enzyme after treatment of 10 k cells with with siRNA nanoparticles. Each well was transfected with 80 ng of siRNA in a polyplex with TetN343 (7) at the N/P ratio shown. Controls were used as described in the Experimental section. DMEM 1 corresponds to an untreated well on the same plate as the first seven samples, DMEM 2 corresponds to an untreated well on the same plate as the second seven samples. Dharmafect 1 and 2 are two separate identically prepared samples. Data shows mean and standard deviation of at least three repeats.

4. *In Vitro* Testing of Nanoparticles

4.5. Transfection efficiency of branched disulfide (16)

Some early data was also obtained for the use of the branched disulfide. No separate siRNA dose optimisation studies were carried out so conditions are the same as for the cyclic TetN343 experiment in Figure 4.3. As shown in Figure 4.4, the most striking thing about this plate is the change in fluorescence reading between the samples from N/P 0 to N/P 10. These samples were on a different plate and the untreated sample from that plate (DMEM 1) is also high. Since the degree of confluency of the cells on both plates (and indeed the plates from the cyclic TetN343 experiment which was run in parallel) were similar it can only be assumed that a measurement error has occurred and given greatly overstated values. This does not prohibit comparison of individual GAPDH/control siRNA readings and it can still be surmised from this data that no knockdown took place at N/P 10 or below.

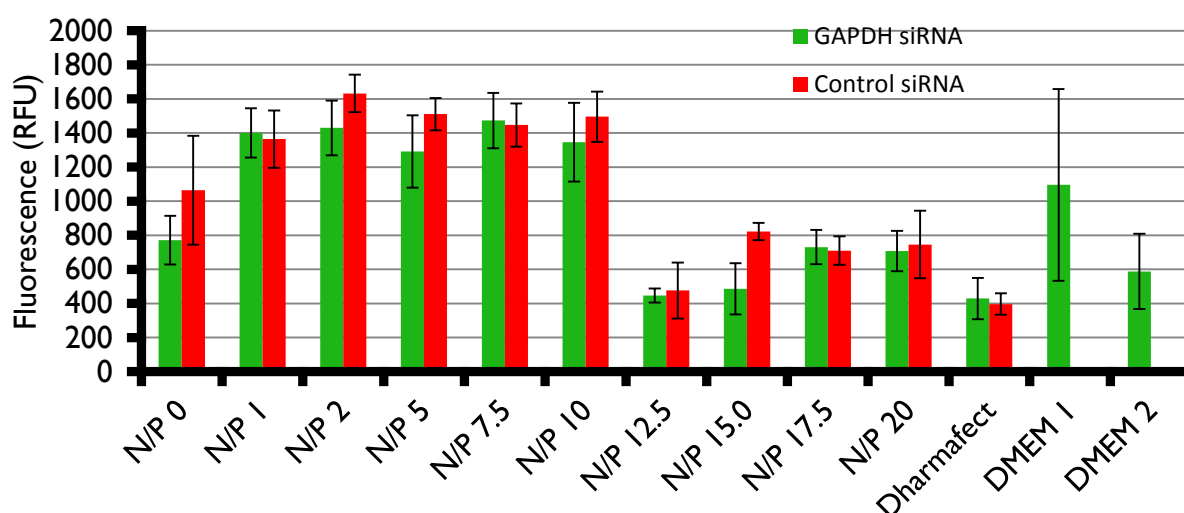


Figure 4.4 – A graph of the relative fluorescence due to the presence of GAPDH enzyme after treatment of 10 k cells with with siRNA nanoparticles. Each well was transfected with 80 ng of siRNA in a polyplex with branched disulfide (16) at the N/P ratio shown. Controls were used as described in the Experimental section. DMEM 1 corresponds to an untreated well on the same plate as the first six samples, DMEM 2 corresponds to an untreated well on the same plate as the second five samples. Data shows mean and standard deviation of at least three repeats.

The values for the rest of the dataset compare well with the cyclic TetN343 dataset. They show a hint of knockdown at N/P 15 in common with the cyclic disulfide material. The degree of knockdown appears to be around 40%. This result is

4. *In Vitro* Testing of Nanoparticles

surprising as these were by no means the smallest particles (~236 nm compared with 163 nm for N/P 10) and it was assumed that this would be beneficial to delivery. These results therefore hint that the charge of the particle has a greater effect than the size though it is not possible to draw any strong conclusions. Another flaw in this experiment is that DharmaFECT® did not appear to mediate any transfection though as this experiment was performed in parallel with the experiment shown in Figure 4.4 – where DharmaFECT® did mediate knockdown – they are not entirely without positive control.

4.6. Summary

It is not clear from the data presented above that these materials are capable of mediating gene knockdown through the delivery of siRNA. However, these preliminary results do suggest that there is a possibility that some successful transfection has taken place. There is clearly still a need for optimisation of transfection conditions – particularly in the case of the oxidised brancher. In addition, for true comparison with DharmaFECT®, branched PEI and jetPEI™ conditions need to be identified where these materials are consistently effective. Also, for a total assessment of the relationship between the nature of the nanoparticles and their transfection efficiency more of the nanoparticle formulations discussed in Chapter 3 need to be tested *in vitro*.

5. Conclusions and Future Work

5. Conclusions and Future Work

5.1. Synthesis

The compound that was originally chosen for study as an siRNA delivery system was successfully prepared and it was also found that a successful delivery agent could be derived from it. The exact preparation of this oxidised brancher is by no means fully understood and it would be preferable to have a more rapid oxidation procedure that could easily be performed on a larger scale. It is possible that, were more control to be gained over the oxidation, a single cyclic with a free thiol could also be prepared (see B, Figure 2.10) and its transfection properties investigated.

There are also many more brancher structures that could be synthesised and tested. Since the original brancher was originally intended for use in conjunction with the spermine-containing cyclic, it was thought to be unnecessary to incorporate this motif into its structure. It would therefore be interesting to prepare a single-compound that combined the features of both of cyclic TetN343 and the oxidised brancher. Such a structure is shown in Figure 5.1 and both the double cyclic motif of the oxidised brancher and the amine spacing of spermine can be seen.

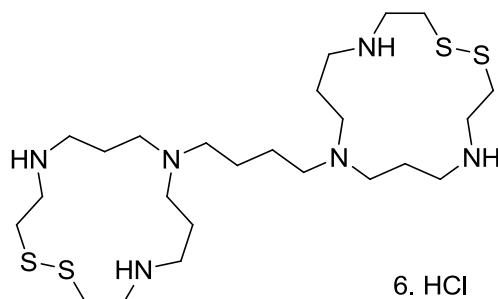


Figure 5.1 – Potential siRNA delivery vector combining the features of cyclic TetN343 and the oxidised brancher.

5.2. Formulation

One of the most fascinating aspects of this project still remains a mystery. It is still unknown how exactly the *in situ* polymerisation of the small-molecule disulfides takes place and no polymer has yet being isolated and characterised. The work described in Section 3.3.4 has furthered our understanding slightly by providing

5. Conclusions and Future Work

evidence dithiols do not polymerise at all and that there is possibly a driving force based on the improved binding of the cyclic disulfide upon ring-opening. While it was outside the scope of this project, it would certainly be possible to investigate this experimentally. Quantitative measurements of the binding strength of linear and cyclic materials with nucleic acids could be carried out. Also, a cyclic material that would be flexible enough such that it gained no binding advantage by ring-opening could be prepared. Such a material is shown in Figure 5.2. The inclusion of the long, PEG-like section would hopefully provide enough flexibility in the ring for the spermine motif to bind unhindered. Polymerisation in this case would disprove the idea that it is an inability of the cyclics to meet the spermine's full binding potential that causes the ring-opening.

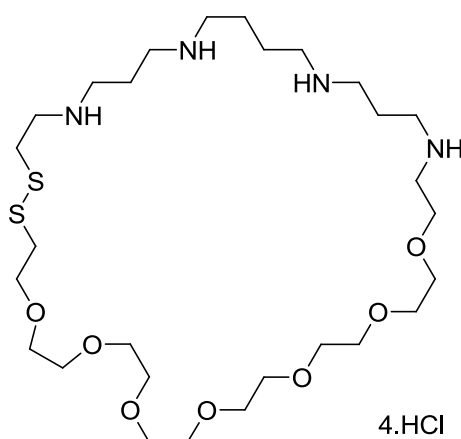


Figure 5.2 – A cyclic compound that would be unlikely to bind more strongly to a nucleic acid when ring-opened. Such a compound could be used to shed light on the polymerisation that takes place with cyclic TetN343

Another interesting result was that obtained when combinations of cyclic TetN343 and oxidised brancher were used to form particles. The size of particles showed a very definite spike when a certain combination was used. This was suggestive that a polymer inappropriate for encapsulation was being formed *in situ*. Isolation of this polymer would be particularly fascinating as it offers a chance of understanding of the subtle structural requirements of a gene delivery polymer.

5. Conclusions and Future Work

5.3. *In vitro* Experiments

Comparing different siRNA knockdown experiments is extremely difficult given the wide range of cell-types, targets and transfection procedures. Our knockdown results hint that transfection of cells with siRNA may be possible with our materials. However, since there are reports of effectively total knockdown in A549 cells in the literature¹⁴⁵ suggesting that there is still room for optimisation. The efficacy of our disulfides do not approach the extent of knockdown in A549 in other recently published data. For example, De Jere *et al*¹⁴⁶ recently reported 70% knockdown of the Akt1 gene in A549 cells using an ester cross-linked branched PEI and Chung *et al*.¹¹⁵ reported around 50% knockdown using an siRNA-lPEI conjugate.

Ultimately, more cell work is required and the results presented here can only be regarded as early data. It would be interesting to test the transfection efficiency of more of the particle formulation methods that were investigated with PCS allowing the relationship between polyplex structure and transfection efficiency to be elucidated.

5.4. Future Scope

The obvious goal of any drug delivery project is effective *in vivo* transfection. The cyclic disulfides had already shown promise in this regard for pDNA delivery¹²⁰ and it would be fascinating to see if the success carries over to siRNA delivery. It would also be interesting to attempt to build a series of compounds that could be tailored for various cell types or disease targets. Modular components that could be combined to provide varying combinations of branched and linear materials would be extremely useful. It can even be envisaged that the system could be expanded to include PEG bearing molecules such as that shown in Figure 5.3. PEGylation of thiols using PEG-maleimide is a known reaction¹⁴⁷ and could be used in conjunction with our branched materials.

5. Conclusions and Future Work

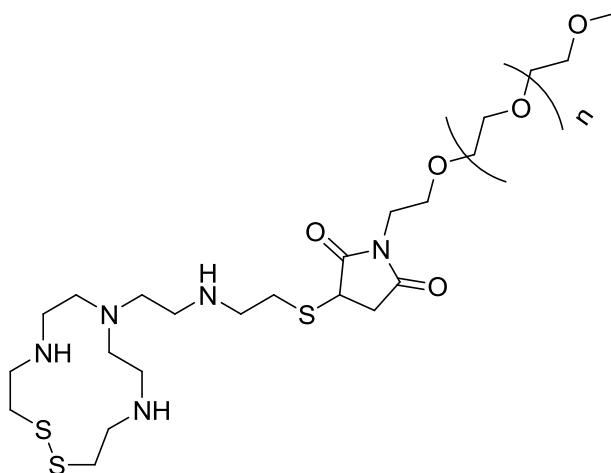


Figure 5.3 – A structure that could be used to incorporate PEG chains into polyplexes

6. Experimental

6. Experimental

6.1. Materials and Methods

6.1.1. Synthesis

All reagents were purchased from Sigma-Aldrich, Fisher, VWR or Acros and were of the highest available purity. Solvents and inorganic salts were purchased from VWR and were of at least analytical grade. Unless otherwise stated, reagents were used as received and without additional purification. Dry methanol, dry DCM and dry toluene were obtained from a pure solv-MD standard design solvent purification system (Innovative Technologies, UK) fitted with columns filled with 3 mm aluminium oxide beads (Sigma-Aldrich, UK). Dry DMF was purchased dry from Sigma-Aldrich and used as received. Acetonitrile, DMSO and DIPEA were dried over calcium hydride then distilled. Acetone was dried over potassium carbonate then distilled. All reactions were carried out under nitrogen unless otherwise stated. Unless specified otherwise solvents were degassed by bubbling nitrogen through for at least 2 h. Piped nitrogen was supplied by BOC Gases and passed through a silica, calcium chloride and sodium hydroxide drying column before use.

Purification over silica refers to flash column chromatography using Silica Gel for Flash Column Chromatography (VWR). TLC was carried out on aluminium backed 60 F₂₅₄ Silica gel sheets (Merck). Unless otherwise stated, solvent mixtures for chromatography are expressed in volume/volume percentages or ratios. Unless specified otherwise, filtration refers to the use of a Buchner funnel fitted to a water aspirator and using Whatman brand filterpaper. 200 nm PTFE syringe filters were purchased from Whatman.

¹H NMR spectra were recorded on Bruker AV400 (400 MHz) spectrometers as homogenous solutions and processed using Topspin software (Ver 1.3, by Bruker BioSpin). Deuterated solvents were purchased from Sigma-Aldrich, Cambridge Isotope Laboratories or Merck and used as received. δ_{H} values are quoted to the nearest 0.01 ppm and coupling constants to the nearest 0.5 Hz. Abbreviations used in NMR assignments are: s (singlet), d (doublet), t (triplet), q (quartet), quin (quintet), m (multiplet), br (broad). A superscript for aromatic protons (eg. ArH²) refers to a number assigned to the attached carbon in the ring where 1 is the carbon with the highest priority substituent according to Cahn-Ingold-Prelog rules with

6. Experimental

numbering continuing around the ring in the direction of the next highest priority substituent.

^{13}C NMR spectra were recorded on Bruker AV400 (100 MHz) spectrometers and processed using Topspin software (Ver 1.3, by Bruker BioSpin). Deuterated solvents were purchased from Sigma-Aldrich, Cambridge Isotope Laboratories or Merck and used as received. δ_{C} values are quoted to the nearest 0.01 ppm. Aromatic carbon numbers are assigned in the same manner as described above for aromatic protons.

High resolution mass spectra were recorded by Imperial College mass spectrometry service.

Elemental Analysis was performed by the Microanalysis service of the University of Cambridge.

Melting points were measured on a Sanyo-Gallenkamp hot stage

pH was measured using either a Eutech Instruments CyberScan pH meter or Merck Universal Indicator paper.

6.1.2. Formulation

HEPES was obtained from Sigma Aldrich and used as received. The pH of HEPES buffer solutions was altered using aqueous sodium hydroxide and aqueous hydrochloric acid. siRNA was purchased from Ambion and used as 50 μM solutions in RNase free water (Invitrogen). pCMV-Luc plasmid DNA was a kind gift of the Imperial College Genetic Therapies Centre. Pre-made Novex 20% TBE polyacrylamide gels, Novex 5x TBE Hi-Density Sample Buffer, Novex 5x TBE Running Buffer, GIBCO RNase Free water, Molecular Probes SYBR® Green II RNA Gel Stain 10,000x in DMSO, and Molecular Probes 1 mgml^{-1} Propidium Iodide were purchased from Invitrogen. Water was obtained from a Purite Prestige Water Purification system containing a C750 Carbon pretreatment column fitted with a Millipore "Synergy 185" Water dispenser.

6. Experimental

Gels were run using an XCell Surelock Gel Electrophoresis Box and VWR Accu Power "300 Power Supply". Gels were photographed using a Uvitec Explorer D-77LS 26MX Auto UV Camera and images were processed using UVIsoft software (Uvitec). Fluorescence was measured using a Thermo Varioskan Flash Platereader and SkanIt software. Particles were sized with a Coulter N4 PLUS Photon Correlation Spectrometer and N4 Plus software.

All data were plotted and analysed using Microsoft Excel.

6.1.3. Cell Biology

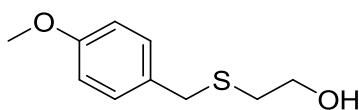
Ambion *Silencer*® GAPDH siRNA, *Silencer*® Negative Control #1 siRNA and KDAAlert™ GAPDH kits were purchased from Applied Biosystems as 50 µM solutions and used as received. DMEM, PBS, FBS, RNase-free water, penicillin-streptomycin and trypsin (used as 1% solution in DMEM) were purchased from Invitrogen and used as received. HEPES was purchased from Sigma-Aldrich and 4 mM pH 7.0 HEPES solutions were prepared using RNase-free water and filter sterilised using pre-sterilised Whatman 0.2 µm syringe-filters. Serum-containing media refers to DMEM containing 10% FBS and 1% penicillin-streptomycin. Serum-free media refers to DMEM containing 1% penicillin-streptomycin. Cation solutions were prepared as 1 mgml⁻¹ in RNase free water and filter sterilised using pre-sterilised Whatman 0.2 µm syringe-filters. Cells were incubated at 37.0 °C in 5% CO₂. All media and PBS was heated to 37 °C in a water-bath prior to use. DharmaFECT® was a gift of the School of Pharmacy of King's College London, jetPEI™ was obtained Polyplus Transfection, 25 kDa bPEI from Sigma-Aldrich and 600 Da bPEI from Polysciences.

All data were plotted and analysed using Microsoft Excel.

6. Experimental

6.2. Synthesis

6.2.1. Synthesis of 2-((4-methoxybenzyl)sulfanyl)ethanol (**1**)¹²⁰

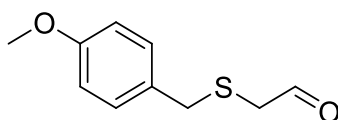


1

Potassium carbonate (20.0 g, 141 mmol, 2.0 eq) and mercaptoethanol (5.00 mL, 71.0 mmol, 1.0 eq) were added to dry methanol (200 mL) under nitrogen with vigorous stirring. 4-methoxybenzylchloride (9.70 mL, 69.0 mmol, 1.0 eq) was added dropwise and the solution was left to stir for 3 h. The solvent was removed on a rotary evaporator and the resulting white solid was dissolved in a 1:1 (v:v) mixture of chloroform and water. The organic phase was separated and the aqueous phase was washed with water (3 x 100 mL) and subsequently brine (3 x 100 mL). The organic phases were combined, dried over MgSO_4 , filtered and evaporated to dryness under reduced pressure to give the alcohol **1** as a white powder (12.8 g, 93% (lit. 96%)).

R_f 0.55 (4:1 (v:v) EtOAc:Hexane); mp 46.6-47.5 °C; ^1H NMR (CDCl_3), (400 MHz): δ_{H} = 2.05 (1H, t, $^3J_{\text{HH}}$ 6.5 Hz, OH), 2.66 (2H, t, $^3J_{\text{HH}}$ 6.0 Hz, $\text{SCH}_2\text{CH}_2\text{OH}$), 3.69 (2H, dt, $^3J_{\text{HH}}$ 6.5 Hz, 6.0 Hz, CH_2OH), 3.70 (2H, s, ArCH_2S), 3.82 (3H, s, OCH_3), 6.87 (2H, d, $^3J_{\text{HH}}$ 8.5 Hz, ArH^2), 7.25 (2H, d, $^3J_{\text{HH}}$ 8.5 Hz, ArH^3). Data consistent with those previously reported.¹²⁰

6.2.2. Synthesis of 2-((4-methoxybenzyl)sulfanyl)acetaldehyde (**2**)¹²⁰



2

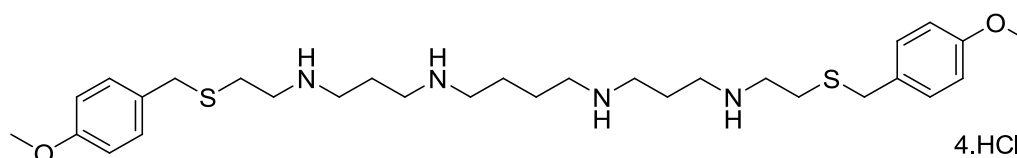
Sulfurtrioxide-pyridine complex (12.0 g, 76.0 mmol, 3.0 eq) was dissolved in dry DMSO (80.0 mL) under nitrogen. After stirring for 15 minutes, dry DCM (40.0 mL) was added. Concurrently, a solution of alcohol **1** (5.00 g, 25.3 mmol, 1.0 eq) and dry DIPEA (26.0 mL, 152.0 mmol, 6.0 eq) in DCM (60 mL) was prepared and added to the DMSO solution after the aforementioned 15 mins stirring. The resulting dark red

6. Experimental

solution was stirred for 18 h followed by addition of ethyl acetate (100 mL). The solution was then washed with water, 1N HCl, 1N NaHCO₃ and brine (3 x 50 mL each). The organic phase was dried over MgSO₄, filtered and evaporated to dryness to give a dark red oil. This oil was purified by chromatography (3:7 (v:v) Et₂O:hexane) isolating the product aldehyde 2 as a yellow oil (1.3 g, 26%(lit. 52 %)).

R_f 0.33 (3:7 (v:v) Et₂O:hexane); ¹H NMR (CDCl₃), (400 MHz): δ_H = 3.08 (d, ³J_{HH} 3.5 Hz, 2H, SCH₂CHO), 3.60 (s, 2H, ArCH₂S), 3.81 (s, 3H, OCH₃), 6.87 (d, ³J_{HH} 7.5 Hz, 2H, ArH²), 7.23 (d, ³J_{HH} 7.5 Hz, 2H, ArH³), 9.42 (t, ³J_{HH} 3.5 Hz, 1H, CHO). Data consistent with those previously reported.¹²⁰

6.2.3. Synthesis of *N,N'*-bis(((2-(4-methoxybenzylsulfanyl)ethyl)amino)propyl)butane-1,2-diamine (tetra-hydrochloride salt) (3)¹²⁰



3

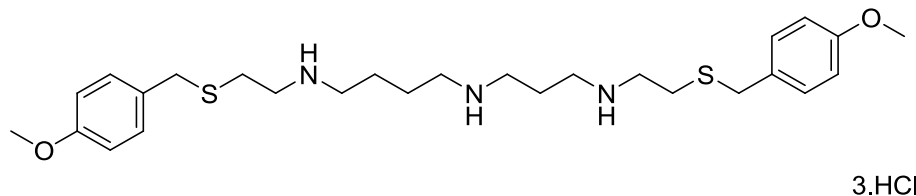
Magnesium sulphate (5.00 g) was suspended in dry DCM (100 mL) with stirring. Aldehyde 2 (1.00 g, 5.10 mmol, 2.1 eq) was added followed by spermine (0.500 g, 2.47 mmol, 1.0 eq). After stirring for 8 h, the suspension was filtered and added to a flask containing dry methanol (100 mL) with stirring. Sodium borohydride (0.950 g, 25.0 mmol, 10.0 eq) was added dropwise and the solution was stirred for another 18 h. After this time, concentrated hydrochloric acid (20 mL) was added causing evolution of gas and formation of a white precipitate. This suspension was refluxed for 6 h at 50 °C. Following concentration to around 10 mL of liquid on a rotary evaporator, the suspension was filtered and washed with ethyl acetate (50 mL). The resulting white solid was recrystallised from water to give a white crystalline solid (0.70 g, 38% (lit. 50%)).

¹H NMR (D₂O), (400 MHz): δ_H = 1.66 (4H, br, NHCH₂CH₂CH₂CH₂NH), 1.94 (4H, quin, ³J_{HH} 8.0 Hz, NHCH₂CH₂CH₂NH), 2.66 (4H, t, ³J_{HH} 6.5 Hz, SCH₂CH₂NH), 2.92-3.01 (16H, m, CH₂NHCH₂), 3.69 (4H, s, ArCH₂S), 3.73 (6H, s, OCH₃), 6.90 (4H,

6. Experimental

d, $^3J_{\text{HH}}$ 8.5 Hz, ArH^2), 7.25 (4H, d, $^3J_{\text{HH}}$ 8.5 Hz, ArH^3). Data consistent with those previously reported.¹²⁰

6.2.4. Synthesis of *N*-(2-(4-methoxybenzylsulfanyl)ethyl)-*N'*-(((2-(4-methoxybenzylsulfanyl)ethyl)amino)propyl)butane-1,4-diamine (tri-hydrochloride salt) (**4**)¹²⁰



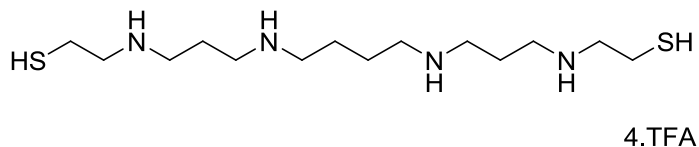
4

Magnesium sulphate (3.0 g) was suspended in dry DCM (100 ml) with stirring. Aldehyde **2** (1.00 g, 5.10 mmol, 2.0 eq) of was added followed by spermidine (0.40 mL, 2.5 mmol, 1.0 eq). After stirring for 8 h, the suspension was filtered and the filtrate rapidly transferred to a flask containing dry methanol (100 mL) with stirring and under nitrogen. Sodium borohydride (0.950 g, 25.0 mmol, 10.0 eq) was added and the solution was stirred for another 18 h. After this time, concentrated Hydrochloric acid (20 mL) was added dropwise causing evolution of gas and formation of a white precipitate. This suspension was refluxed for six hours at 50 °C. Following concentration to around 10 ml of liquid on a rotary evaporator, the suspension was filtered and washed with ethyl acetate (50 mL). The resulting white solid was recrystallised from water to give a white crystalline solid (0.67 g, 44% (lit. 53%)).

^1H NMR (D_2O), (400 MHz): δ_{H} = 1.60 (br, 4H, $\text{NHCH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{NH}$), 1.95 (quin, $^3J_{\text{HH}}$ 8.0 Hz, 2H, $\text{NHCH}_2\text{CH}_2\text{CH}_2\text{NH}$), 2.66 (t, $^3J_{\text{HH}}$ 7.0 Hz, 4H, $\text{SCH}_2\text{CH}_2\text{NH}$), 2.88-3.02 (m, 12H, CH_2NHCH_2), 3.69 (s, 4H, ArCH_2S), 3.73 (s, 6H, OCH_3), 6.90 (4H, d, $^3J_{\text{HH}}$ 8.0 Hz, ArH^2), 7.26 (4H, d, $^3J_{\text{HH}}$ 8.0 Hz, ArH^3). Data consistent with those previously reported.¹²⁰

6. Experimental

6.2.5. Synthesis of 3,7,12,16-tetraazaoctadecane-1,18-dithiol (tetra-trifluoro acetate salt) (**5**)¹²⁰

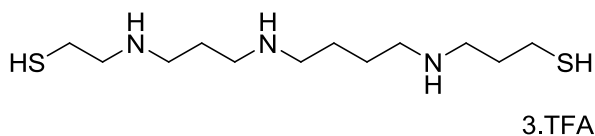


5

A solution of anisole (1.10 ml 10.1 mmol, 10.2 eq) in trifluoroacetic acid (30 mL) was degassed via the freeze-pump-thaw method. With stirring, protected dithiol **3** (0.700 g, 0.988 mmol, 1.0 eq) was added and the solution was heated at 90 °C for 3 days. This was followed by concentration to approximately 2 mL on a rotary evaporator. The resulting suspension was washed with diethyl ether (50 mL) and dissolved in the minimum amount of methanol. The product was precipitated with diethyl ether and filtered to give an off-white solid (0.37 g, 37% (lit. 64%)).

¹H NMR (*d*₄-MeOH), (400 MHz): 1.83 (4H, br, NHCH₂CH₂CH₂CH₂NH), 2.18 (4H, br, NHCH₂CH₂CH₂NH), 2.83 (4H, t, ³*J*_{HH} 7.0 Hz, HSCH₂), 3.1-3.3 (16H, m, CH₂NHCH₂). Data consistent with those previously reported.¹²⁰

6.2.6. Synthesis of 2-((3-((4-((2-sulfanylethyl)amino)butyl)amino)propyl) amino) ethanethiol (tri-trifluoroacetate salt) (**6**)¹²⁰



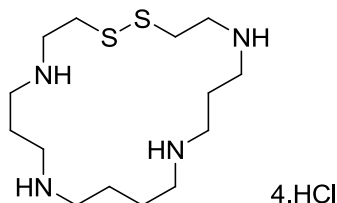
6

A solution of anisole (0.90 mL, 8.2 mmol, 10 eq) in trifluoroacetic acid (30 mL) was degassed via the freeze-pump-thaw method. With stirring, the protected dithiol **4** (0.490 g, 0.798 mmol, 1.0 eq) was added and the solution was refluxed at 90°C for 3 days. This was followed by concentration on a rotary evaporator. The resulting suspension was washed with diethyl ether (50 mL) and dissolved in the minimum amount of methanol. The product was precipitated with diethyl ether and filtered to give an off-white solid (0.15 g, 30% (lit.34%)).

6. Experimental

^1H NMR (d_4 -MeOH), (400 MHz): 1.83 (4H, br, $\text{NHCH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{NH}$), 2.16 (2H, br, $\text{NHCH}_2\text{CH}_2\text{CH}_2\text{NH}$), 2.84 (4H, dt, $^3J_{\text{HH}}$ 7.0 Hz 3.0 Hz, HSCCH_2), 3.0-3.3 (12H, m, CH_2NHCH_2).¹²⁰

6.2.7. Synthesis of di(1,2-dithia-5,9,14,18-tetraazacycloeicosane(tetra-hydro chloride salt)] (“Cyclic TetN343”) (7)¹²⁰



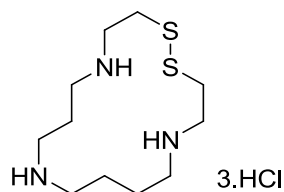
7

Dithiol 5 (0.360 g, 0.462 mmol, 1.0 eq) and DMSO (0.1 ml) were dissolved in concentrated hydrochloric acid (10 mL) and stirred for 24 h. The resulting suspension was concentrated on a rotary evaporator to approximately 1 mL. Acetone (25 mL) was added to further precipitate the suspension which was then filtered and washed with portions of acetone (2 x 25 mL). The solid was then dried *in vacuo* for 1 week at 95 °C to give an off-white solid (0.18 g, 88% (lit. 89%)).

^1H NMR (D_2O), (400 MHz): 1.67 (4H, br, $\text{NHCH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{NH}$), 2.02 (4H, br, $\text{NHCH}_2\text{CH}_2\text{CH}_2\text{NH}$), 2.9-3.1 (16H, m, CH_2NHCH_2), 3.34 (4H, br, CH_2SSCH_2). m/z (ES) Found: 321.2189; $\text{C}_{14}\text{H}_{32}\text{N}_4\text{S}_2$ requires $[\text{M}+\text{H}]^+$ 321.2068. Data consistent with those previously reported⁹².

6. Experimental

6.2.8. Synthesis of di[1,2-dithia-5,9,14-triazacyclohexadecane (tri-hydrochloride salt)] (8)⁹²

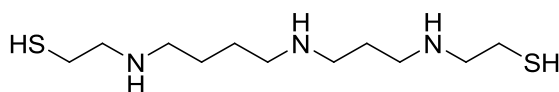


8

Dithiol **6** (75 mg, 0.12 mmol, 1.0 eq) and DMSO (0.1 mL) were dissolved in concentrated hydrochloric acid (10 mL) and stirred for 24 h. The resulting suspension was concentrated on a rotary evaporator to give a suspension of white solid. Acetone (25 mL) was added to further precipitate the suspension which was then filtered and washed with portions of acetone (2 x 25 mL). The solid was then dried *in vacuo* for 1 week at 95 °C to give an off-white solid (10 mg, 22% (lit. 87%)).

¹H NMR (D₂O), (400 MHz): 1.69 (4H, br, NHCH₂CH₂CH₂CH₂NH), 2.00 (4H, br, NHCH₂CH₂CH₂NH), 2.84-3.17 (12H, m, CH₂NHCH₂), 3.34 (4H, br, CH₂SSCH₂). Data consistent with those previously reported.¹²⁰

6.2.9. Attempted synthesis of 2-((3-((4-((2-sulfanylethyl) amino)butyl) amino)propyl)amino) ethanethiol using ethylene sulfide (**9**)

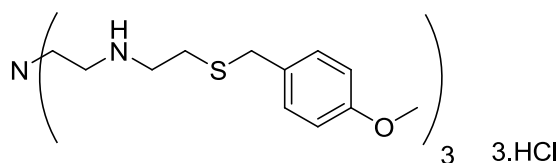


9

Spermidine (0.78 mL, 5.0 mmol, 1.0 eq) and ethylene sulphide (0.60 mL, 10 mmol, 2.0 eq) were added to dry toluene (25 mL) and refluxed for 24 h. During this time a white precipitate developed. The solution was filtered and solvent removed on a rotary evaporator to leave a colourless oil. No reaction had taken place by NMR. The white precipitate was insoluble in all NMR solvents.

6. Experimental

6.2.10. Attempted Synthesis of *N*¹-(2-(4-methoxybenzylthio)ethyl)-*N*²,*N*²-bis(2-(2-(4-methoxybenzylthio)ethylamino)ethyl)ethane-1,2-diamine (tri-hydrochloride salt)(**10**)

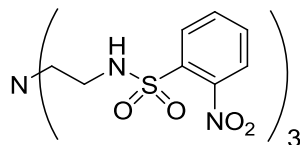


Magnesium sulphate (5.00 g) was suspended in dry DCM (100 mL) with stirring. Aldehyde **2** (1.10 g, 5.60 mmol, 3.5 eq) was added followed by (0.26 mL, 1.6 mmol, 1.0 eq) of tris(2-aminoethyl)amine. After stirring for 18 h, the suspension was filtered and added to a flask containing dry methanol (100 mL) with stirring. Sodium borohydride (2.20 g, 16.0 mmol, 10.0 eq) was added and the solution was stirred for another 18 h. After this time, concentrated hydrochloric acid (20 mL) was added causing evolution of gas and formation of a white precipitate. This suspension was heated for six hours at 50 °C. Following evaporation of dryness on a rotary evaporator, the suspension was filtered and washed with ethyl acetate (50 mL). The resulting white solid was incompletely dissolved in hot water and the subsequent attempt at recrystallisation failed. Characterisation of crude material showed incomplete substitution.

¹H NMR (D₂O), (400 MHz): δ_{H} = 2.90 (t, NHCH₂CH₂S), 2.99 (m, NCH₂CH₂NH), 3.27 (m, NCH₂CH₂NHCH₂), 3.91 (s, ArCH₂S), 3.92 (s, CH₃O), 7.09 (d, CCHCHCOMeCHCH), 7.47 (d, CCHCHCOMeCHCH). MS (ES): Found 685.33 (tri-substituted, 93%), 507.28 (di-substituted, 100%), 326.21 (mono-substituted, 48%).

6. Experimental

6.2.11. Synthesis of *N,N,N*-tris(2-(*N'*-o-nitrobenzenesulfonyl)aminoethyl) amine (**11**)

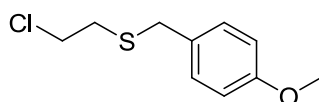


11

Tris(2-aminoethyl)amine (2.00 g, 13.7 mmol, 1.0 eq) and dry triethylamine (7.00 mL, 50.1 mmol, 3.6 eq) were dissolved in dry DCM (200 mL). The solution was cooled in an ice bath and a solution of 2-nitrobenzenesulfonyl chloride (9.40 g, 42.4 mmol, 3.1 eq) in DCM (200 mL) was added dropwise over 15 mins. The reaction was left stirring overnight. The reaction mixture was extracted with water (6 x 100 mL) dried over MgSO_4 , filtered and reduced to dryness on a rotary evaporator. The material was then purified by chromatography (4:1 (v:v), DCM:EtOAc) to give a yellow amorphous solid after solvent had been removed. Repeated dissolution in DCM and evaporation to dryness (~3 cycles) ultimately led to a pale yellow powder (5.3 g, 55%).

$R_f=0.38$ (4:1 (v:v), DCM:EtOAc), ^1H NMR (CDCl_3), (400 MHz): $\delta_{\text{H}} = 2.67$ (6H, br, $\text{NCH}_2\text{CH}_2\text{NH}$), 3.14 (6H, br, $\text{NCH}_2\text{CH}_2\text{NH}$), 5.85 (3H, br s, NH), 7.76 (6H, quin d, $^3J_{\text{HH}} 7.5 \text{ Hz } 1.5 \text{ Hz}$, $\text{ArH}^4 \text{ ArH}^5$), 7.84 (3H, dd, $^3J_{\text{HH}} 7.5 \text{ Hz } 1.5 \text{ Hz}$, ArH^6), 8.12 (3H, dd, $^3J_{\text{HH}} 7.5 \text{ Hz } 1.5 \text{ Hz}$, ArH^3). ^{13}C NMR (CDCl_3), (400 MHz): $\delta_{\text{C}} = 41.66$ ($\text{NCH}_2\text{CH}_2\text{NH}$), 54.81 ($\text{NCH}_2\text{CH}_2\text{NH}$), 125.57 ($\text{SO}_2\text{C}_6\text{H}_4\text{NO}_2$ C³), 130.92, 132.99, 133.07, 133.77, 148.07 ($\text{SO}_2\text{C}_6\text{H}_4\text{NO}_2$ C²), m/z (ES) Found: 702.0945; $\text{C}_{24}\text{H}_{27}\text{N}_7\text{O}_{12}\text{S}_3$ requires $[\text{M}+\text{H}]^+$ 702.088. Data consistent with those previously reported.¹²²

6.2.12. Synthesis of 1-(2-chloro-ethylsulfanylmethyl)-4-methoxy-benzene (**12**)¹²⁰



12

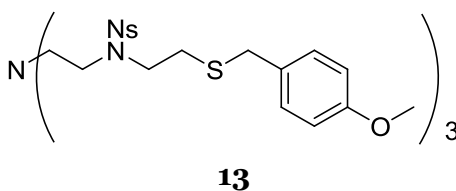
Alcohol 1 (5.0 g, 25.2 mmol, 1.0 eq) and dry pyridine (2.2 mL, 27.7 mmol, 1.1 eq) were dissolved in 100 mL of dry chloroform with stirring under nitrogen at 0°C in an ice-bath. Thionyl chloride (20.0 mL, 27.7 mmol, 1.1 eq) was added dropwise. The ice

6. Experimental

bath was replaced with an oil bath and the reaction was heated at reflux for 18 h. 9:1 (v:v) DCM:H₂O (10 mL) was added and the organic phase was separated, washed with brine (3x25 mL), dried over MgSO₄, filtered and evaporated to dryness to give 3.4 g of yellow-orange liquid (3.4 g, 62%). No attempt at purification was made due to the potentially vesicant nature of the material.¹²⁰

¹H NMR (CDCl₃), (400 MHz): δ_{H} = 2.78 (2H, m, SCH₂CH₂), 3.55 (2H, m, SCH₂CH₂), 3.74 (2H, s, ArCH₂S), 3.83 (2H, s, CH₃O), 6.90 (2H, d, $^3J_{\text{HH}}$ 8.6 Hz, CHC(OMe)CH), 7.27 (2H, d, $^3J_{\text{HH}}$ 8.6 Hz, CHC(CH₂S)CH). Data consistent with those previously reported.¹²⁰

6.2.13. *N,N,N'*-(2,2',2''-nitrilotris(ethane-2,1-diyl)) tris(*N*-(2-(4-methoxybenzylthio)ethyl)-2-nitrobenzene-sulfonamide) (**13**)

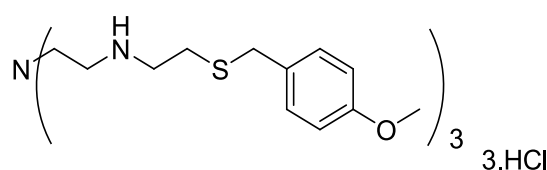


To dry DMF (200 mL) were added protected amine **11** (5.40 g, 7.70 mmol, 1.0 eq) and Cs₂CO₃ (10.0 g, 30.7 mmol, 4.0 eq) under nitrogen with stirring. Chloride **12** (5.40 mL, 30.6 mmol, 4.0 eq) was added and the solution was stirred at 90 °C for 3 days during which time the solution turned from yellow to orange. 500 mL of 1N aqueous NaHCO₃ was added in one portion increasing the cloudiness of the suspension. The mixture was left to stand overnight during which time a thick brown oil formed. Following decantation of the aqueous supernatant, the oil was dissolved in DCM (100 mL). The resulting orange solution was washed with 1N aqueous NaHCO₃ (200 mL in three portions), dried over MgSO₄, filtered and reduced to dryness on a rotary evaporator. The material was then purified by chromatography (5% EtOAc/DCM (v/v)) to give an orange oil after solvent had been removed under reduced pressure. Repeated dissolution in DCM and evaporation to dryness (~3 cycles) ultimately led to a yellow amorphous solid (3.08 g, 32% (90% pure by ¹H NMR)).

6. Experimental

R_f 0.43 (5% EtOAc/DCM (v/v)) ^1H NMR (CDCl_3), (400 MHz): δ_{H} = 2.53-2.74 (12H, m, $\text{SCH}_2\text{CH}_2\text{N}$), 3.25 – 3.47 (12H, m, $\text{CH}_2\text{NNsCH}_2$), 3.68 (6H, s, SCH_2Ar), 3.81 (9H, s, CH_3O), 6.85 (d, 2H, $^3J_{\text{HH}}$ 8 Hz, MeOBn ArH^2), 7.24 (d, 2H, $^3J_{\text{HH}}$ 8 Hz, MeOBn ArH^3), 7.60-7.75 (m, 9H, nosyl ArH^4 ArH^5 ArH^6), 7.89-7.95 (m, 3H, nosyl ArH^3); m/z (ES) Found: 1242.2832; $\text{C}_{54}\text{H}_{63}\text{N}_7\text{O}_{15}\text{S}_6$ requires $[\text{M}+\text{H}]^+$ 1242.2706.

6.2.14. *N*-(2-(4-methoxybenzylthio)ethyl)-*N*',*N*'-bis(2-(2-(4-methoxybenzylthio)ethylamino)ethyl)ethane-1,2-diamine (tri-hydrochloride salt) (**14**)



14

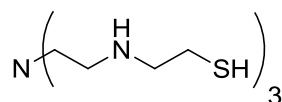
Protected thiol **13** (3.00 g, 2.41 mmol, 1.0 eq) was dissolved in acetonitrile (50 mL) with stirring. Potassium carbonate (2.00 g, 14.5 mmol, 6.0 eq) and thiophenol (1.05 mL, 14.5 mmol, 6.0 eq) were added and the reaction was left stirring in a sealed flask at room temperature for 3 days. After this time, the solvent was removed on a rotary evaporator and the resulting residue was dissolved in a 1:1 (v:v) mixture of DCM and water (100 mL). Following separation, the aqueous layer was washed with DCM (3 x 50 mL) and then the combined organic portions were washed with 1N aqueous NaHCO_3 (200 mL in three portions), dried over MgSO_4 , filtered and reduced to dryness on a rotary evaporator. The resulting oil was dissolved in 1.5 M HCl in methanol (100 mL) and reduced to dryness. Now solid, the material was added to water (500 mL), shaken briefly and left overnight. The suspension was filtered and the supernatant evaporated to dryness to give a residue. The bright yellow filtrate was washed with acetone (300 mL) and the remaining solid combined with the residue obtained earlier from the removal of water. This combination was then crystallised from methanol and recrystallised until all the nitrobenzyl resonances were absent from ^1H NMR (typically 3 recrystallisations) to give a white solid (39%).

^1H NMR (CDCl_3), (400 MHz): δ_{H} = 2.95-3.28 (24H, m, $\text{NCH}_2\text{CH}_2\text{N}$ $\text{CH}_2\text{CH}_2\text{S}$), 3.78 (9H, s, CH_3O), 3.80 (6H, s, SCH_2Ar), 6.87 (d, 2H, $^3J_{\text{HH}}$ 8.0 Hz, MeOBn ArH^2), 7.30

6. Experimental

(d, 2H, $^3J_{HH}$ 8.0 Hz, MeOBn ArH³), 9.78 (br, 6H, +NH₂). *m/z* (ES) Found: 687.3463; C₃₆H₅₄N₄O₃S₃ requires [M+H]⁺ 687.3358.

6.2.15. Synthesis of 2,2',2''-(2,2',2''-nitrilotris(ethane-2,1-diyl) tris(azanediyl)) triethanethiol (tri-hydrochloride salt) ("Brancher") (**15**)



3.HCl

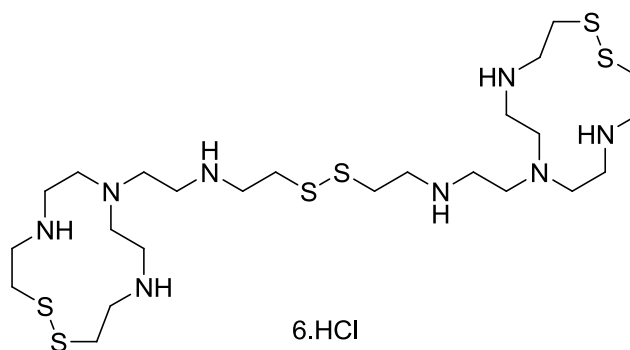
15

A solution of anisole (0.68 mL, 6.2 mmol, 10.0 eq) in TFA (50 mL) was degassed using the freeze-pump-thaw method. Under N₂ with stirring the protected trithiol **14** (0.500 g, 0.628 mmol, 1.0 eq) was added and the solution was heated at 70 °C for three days. The solvent was removed under reduced pressure and the resulting residue was washed with dry, degassed diethyl ether (50 mL then 10 mL). After drying the residue under high-vacuum, deoxygenated conc. HCl (50 mL) was added and the flask shaken. The acidic solution was decanted and reduced to dryness on a rotary evaporator followed by high-vacuum to give an off-white solid (0.17 g, 62%).

¹H NMR (DCl/D₂O, Acetone in capillary used for lock), (400 MHz): δ_H = 1.96 (6H, t, $^3J_{HH}$ 6.0 Hz, CH₂SH), 2.45 (6H, t, $^3J_{HH}$ 6.0 Hz, CH₂CH₂SH), 2.83 (6H, br t, NCH₂CH₂NH), 2.95 (6H, br t, NCH₂CH₂NH). *m/z* (ESI) Calculated [MH⁺] 327.16326, Observed [MH⁺] 327.1696.

6. Experimental

6.2.16. Synthesis of [2-(1,2-Dithia-5,8,11-triaza-cyclotridec-8-yl)-ethyl]-(2-{2-[2-(1,2-dithia-5,8,11-triaza-cyclotridec-8-yl)-ethylamino]-ethyl}disulfanyl)-ethyl)-amine (hexa-hydrochloride salt) (“Oxidised Brancher”) (**16**)

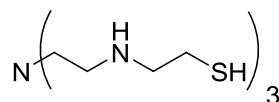


16

Trithiol **15** (1.0 mg) was dissolved in D₂O (1.0 mL). The solution was left in a sealed NMR tube for 45 days. After this time, the solution was transferred to a flask and freeze-dried. The resulting white solid was immediately redissolved in water for use in biological experiments.

¹H NMR (D₂O), (400 MHz): $\delta_{\text{H}} = 2.78$ (11H, t, $^3J_{\text{HH}} 6.0$ Hz, NCH₂CH₂NH), 3.06 (12H, t, $^3J_{\text{HH}} 7.5$ Hz, CH₂CH₂SS), 3.19 (12H, t, $^3J_{\text{HH}} 6.0$ Hz, NCH₂CH₂NH), 3.44 (12H, t, $^3J_{\text{HH}} 7.5$ Hz, CH₂CH₂SH). *m/z* (ESI) Calculated [MH⁺] 647.2796, Observed [MH⁺] 647.2924.

6.2.17. Attempted synthesis of 2,2',2''-(2,2',2''-nitrilotris(ethane-2,1-diyl)tris(azanediyl))triethanethiol using ethylene sulfide (**17**)



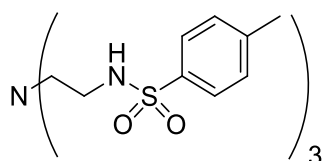
17

Tris(2-aminoethyl)amine (1.00 g, 6.83 mmol, 1 eq) was dissolved in water and the pH regulated to 7.1 (by pH meter) using 1N HCl. The water was removed on a rotary evaporator to give a white solid. The solid was redissolved in water and a solution of aqueous NaBPH₄ was added. A white precipitate formed immediately, was collected

6. Experimental

by filtration and dried overnight in an oven. This was dissolved in degassed methanol (20 mL) and ethylene sulfide (1.40 mL, 23.1 mmol, 3.4 eq) was added. The reaction was left stirring for 72 h at room temperature under nitrogen. No reaction was observed by NMR.

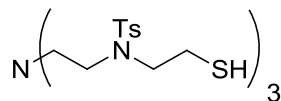
6.2.18. Synthesis of *N,N',N''*-(2,2',2''-nitrilotris(ethane-2,1-diyl))tris(4-methylbenzenesulfonamide) (**18**)



18

Tris(2-aminoethyl)amine (4.00 g, 27.4 mmol, 1.0 eq) was dissolved in a solution of NaOH (3.39 g, 84.8 mmol, 3.1 eq) in water (70 mL). To this solution was added dropwise tosyl chloride (16.2 g, 84.8 mmol, 3.1 eq) in diethyl ether (60 mL) with stirring under nitrogen. Stirring was continued for 2 h and then left to stand for 12 h. The resulting white precipitate was collected and washed with copious amounts of water. After drying under high-vacuum a white solid was collected (13.8 g, 84 %). ¹H NMR (CDCl₃), (400 MHz): δ_H = 2.45 (s, 9H, ArCH₃), 2.51 (t, 6H, NCH₂CH₂NArH), 2.94 (dt, 6H, NCH₂CH₂NArH), 6.10 (t, 3H, ArNH), 7.33 (d, 6H, ³J_{HH} 8 Hz, ArH₂ ArH₆), 7.83 (d, 6H, ³J_{HH} 8 Hz, ArH₃ ArH₅). Consistent with data previously reported.¹²²

6.2.19. Synthesis of *N,N',N''*-(2,2',2''-nitrilotris(ethane-2,1-diyl))tris(*N*-(2-mercaptoethyl)-4-methylbenzenesulfonamide) (**19**)



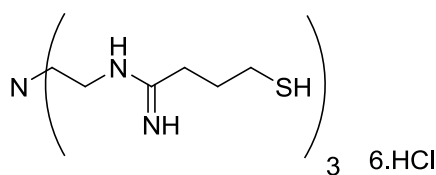
19

Tosylated amine **18** (0.500 g, 0.821 mmol, 1.0 eq) was dissolved in either degassed dry methanol or degassed dry DMF under nitrogen with stirring. For both solvents,

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ethylene sulfide (0.200 g, 3.33 mmol, 4.0 eq) was added. For basic conditions, catalytic amounts of Cs_2CO_3 were added. The solutions were left to stir at RT until either polymer formation was observed or for 5 days (methanol). The DMF experiment was heated at 90 °C for 2 days after which temperature was increased to 115 °C. No reactions were observed by TLC for either set of conditions.

6.2.20. Attempted NMR scale synthesis of *N,N',N''*-(2,2',2''-nitrilotris(ethane-2,1-diyl)tris(4-mercaptobutanimidamide) (hexa-hydrochloride salt) (**20**)

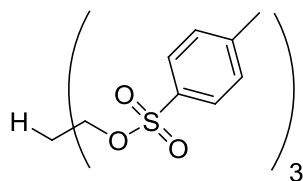


Tris(2-aminoethyl)amine (25.6 μL , 0.171 mmol, 1.0 eq) of was dissolved in degassed 1% aqueous acetic acid (7.0 mL) followed by 2-iminothiolane (73 mg, 0.53 mmol, 3.1 eq). 0.1N NaOH was used to adjust the pH to 7.0 and the mixture was left to stir at room temperature for 2 h. After this time 0.75 mL of the solution was added to concentrated HCl (0.5 mL) and evaporated to dryness on a rotary evaporator. The resultant white crystals were dissolved in degassed D_2O and left for 2 weeks over which time the reaction appeared to proceed to near-completion as discussed in Section 2.11.

^1H NMR (D_2O), (400 MHz): δ_{H} 2.24 (2H, tt, $^3J_{\text{HH}}$ 7.3 Hz 6.6 Hz, $\text{CH}_2\text{CH}_2\text{CH}_2$), 2.54 (2H, t, $^3J_{\text{HH}}$ 7.2 Hz, CH_2SH), 2.85 (2H, t, $^3J_{\text{HH}}$ 6.9 Hz, $\text{NCH}_2\text{CH}_2\text{NH}$), 3.11 (2H, t, $^3J_{\text{HH}}$ 6.9 Hz, $\text{NCH}_2\text{CH}_2\text{NH}$), 3.42 (2H, t, $^3J_{\text{HH}}$ 6.6 Hz, $\text{NHCH}_2\text{CH}_2\text{CH}_2\text{SH}$). Concentration too low for ^{13}C NMR.

6. Experimental

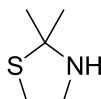
6.2.21. Attempted synthesis of toluene-4-sulfonic acid 3,2-bis(toluene-4-sulfonyl methoxy)–propyl ester (**21**)



21

2-(hydroxymethyl)-1,3-propane diol (0.500 g, 4.71 mmol, 1.0 eq), dry pyridine (1.40 mL, 16.9 mmol, 3.6 eq) and DMAP (60 mg, 0.47 mmol, 0.1 eq) were dissolved in dry DCM (50 mL) and cooled to 0°C under N₂ with stirring. A solution of tosyl chloride (3.00 g, 15.5 mmol, 3.3 eq) in DCM (20 mL) was added dropwise. The reaction was stirred for 18 h. After this, the reaction mixture was washed with H₂O (3x 15 mL), Dried with MgSO₄, filtered and evaporated to dryness to give a colourless oil, almost freezing at RT to give a white solid. A TLC on silica eluted with 100% DCM gave spots with R_f = 0.82, 0.49, 0.13 and 0. NMR showed mixture of tosyl chloride (51%), and mono- (10%), di- (31%) and tri- (8%) substituted product.

6.2.22. Synthesis of 2,2-dimethyl-thiazolidine (**22**)



22

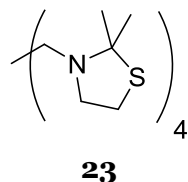
Cysteamine (1.00 g, 13.0 mmol, 1.0 eq) was stirred at 35 °C in dry acetone (100 mL) over 3 h. After this time, heating was removed and MgSO₄ (5.00 g) and dry DCM (150 mL) were added. The reaction was stirred under nitrogen for a further 5 days at room temperature. It was then filtered and solvent reduced to around 2 mL under reduced pressure. The cloudy suspension was filtered with a 200 nm PTFE syringe filter and the filtrate evaporated to dryness under reduced pressure to give a colourless oil (0.52 g, 34%) which was used immediately without purification.

¹H NMR (CDCl₃), (400 MHz): δ_H = 1.58 (s, 6H, CH₃), 2.26 (br, 1H, NH), 3.06 (t, ³J_{HH} = 6.3 Hz, 2H, CH₂S), 3.34 (t, ³J_{HH} = 6.3 Hz, 2H, CH₂NH). ¹³C NMR (CDCl₃), (400

6. Experimental

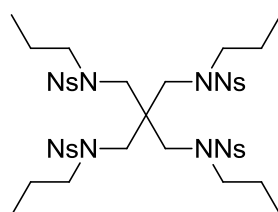
MHz): δ_c = 31.5 (q), 37.6 (t), 51.3 (t), 76.3 (q). Data consistent with those previously reported.¹³⁹

6.2.23. Attempted Synthesis of 3,3'-(2,2-bis((2,2-dimethylthiazolidin-3-yl)propane-1,3-diyl)bis(2,2-dimethylthiazolidine) (**23**)



2,2-dimethylthiazolidine (**22**) (0.500 g, 4.27 mmol, 3.3 eq) was dissolved in dry acetonitrile (100 mL) and K_2CO_3 (0.640 g, 4.63 mmol, 3.6 eq) was added with stirring. Pentaerythritol tetrabromide (0.500 g, 1.29 mmol, 1.0 eq) in dry acetonitrile (50 mL) was then added dropwise. No reaction was observed by TLC or NMR after 3 days.

6.2.24. Attempted synthesis of *N,N'*-(2,2-bis((*N*-propyl-2-nitrophenylsulfonamido)methyl)propane-1,3-diyl)bis(*N*-propyl-2-nitrobenzenesulfonamide) (**24**)

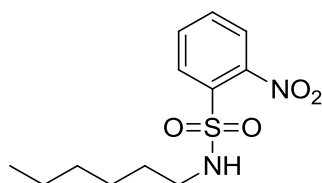


24

N-Nosyl propylamine (0.200 g, 0.819 mmol, 4.4 eq) was dissolved in dry acetonitrile (25 mL) with potassium carbonate (0.130 g, 0.941 mmol, 5.0 eq) with stirring under nitrogen. A solution of pentaerythritol tetrabromide (72 mg, 0.19 mmol, 1.0 eq) in acetonitrile (10 mL) was added dropwise. The vessel was then heated to reflux. No reaction was observed by TLC or aliquot NMR over 48 h.

6. Experimental

6.2.25. Synthesis of *N*-nitrobenzenesulfonyl hexylamine (**25**)

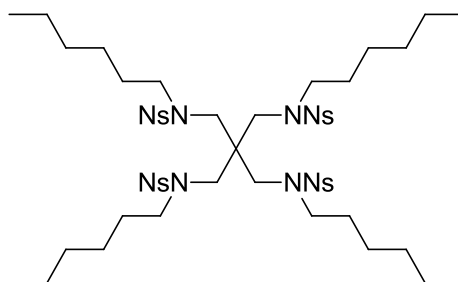


25

Hexylamine (0.60 mL, 4.2 mmol, 1.0 eq) and dry triethylamine (0.70 mL, 5.0 mmol, 1.2 eq) were dissolved in dry DCM (50 mL). The solution was cooled in an ice bath and a solution of 2-nitrobenzenesulfonyl chloride (1.0 g, 4.51 mmol, 1.1 eq) in DCM (50 mL) was added dropwise over 15 mins. The reaction was left stirring overnight. The reaction mixture was extracted with water (6 x 100 mL), dried over MgSO_4 , filtered and reduced to dryness on a rotary evaporator. The material was then purified by chromatography (2:1 (v:v), EtOAc:Hexane) to give a yellow amorphous solid after solvent had been removed. Repeated dissolution in DCM and evaporation to dryness (~3 cycles) ultimately led to a pale yellow powder (1.0 g, 83%).

$R_f=0.83$ (2:1 (v:v), EtOAc:hexane), ^1H NMR (CDCl_3), (400 MHz): $\delta_{\text{H}} = 0.86$ (3H, t, $^3J_{\text{HH}} 7.0$ Hz, CH_3), 1.18-1.35 (6H, m, $\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_3$), 1.53 (2H, quin, NH), 3.11 (2H, q, $^3J_{\text{HH}} 6.0$ Hz,), 5.27 (1H, br t, NH), 7.76 (2H, quin, NH), 7.88 (1H, m, ArH^6), 8.16 (1H, m, ArH^3).

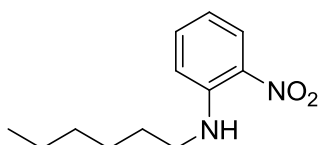
6.2.26. Attempted synthesis of *N,N'*-(2,2-bis((*N*-hexyl-2-nitrophenylsulfonamido)methyl)propane-1,3-diyl)bis(*N*-hexyl-2-nitrobenzenesulfonamide) (**26**)



26

6. Experimental

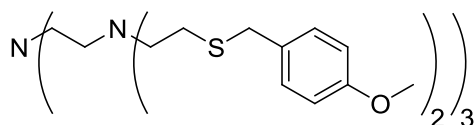
To a solution of pentaerythritol tetrabromide (55 mg, 0.14 mmol, 1.0 eq) in DMF (10 ml) was added potassium carbonate (99 mg, 0.72 mmol, 5.0 eq) and sodium iodide (2 mg, 0.02 mmol, 0.1 eq) with stirring under nitrogen was added a solution of **25** (180 mg, 0.62 mmol, 4.4 eq) in dry DMF (25 mL). The vessel was then heated at 120°C. After addition of 1M NaOH (25 mL) and extraction with CHCl₃ (25 mL) the organic phase was dried over MgSO₄ and solvent removed. The resulting orange oil was purified over silica. The product of the reaction was identified as N-hexyl 2-nitroaniline (**27**).



27

¹H NMR (CDCl₃), (400 MHz): δ_{H} = 0.94 (t, 3H, CH₃), 1.27-1.53 (m, 6H, NHCH₂CH₂(CH₂)₃CH₃), 1.76 (m, ³J_{HH} = 6.3 Hz, 2H, NHCH₂CH₂), 3.32 (m, 2H, NHCH₂), 6.65 (t, 1H, Ar-H), 6.87 (d, 1H, Ar-H), 7.43 (t, 1H, Ar-H), 8.09 (br s, 1H, NH), 8.19 (t, 1H, Ar-H). *m/z* (ES): Found 223.1 Calculated (MH⁺) 222.1368. Data consistent with those previously reported.¹⁴⁰

6.2.27. Attempted synthesis of *N*¹,*N*¹-bis(2-(bis(2-(4-methoxybenzylthio)ethyl)amino)ethyl)-*N*²,*N*²-bis(2-(4-methoxybenzylthio)ethyl)ethane-1,2-diamine (**28**)

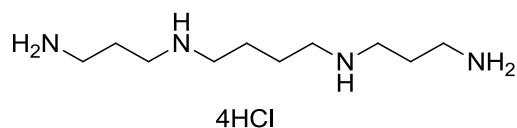


28

Tris(2-aminoethyl)amine (0.310 g, 2.12 mmol, 1.0 eq) was dissolved in DMF (100 mL) under nitrogen with stirring. Following the addition of Cs₂CO₃ (4.50 g, 13.9 mmol, 6.6 eq), chloride **12** (3.00 g, 13.9 mmol, 6.6 eq) was added dropwise and the reaction was heated at 90 °C for three days. After this time, reduction to dryness and NMR showed that no reaction had taken place between chloride and amine.

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6.2.28. Preparation of spermine tetrahydrochloride (**29**)

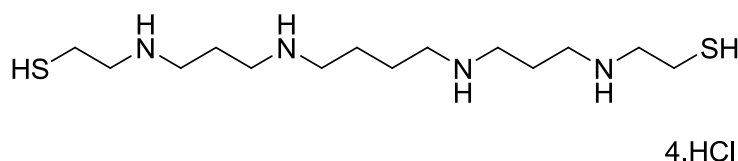


29

Spermine (0.100 g, 0.494 mmol, 1.0 eq) was dissolved in conc. HCl (10 mL) and stirred at room temperature for 1 hr. After this time it was dried thoroughly on a rotavap until a free flowing powder was obtained. A white solid was collected (0.17 g, 98 %).

^1H NMR (D_2O), (400 MHz): $\delta_{\text{H}} = 1.68$ (m, 4H, $\text{NHCH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{NH}$), 1.98 (m, 4H, $\text{NHCH}_2\text{CH}_2\text{CH}_2\text{NH}$), 2.92 – 3.12 (m, 12H, CH_2NHCH_2).

6.2.29. Preparation of 3,7,12,16-tetraazaoctadecane-1,18-dithiol (tetrahydrochloride salt) (“Linear TetN343”)(**30**)



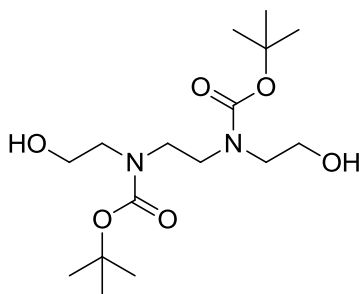
30

The tetrafluoroacetate salt **5** (0.100 g, 0.494 mmol) was dissolved in conc. HCl (10 mL) and stirred at room temperature for 1 hr. After this time it was dried thoroughly on a rotavap until a free flowing powder was obtained. A white solid was collected (0.17 g, 98 %).

^1H NMR (d_4 -MeOH), (400 MHz): 1.68 (4H, br, $\text{NHCH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{NH}$), 2.03 (4H, br, $\text{NHCH}_2\text{CH}_2\text{CH}_2\text{NH}$), 2.76 (4H, t, $^3J_{\text{HH}}$ 7.0 Hz, HSCH_2), 2.93-3.23 (16H, m, CH_2NHCH_2).

6. Experimental

6.2.30. Synthesis of *N,N'*-di(*tert*-butoxycarbonyl)-3,6-diazaoctane (**31**)

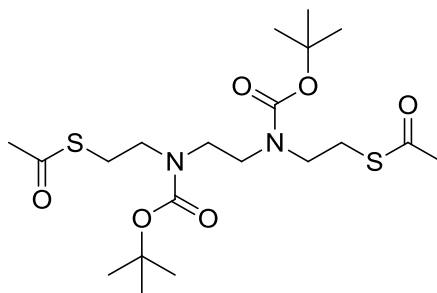


31

Bis(2-hydroxyethyl)ethylenediamine (2.96 g, 20.0 mmol, 1.0 eq) was dissolved in chloroform (200 ml). Di-*t*-butyl dicarbonate (9.14 g, 42.0 mmol, 2.1 eq) was added followed by saturated, aqueous sodium bicarbonate solution (10 mL). The resulting biphasic solution was stirred for 5 h. After this time the organic layer was separated, washed with water (3 x 50 mL), dried over MgSO_4 and reduced to dryness on a rotavap. The crude product was then washed with hexane to give a white solid (4.75 g, 75 % (lit. 48%)).

^1H NMR (CDCl_3), (400 MHz): $\delta_{\text{H}} = 1.49$ (s, 18H, $\text{C}(\text{CH}_3)_3$), 3.08 (br s, 2H, OH), 3.32 - 3.56 (m, 8H, $\text{CH}_2\text{N}(\text{COOC}(\text{CH}_3)_3)\text{CH}_2$), 3.76 (t, 4H, $^3J_{\text{HH}}$ 5.0 Hz, CH_2OH). Data consistent with those previously reported.¹²⁰

6.2.31. *N,N'*-Di(*tert*-butoxycarbonyl)-1,8-dithioacetate-3,6-diazaoctane (**32**)



32

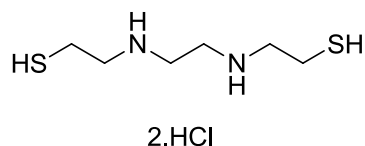
Triphenylphosphine (6.02 g, 22.1 mmol, 1.0 eq) was dissolved in dry THF (200 mL) and cooled to 0 °C. DIAD (4.52 mL, 22.1 mmol, 1.0 eq) was added and the resulting solution was stirred for 45 mins during which time a precipitate formed. Protected amine **31** (1.75 g, 22.1 mmol, 1 eq) and thioacetic acid (1.64 mL, 22.1 mmol, 1.0 eq)

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(as a solution in dry THF (50 mL)) were added dropwise over 5 mins. The resulting suspension was stirred for 1 h in an ice bath and for a further 15 h during which time the precipitate disappeared. After the removal of the solvent, a yellow solid was obtained and purified over silica (4:1 (v:v) Hexane:EtOAc, R_f 0.30) (0.54 g, 23% (lit. 65%)).

^1H NMR (CDCl_3), (400 MHz): $\delta_{\text{H}} = 1.49$ (s, 18H, $\text{C}(\text{CH}_3)_3$), 2.37 (s, 6H, SCOCH_3), 3.00 - 3.10 (m, 4H, SCH_3), 3.27-3.43 (m, 8H, $\text{CH}_2\text{N}(\text{COOC}(\text{CH}_3)_3)\text{CH}_2$). Data consistent with those previously reported.¹²⁰

6.2.32. Synthesis of *N,N'*-diazaoctane-1,8-dithiol (bis-hydrochloride salt) (**33**)

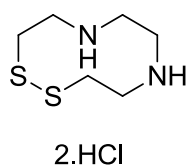


33

33 (0.540 g, 1.25 mmol, 1.0 eq) was dissolved in conc. HCl (50 ml) and stirred for 1 h at reflux. After this time, solvent was removed to give a white solid (0.26 g, 72% (lit. 84%)).

^1H NMR (D_2O), (400 MHz): $\delta_{\text{H}} = 2.76$ (t, 4H, $^3J_{\text{HH}}$ 6.5 Hz, CH_2SH), 3.23 (t, 4H, $^3J_{\text{HH}}$ 6.5 Hz, $\text{NHCH}_2\text{CH}_2\text{SH}$), 3.40 (s, 4H, $\text{NHCH}_2\text{CH}_2\text{NH}$). Data consistent with those previously reported.¹²⁰

6.2.33. Synthesis of 1,2-dithia-5,8,-diazacyclodecane (bis-hydrochloride salt) (**34**)



34

Dithiol (**33**) (130 mg, 0.513 mmol, 1.0 eq) and DMSO (0.1 mL) were dissolved in concentrated hydrochloric acid (10 mL) and stirred for 24 h. The resulting

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suspension was concentrated on a rotary evaporator to give a suspension of white solid. Acetone (25 mL) was added to precipitate the suspension fully which was then filtered and washed with portions of acetone (2x25 mL).

^1H NMR (D_2O), (400 MHz): δ_{H} = 2.85-3.03 (m, 6.81H) 3.08 (t, 4H, $^3J_{\text{HH}}$ 6.5 Hz, CH_2S), 3.32-3.53 (m, 21.1H). m/z (ESI) Calculated $[\text{MH}^+]$ 179.05984, Observed $[\text{MH}^+]$ 179.0672 (2%), 357.1338 (100%). Calculated $[\text{MH}^+]$ for dimeric species is 357.11968.

Literature spectrum: δ_{H} = 3.06 (t, 4H, $^3J_{\text{HH}}$ = 6.5 Hz, CH_2S), 3.53 (m, 8H, NHCH_2), 3.32-3.53 (m, 21.1H).

6.3. Formulation

6.3.1. Polyacrylamide Gel Electrophoresis

Polyplexes were prepared by adding solution of cation (as 1 mg mL^{-1} in RNase free water made up to $4 \mu\text{L}$ with buffer) to a solution of siRNA ($1.0 \mu\text{g}$ in $4.0 \mu\text{L}$ of buffer). Polyplexes were prepared at an N/P ratio of 0, 1, 2, 5, 10, 15, 20, 30 and 40 using cyclic TetN343 (7). After vortexing for five seconds, the solutions were left at room temperature for one hour. Immediately prior to being run on the gel, $2.0 \mu\text{L}$ of sample buffer was added. Gels were run at fixed voltage (200 V) for 1 h. Gels were developed by soaking in 1x aqueous SYBR GREEN for 1 hr and subsequently photographed with a UV camera.

6.3.2. Propidium Iodide Exclusion Assay

6.3.2.1. General Procedure

Unless otherwise specified, polyplexes were prepared by adding a solution of cation (prepared by dissolving a 1 mg mL^{-1} aqueous solution of cation in sufficient buffer to give a final volume of $100.0 \mu\text{L}$) to a solution of siRNA ($0.69 \mu\text{g}$ in $100.0 \mu\text{L}$ of buffer). “Blank” solutions containing no siRNA but with otherwise identical compositions were also prepared. After vortexing for five seconds, the solutions were left to incubate at room temperature. $2.5 \mu\text{M}$ aqueous propidium iodide solution ($200 \mu\text{L}$) was added to each sample and left for 5 mins. $220 \mu\text{L}$ of each solution was added to a

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black 96-well plate and fluorescence measured at 536 nm excitation and 617 nm emission. Between three and six measurements were made per experiment.

6.3.2.2. Encapsulation efficiency of cyclic TetN343 with respect to N/P Ratio

Polyplex solutions were prepared using cyclic TetN343 (**7**) as the cation and pH 7, 4 mM HEPES as buffer at the following N/P ratios: 0, 1, 2, 5, 10, 15, 20, 30, 40. Each sample was incubated for 1 h after mixing. Fluorescence was measured according to the general procedure described in Section 6.3.2.1.

6.3.2.3. Encapsulation efficiency of linear TetN343 with respect to N/P Ratio

Polyplex solutions were prepared using linear TetN343 (**30**) as the cation and pH 7, 4 mM HEPES as buffer at the following N/P ratios: 0, 1, 2, 5, 10, 15, 20, 30, 40. Each sample was incubated for 1 h after mixing. Fluorescence was measured according to the general procedure described in Section 6.3.2.1.

6.3.2.4. Encapsulation efficiency of cyclic TetN343 at N/P 10 with respect to time

Polyplex solutions were prepared using cyclic TetN343 (**7**) as the cation and pH 7, 4 mM HEPES as buffer. Six samples were prepared at N/P 10 and one sample incubated for each 10, 20, 30, 40, 50 and 60 minutes before addition of the propidium iodide solution. Fluorescence was measured according to the general procedure described in Section 6.3.2.1.

6.3.2.5. Encapsulation efficiency of linear TetN343 at N/P 10 with respect to time

Polyplex solutions were prepared using linear TetN343 (**30**) as the cation and pH 7, 4 mM HEPES as buffer. Six samples were prepared at N/P 10 and one sample incubated for each 10, 20, 30, 40, 50 and 60 minutes before addition of the propidium iodide solution. Fluorescence was measured according to the general procedure described in Section 6.3.2.1.

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6.3.2.6. Encapsulation efficiency using combinations of linear and cyclic TetN343 at N/P 10

Cation solutions were prepared by dissolving cyclic TetN343 in pH 7, 4 mM HEPES followed by linear TetN343 (**30**). The proportion of each cation was varied so that samples containing 0, 1, 5, 10, 25, 50 and 100% of the total moles of secondary amine were contributed by linear TetN343 while preserving an N/P ratio of 10. Once prepared, the cation solutions were added to siRNA solution as described in the general procedure. All samples were incubated for 1 h before addition of the propidium iodide solution. Fluorescence was measured according to the general procedure described in Section 6.3.2.1.

6.3.2.7. Encapsulation efficiency of linear TetN343 At higher N/P ratios

Polyplex solutions were prepared using linear TetN343 (**30**) as the cation and pH 7, 4 mM HEPES as buffer at the following N/P ratios: 0, 10, 25, 50, 100, 200, 400 and 1000. For N/P ratios 100 and above, the aqueous cation solution used was 10 mgmL⁻¹ as opposed to the 1 mgmL⁻¹ solution described in the general procedure. Each sample was incubated for 1 h after mixing. A sample was also prepared using cyclic TetN343 at N/P 10 for use as a positive control. Fluorescence was measured according to the general procedure described in Section 6.3.2.1.

6.3.2.8. Encapsulation efficiency of spermine tetrahydrochloride with respect to N/P ratio

Polyplex solutions were prepared using spermine tetrahydrochloride (**29**) as the cation and pH 7, 4 mM HEPES as buffer at the following N/P ratios: 0, 1, 2, 5, 10, 15, 20, 30, 40. Each sample was incubated for 1 h after mixing. A sample was also prepared using cyclic TetN343 at N/P 10 for use as a positive control. Fluorescence was measured according to the general procedure described in Section 6.3.2.1.

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6.3.2.9. Encapsulation efficiency using combinations of linear and cyclic TetN343

Cation solutions were prepared by dissolving cyclic TetN343 in pH 7, 4 mM HEPES followed by linear TetN343 (**30**). The proportion of each cation in each sample was varied so that 0, 25, 35, 45, 55, 65, 75 or 100% of the total moles of secondary amine were contributed by linear TetN343 while preserving an N/P ratio of 10. Once prepared, the cation solutions were added to siRNA solution as described in the general procedure. All samples were N/P ratio 10 and were incubated for 1 h after mixing. Fluorescence was measured according to the general procedure described in Section 6.3.2.1.

6.3.2.10. Encapsulation efficiency of cyclic TetN343 with respect to pH

Polyplex solutions were prepared using cyclic TetN343 (**7**) as the cation. 4 mM HEPES was used as buffer at pH 6.3, 7.0, 7.4 and 8.0. Three samples were prepared at each pH and incubated for 1 h after mixing. A sample was also prepared using cyclic TetN343 at N/P 10 for use as a positive control. Fluorescence was measured according to the general procedure described in Section 6.3.2.1.

6.3.2.11. Encapsulation efficiency of linear TetN343 with respect to pH

Polyplex solutions were prepared using linear TetN343 (**30**) as the cation. 4 mM HEPES was used as buffer at pH 6.3, 7.0, 7.4 and 8.0. Three samples were prepared at each pH and incubated for 1 h after mixing. Three samples using cyclic TetN343 at N/P 10 and three N/P 0 samples were also prepared for use as positive and negative controls, respectively. Fluorescence was measured according to the general procedure described in Section 6.3.2.1.

6.3.2.12. Encapsulation efficiency of a combination of linear and cyclic TetN343 with respect to pH

Cation solutions were prepared by dissolving cyclic TetN343 (**7**) in buffer followed by linear TetN343 (**30**) in a proportion such that 50% of the total moles of secondary

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amine were contributed by linear TetN343. Once prepared, the cation solutions were added to siRNA solution as described in the general procedure. 4 mM HEPES was used as buffer at pH 6.3, 7.0, 7.4 and 8.0. Three samples were prepared at each pH and incubated for 1 h after mixing. Three samples using cyclic TetN343 at N/P 10 and three N/P 0 samples were also prepared for use as positive and negative controls, respectively. Fluorescence was measured according to the general procedure described in Section 6.3.2.1.

6.3.2.13. Encapsulation efficiency using combinations of brancher and cyclic TetN343

Cation solutions were prepared by dissolving cyclic TetN343 (**7**) in pH 7, 4 mM HEPES followed by brancher (**15**). The proportion of each cation in each sample was varied so that either 0, 1, 2.5, 5, 10, 15, 25, 50, 75 and 100% of the total moles of secondary amine were contributed by brancher. Once prepared, the cation solutions were added to siRNA solution as described in the general procedure. All samples were N/P ratio 10 and were incubated for one hour after mixing. Three samples using cyclic TetN343 at N/P 10 and three N/P 0 samples were also prepared for use as positive and negative controls, respectively. Fluorescence was measured according to the standard procedure. Fluorescence was measured according to the general procedure described in Section 6.3.2.1.

6.3.2.14. Encapsulation efficiency using combinations of brancher of different degrees of oxidation and cyclic TetN343

Cation solutions were prepared by dissolving cyclic TetN343 (**7**) in buffer followed by brancher (**15**) in a proportion such that 50% of the total moles of secondary amine were contributed by brancher. Brancher that had been stored as 1 mgmL⁻¹ solutions at 4 °C for 6, 8 and 14 days were used (by ¹H NMR: degrees of oxidation of 86, 89 and 99%, respectively) as well as a freshly prepared solution. Once prepared, the cation solutions were added to siRNA solution as described in the general procedure. 4 mM HEPES was used as buffer at pH 7.0. Three samples were prepared with each sample of brancher and incubated for one hour. Three samples using cyclic TetN343

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at N/P 10 and three N/P 0 samples were also prepared for use as positive and negative controls, respectively. Fluorescence was measured according to the general procedure described in Section 6.3.2.1.

6.3.2.15. Encapsulation efficiency using combinations of fully oxidised brancher and cyclic TetN343

Cation solutions were prepared by dissolving cyclic TetN343 (**7**) in pH 7, 4 mM HEPES followed by oxidised brancher (**16**) that had been prepared by storing as an aqueous 1 mgml⁻¹ solution at 4 °C for 14 days. The proportion of each cation was varied so that samples containing 0, 1, 2.5, 5, 10, 15, 25, 50, 75 and 100% of the total moles of secondary amine were contributed by brancher. Once prepared, the cation solutions were added to siRNA solution as described in the general procedure. All samples were N/P ratio 10 and were incubated for 60 minutes before addition of the propidium iodide solution. Three samples using cyclic TetN343 at N/P 10 and three N/P 0 samples were also prepared for use as positive and negative controls, respectively. Fluorescence was measured according to the general procedure described in Section 6.3.2.1.

6.3.2.16. Encapsulation efficiency of partially oxidised brancher with respect to N/P ratio

Polyplex solutions were prepared using oxidised brancher (**16**) that had been prepared by storing as a 1 mgml⁻¹ solution at 4 °C for 6 days as the cation and pH 7, 4 mM HEPES as buffer at the following N/P ratios: 0, 1, 2, 5, 10, 15, 20 and 40. Each sample was incubated for 1 h after mixing. Fluorescence was measured according to the general procedure described in Section 6.3.2.1.

6.3.2.17. Encapsulation efficiency of fully oxidised brancher with respect to N/P Ratio

Polyplex solutions were prepared using oxidised brancher (**16**) that had been prepared by storing as a 1 mgml⁻¹ solution at 4 °C for 14 days as the cation and pH 7,

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4 mM HEPES as buffer at the following N/P ratios: 0, 1, 2, 5, 10, 15, 20 and 40. Each sample was incubated for 1 h after mixing. Fluorescence was measured according to the general procedure described in Section 6.3.2.1.

6.3.2.18. Efficiency of pDNA encapsulation using cyclic TetN343 with respect to N/P ratio

Polyplex solutions were prepared using cyclic TetN343 (7) as the cation and pH 7, 4 mM HEPES as buffer at the following N/P ratios: 0, 1, 2, 5, 10, 15, 20, 30, 40. Instead of siRNA, 0.129 mgmL⁻¹ solutions of pCMV-Luc plasmid DNA were used though the general procedure remained unchanged. Each sample was incubated for 1 h after mixing. Fluorescence was measured according to the general procedure described in Section 6.3.2.1.

6.3.2.19. Efficiency of pDNA encapsulation using cyclic TetN343 with respect to N/P ratio

Polyplex solutions were prepared using cyclic TetN343 (7) as the cation and pH 7, 4 mM HEPES as buffer at the following N/P ratios: 0, 1, 2, 5, 10, 15, 20, 30, 40. Instead of siRNA, 0.129 mgmL⁻¹ solutions of pCMV-Luc plasmid DNA were used though the general procedure remained unchanged. Each sample was incubated for 1 h after mixing. Fluorescence was measured according to the general procedure described in Section 6.3.2.1.

6.3.3. Photon Correlation Spectroscopy

6.3.3.1. General Procedure

Unless otherwise specified, polyplexes were prepared by adding a particular solution of cation (prepared by dissolving a 1 mgmL⁻¹ aqueous solution of cation in sufficient buffer to give a final volume of 120.0 μ L) to a solution of siRNA (5.0 μ g in 120.0 μ L of buffer). Particle size was measured in a quartz cell at 25 °C. Solvent was set to “water”. After a one minute equilibration period, three measurements of three

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minutes each were carried out at a scattering angle of 62.5° . Unless otherwise specified, all count rates were within the manufacturer's recommended limits ($5 \times 10^4 - 1 \times 10^6$ counts/second).

6.3.3.2. Particle size as a function of N/P ratio using cyclic TetN343

Polyplex solutions were prepared using cyclic TetN343 (7) as the cation and pH 7, 4 mM HEPES as buffer at the following N/P ratios: 0, 1, 2, 5, 10, 15, 20, 30, 40. After mixing, each sample was vortexed for 5 s and incubated for 1 h at room temperature. Size was measured according to the general procedure described in Section 6.3.3.1.

6.3.3.3. Particle size as a function of time using cyclic TetN343

A polyplex solution was prepared using cyclic TetN343 (7) at an N/P ratio of 10 in pH 7, 4 mmol HEPES buffer. After mixing the two components, the solution was vortexed for 5 s and transferred to the quartz cell. Measurements were taken every 10 mins for 70 mins. A final measurement was recorded after 270 mins. Size was measured according to the general procedure described in Section 6.3.3.1..

6.3.3.4. Particle formation over time using a gradual addition of cyclic TetN343 solution

A solution of siRNA (0.5 μg in 222.4 μL pH 7, 4 mM HEPES) was placed in a quartz cell. After measuring particle size (Time = 0), 2.95 μL of a 1 mgmL^{-1} solution of cyclic TetN343 (7) was added (one sixth of the total). The size was measured again, followed by the addition of another 2.95 μL portion of polymer solution. This was continued until 17.7 μL had been added (time = 60). Size was measured according to the general procedure described in Section 6.3.3.1..

6.3.3.5. Particle formation by adding siRNA to cyclic TetN343

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Three identical polyplex solutions were prepared at an N/P ratio of 10 using cyclic TetN343 (7) in pH 7, 4 mmol HEPES buffer by adding the siRNA solution to the cation solution. A single sample was also prepared using the same materials but using the standard order of mixing (i.e. cation added to siRNA). Size was measured according to the general procedure described in Section 6.3.3.1..

6.3.3.6. Particle size as a function of time using linear TetN343

A polyplex solution was prepared at an N/P ratio of 10 using linear TetN343 (**30**) in pH 7, 4 mM HEPES buffer. After mixing the two components, the solution was vortexed for 5 s and transferred to a quartz cell. Measurements were taken every 10 mins for 60 mins. Size was measured according to the general procedure described in Section 6.3.3.1.

6.3.3.7. Attempted reduction of polyplexes formed using linear TetN343

A polyplex solution was prepared using linear TetN343 (**30**) at an N/P ratio of 10 in pH 7, 4 mM HEPES buffer. After mixing the two components, the solution was vortexed for 5 s and transferred to a quartz cell. Measurements were taken every 10 mins for 60 mins. At 60 mins, and every 10 mins after was added a portion of aqueous mercaptoethanol immediately followed by a measurement. The amounts/concentrations are summarised in Table 2. Size was measured according to the general procedure described in Section 6.3.3.1.

6.3.3.8. Attempted reduction of polyplexes formed using cyclic TetN343

A polyplex solution was prepared using cyclic TetN343 (7) at an N/P ratio of 10 in pH 7, 4 mM HEPES buffer. After mixing the two components, the solution was vortexed for 5 s and transferred to a quartz cell. Measurements were taken every 10 mins for 60 mins. At 60 mins, and every 10 minutes after was added a portion of aqueous

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mercaptoethanol immediately followed by a measurement. The amounts/concentrations are summarised in Table 2. Size was measured according to the general procedure described in Section 6.3.3.1.

Time (min)	60	70	80	90	100	110
Concentration of Solution Added(M)	0.07857	0.07857	0.07857	0.7857	0.7857	7.587
Volume Added (μL)	0.5	2.0	2.5	2.0	2.5	2.0
Total Equivalents in Sample (cumulative)	0.001	0.005	0.01	0.05	0.1	0.5

Table 2 – Concentrations and amounts of mecaptoethanol solutions added in procedures 6.3.3.7 and 6.3.3.8.

6.3.3.9. Particle size using combinations of cyclic TetN343 and brancher

A polyplex solution was prepared by adding cyclic TetN343 (7) (1 mgmL^{-1} solution in water) to pH 7, 4 mM HEPES (to make a final volume of 120 μL) followed by brancher (**15**) (as 1 mgmL^{-1} solution in water). The specific quantities of each cation were varied such that the N/P ratio remained constant at 10 but the percentage of total protonatable nitrogen atoms from the brancher was either 0, 1, 2.5, 5, 7.5 or 10. The solution of cations was added to a solution of siRNA (5 μg as 50 μM solution made to 120 μL with pH 7, 4 mM HEPES) in one portion, vortexed for 5 s and incubated for 1 h. Size was measured according to the general procedure described in Section 6.3.3.1.

6.3.3.10. Particle size using combinations of TetN343-CDS and trithiol revised

A polyplex solution was prepared by adding cyclic TetN343 (7) (1 mgmL^{-1} solution in water) to brancher (**15**) (as 1 mgmL^{-1} solution in water) and leaving for 15 mins at room temperature. After this time, pH 7, 4 mM HEPES (to make a final volume of 120 μL) was added. The specific quantities of each cation were varied such that the N/P ratio remained constant at 10 but the percentage of total protonatable nitrogen atoms from the brancher was either 0, 1, 2.5, 5, 7.5 or 10. The solution of cations was added to a solution of siRNA (5 μg as 50 μM solution made to 120 μL with pH 7, 4

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mM HEPES) in one portion, vortexed for 5 s and incubated for 1 h. Size was measured according to the general procedure described in Section 6.3.3.1.

6.3.3.11. Particle size using combinations of cyclic TetN343 and tris(2-aminoethyl) amine hydrochloride.

A polyplex solution was prepared by adding cyclic TetN343 (**7**) (1 mgmL⁻¹ solution in water) to tren hydrochloride (as 1 mgmL⁻¹ solution in water) and leaving for 15 mins at room temperature. After this time, pH 7, 4 mM HEPES (to make a final volume of 120 μ L) was added. The specific quantities of each cation were such that the N/P ratio remained constant at 10 but the percentage of total protonatable nitrogen atoms from the tren was either 0, 1, 2.5, 5, 7.5 or 10. The solution of cations was added to a solution of siRNA (5 μ g as 50 μ M solution made to 120 μ L with pH 7, 4 mM HEPES) in one portion, vortexed for 5 s and incubated for 1 h. Size was measured according to the general procedure described in Section 6.3.3.1.

6.3.3.12. Particle size using combinations of cyclic TetN343 and trithiol over time

A polyplex solution was prepared by adding cyclic TetN343 (**7**) (1 mgmL⁻¹ solution in water) to brancher (**15**) (as 1 mgmL⁻¹ solution in water) and leaving for 15 mins at room temperature. After this time, pH 7, 4 mM HEPES (to make a final volume of 120 μ L) was added. The specific quantities of each cation were varied such that 1% of total protonatable nitrogen atoms were from the brancher and N/P was 10. The solution of cations was added to a solution of siRNA (5 μ g as 50 μ M solution made to 120 μ L with pH 7, 4 mM HEPES) in one portion, vortexed for 5 s and after this time particle size was measured every 10 mins for 100 mins according to the general procedure described in Section 6.3.3.1.

6.3.3.13. Particle size as a function of N/P ratio using oxidised brancher

Polyplex solutions were prepared using oxidised brancher (**16**) as the cation and pH 7, 4 mM HEPES as buffer at the following N/P ratios: 0, 1, 2, 5, 7.5, 10, 12.5, 15, 17.5 and 20. After mixing, each sample was vortexed for 5 s and incubated for 1 h at room

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temperature. Size was measured according to the general procedure described in Section 6.3.3.1.

6.3.3.14. Particle size using combinations of cyclic TetN343 and oxidised brancher

A polyplex solution was prepared by adding cyclic TetN343 (**7**) (1 mgmL⁻¹ solution in water) to pH 7, 4 mM HEPES (to make a final volume of 120 µL) followed by oxidised brancher (**16**) (as 1 mgmL⁻¹ solution in water). The specific quantities of each cation were varied such that the N/P ratio remained constant at 10 but the percentage of total protonatable nitrogen atoms from the brancher was either 0, 12.5, 25, 37.5, 50, 62.5, 75, 87.5 or 100. The solution of cations was added to a solution of siRNA (5 µg as 50 µM solution made to 120 µL with pH 7, 4 mM HEPES) in one portion, vortexed for 5 s and incubated for 1 h. Size was measured according to the general procedure described in Section 6.3.3.1.

6.3.3.15. Particle size using combinations of TetN343-CDS and oxidised brancher over narrow range

A polyplex solution was prepared by adding TetN343-CDS (**7**) (1 mgmL⁻¹ solution in water) to pH 7, 4 mM HEPES (to make a final volume of 120 µL) followed by oxidised brancher (**16**) (as 1 mgmL⁻¹ solution in water). The specific quantities of each cation were varied such that the N/P ratio remained constant at 10 but the percentage of total protonatable nitrogen atoms from the brancher was either 65, 70, 75, 80, 85, 90, 95 or 100. The solution of cations was added to a solution of siRNA (5 µg as 50 µM solution made to 120 µL with pH 7, 4 mM HEPES) in one portion, vortexed for 5 s and incubated for 1 h. Size was measured according to the general procedure described in Section 6.3.3.1.

6.3.3.16. Particle size as a function of time using oxidised brancher

A polyplex solution was prepared at an N/P ratio of 10 using oxidised brancher (**16**) in pH 7, 4 mmol HEPES buffer. After mixing the two components, the solution was vortexed for 5 s and transferred to the quartz cell. Measurements were taken every 10

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mins for 120 mins and again at 210 and 360 mins. After covering with foil and leaving for 1360 mins (during which time approx. one third of the solvent evaporated) size was measured again every 10 mins for 50 mins. Size was measured according to the general procedure described in Section 6.3.3.1.

6.3.3.17. Particle size as a function of N/P ratio using oxidised brancher in pH 7, 20 mM HEPES

Polyplex solutions were prepared using oxidised brancher (**16**) as the cation and pH 7, 20 mM HEPES as buffer at the following N/P ratios: 0, 1, 2, 5, 7.5, 10, 12.5, 15, 17.5 and 20. After mixing, each sample was vortexed for 5 s and incubated for one hour at room temperature. Size was measured according to the general procedure described in Section 6.3.3.1. A range of samples at the same N/P ratios in pH 7, 4 mM HEPES was prepared and run using the same materials for comparison.

6.3.3.18. Particle size as a function of time using oxidised brancher in Opti-MEM buffer

A polyplex solution was prepared at an N/P ratio of 10 using oxidised brancher (**16**) as cation and Opti-MEM as buffer. After mixing the two components, the solution was vortexed for 5 s and transferred to the quartz cell. Measurements were taken every 10 mins for 120 mins. Size was measured according to the general procedure described in Section 6.3.3.1.

6.3.3.19. Particle size as a function of time using cyclic TetN343 in Opti-MEM buffer

A polyplex solution was prepared at an N/P ratio of 10 using cyclic TetN343 (**7**) as cation and Opti-MEM as buffer. After mixing the two components, the solution was vortexed for 5 s and transferred to the quartz cell. Measurements were taken every 10 mins for 60 mins. Size was measured according to the general procedure described in Section 6.3.3.1.

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6.4. *In Vitro* Testing of Nanoparticles

6.4.1. GAPDH Assay

6.4.1.1. General Procedure

To all but the circumferential wells in a 96-well plate was added 200 μ L of serum-containing media containing a specific number of A549 cells. The plate was then incubated for 48 h. Polyplexes were prepared by adding 125 μ L of a solution of cation (cyclic TetN343 (7), oxidised brancher (**16**) or bPEI) in HEPES to 125 μ L of a solution of siRNA in HEPES, vortexing for 5 s and incubating for 1 h at room temperature. The amount of cation and siRNA was calculated such that 50 μ L of the solution contained the desired amount of siRNA (typically 80 ng) and a quantity of cation that would give the desired N/P ratio. DharmaFECT® polyplexes were prepared in a similar fashion although only incubated for 30 mins in accordance with the manufacturer's instructions. jetPEI™ polyplexes were prepared in saline solution according to the manufacturer's instructions. 185 μ L of the media was removed from each well and 50 μ L of serum-free media added. 50 μ L of polyplex solution was then added (each sample in quadruplicate) and the plate returned to the incubator for 24 h. As negative controls, some wells contained 100 μ L of serum-free.

Media was removed and replaced with 100 μ L of serum-containing DMEM and the plate was returned to the incubator. After 24 h, media was removed and the plate was frozen at -80°C until assaying. GAPDH assays were carried out according to manufacturer's recommendation (briefly, cells were lysed for 20 mins at 4°C before plating 10 μ L in a black 96-well plate, adding 100 μ L of KDAAlert™ master mix and recording fluorescence (Ex. 560 nm, Em. 590 nm) immediately, and then again after 4 mins. Results presented are the mean and standard deviation of the difference between the readings at $t=0$ mins and $t=4$ mins. All measurements include at least three repeats.

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6.4.1.2. Determination of optimum dose of siRNA

In accordance with the general procedure described in Section 6.4.1.1, cells were plated to either 5000 cells per well or 10000 cells per well. Polyplexes were prepared containing either 20, 40 or 80 ng of siRNA at N/P 20. An additional sample of polyplexes prepared using 40 ng of siRNA at N/P 20 but prepared doubly concentrated in HEPES buffer was also prepared (such that 25 μ L of solution as opposed to 50 μ L was required per well). Cells were transfected and assayed in accordance with the general procedure described in Section 6.4.1.1.

6.4.1.3. Determination of optimum DharmaFECT® conditions

In accordance with the general procedure described in Section 6.4.1.1, cells were plated to 10000 cells per well. Lipoplexes were prepared containing 80 ng of siRNA and a quantity of DharmaFECT® that would give either 0.05, 0.25 or 0.5 μ L of transfection reagent per well. Lipoplex solutions were prepared either in HEPES buffer and added to wells containing 50 μ L of serum-free media (the procedure used for the cations in other experiments) or prepared in serum-free media and added to wells with serum-containing media (as per the manufacturer's recommendation). Wells containing only serum-free DMEM, serum containing DMEM or a mixture (50% v/v) of serum-free DMEM and HEPES were also prepared as negative controls. Further treatment of cells and assay were carried out in accordance with the general procedure described in Section 6.4.1.1.

6.4.1.4. Transfection efficiency of cyclic TetN343 (7)

In accordance with the general procedure described in Section 6.4.1.1, cells were plated to 10000 cells per well. Polyplexes were prepared containing 80 ng of siRNA. Polyplexes were prepared at N/P 0 (i.e. naked siRNA), using cyclic TetN343 (7) at N/P 1, 2, 5, 10, 15 and 20 and using 600 Da or 25 kDa bPEI at N/P 8. DharmaFECT® and jetPEI™ positive controls were prepared in accordance with the general procedure described in Section 6.4.1.1. Cells were transfected and assayed in accordance with the general procedure described in Section 6.4.1.1.

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6.4.1.5. Transfection efficiency of oxidised brancher (**16**)

ReferencesIn accordance with the general procedure described in Section 6.4.1.1, cells were plated to 10000 cells per well. Polyplexes were prepared containing 80 ng of siRNA. Polyplexes were prepared at N/P 0 (i.e. naked siRNA), using oxidised brancher (**16**) at N/P 1, 2, 5, 7.5, 10, 12.5, 15, 17.5 and 20. DharmaFECT® controls were prepared in accordance with the general procedure described in Section 6.4.1.1. No other controls were used although experiment was conducted in parallel with the one described in Section 6.4.1.4. Cells were transfected and assayed in accordance with the general procedure described in Section 6.4.1.1.

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