Synthesis of hydrophobically modified disulphide containing poly (ethylene imines) using the split-Ugi reaction

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In this work we expand upon a novel approach to poly (ethylene imine) modification by applying it to polymers containing potentially bioreducible disulphide bonds. We successfully used the split-Ugi reaction to synthesize disulphide-containing PEI modified with decanal, dodecylisocyanide and acetic acid, which have been previously shown to greatly improve gene carrier properties of the polymer. We also introduced poly (ethylene glycol)-based modifications to PEI using the same approach to highlight its' versatility. Our findings confirmed that disulphide-containing polymers can be hydrophobically modified by the split-Ugi reaction, laying the foundation for further studies of structure-property relationship in PEI derivatives. Moreover, we have looked into the relationship between reaction conditions and efficiency for the one-pot thiol deprotection and oxidation which leads to the formation of disulphide bonds. We identified THF as the best performing solvent for this reaction, leading to high molecular weights and reaction efficiency. The role of degassing in improving reaction outcomes by preventing premature oxidation was also highlighted.

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List of abbreviations

a.u. – arbitrary units

DMAP – 4-dimethylaminopyridine

DMF – dimethylformamide

DNA – desoxyribonucleic acid

DTNB – 5, 5'-dithiobis-(2-nitrozobenzoic acid)

EtOx – 2-ethyl-2-oxazoline

GSH – reduced glutathione

GSSG – oxidized glutathione dimer

GPC – gel permeation chromatography

HFIP - hexafluoroisopropanol

HMW – high molecular weight

LMW – low molecular weight

MALDI – matrix-assisted laser desorption/ionization

MeCN – acetonitrile

mRNA – matrix ribonucleic acid

MW – molecular weight

MWCO – molecular weight cut-off

NMR – nuclear magnetic resonance

PAMAM – poly (amidoamine)

PEI – poly (ethylene imine)

PEtOx – poly(2-ethyl-2-oxazoline)

PLL – poly (L-lysine)

POx – poly(oxazoline)

SEC – size exclusion chromatography

siRNA – signalling ribonucleic acid

THF – tetrahydrofuran

TOF – time of flight

UV-vis – ultraviolet and visible

1. Introduction

Therapies based on nucleic acids have shown great promise for treating genetic[1–3], neurological[4], cardiovascular[5] and infectious diseases[6], as well as certain types of cancers[7]. Their clinical use, however, faces challenges due to their instability, poor cellular uptake and immunogenicity, necessitating the use of carriers. Often they have to be tailored to the specific cargo, as properties of nucleic acids may vary drastically[8]. This hinders the development of individual treatments, as well as the overall research in the field. Therefore, designing a universal carrier system which can be applied to many types of cargo without requiring additional modifications may be a significant step towards further developments in gene therapeutics.

Carriers based on cationic polymers are currently considered very promising[9]. Poly (ethylene imine), or PEI, is especially interesting to the researchers due to its' strong ability to condense nucleic acid and facilitate cellular uptake. However, its' high charge density also leads to cytotoxicity, which is critical for clinical applications. To avoid this trade-off, polymers can be modified, for example, by introduction of hydrophobic moieties[10–13] or bioreducible fragments into the backbone[14,15]. Such modifications can greatly affect the properties of PEI as a gene carrier, and several studies have been performed to explore their effects[16,17]. However, until recently it was difficult to carry out a comprehensive analysis due to a lack of a quick and efficient way to synthesize diverse libraries of PEI derivatives.

In 2025 a novel high-throughput method for PEI modification using a Ugi-type multicomponent reaction was described[18], which allows the introduction of a large variety of side groups into the polymer. Compounds with notable transfection efficiency, low cytotoxicity and capability to deliver complex cargo, such as CRISPR-Cas9-RNA systems, were synthesized and studied, clearly demonstrating the potential of this approach. Since this study focused primarily on the effect of hydrophobic moieties introduced by the Ugi reaction, exploring the applicability of this novel approach to PEI with other modifications is the next step towards establishing it as a common method of PEI-based gene carrier synthesis.

In this work, the compatibility of the Ugi reaction-based derivatization approach with bioreducible PEI will be explored.

1 Literature review

1.1 Cationic polymers in gene delivery

Use of nucleic acids, such as DNA and RNA, in disease treatments *in vivo* can be hindered by their properties and behaviour in the organism. Exogenous DNA and RNA are readily degraded by a number of nucleases that are present in most tissues, including the bloodstream, to maintain homeostasis and protect the organism[19]. In addition, the overall negative charge and high hydrophilicity of nucleic acids do not facilitate their efficient transfer across negatively charged hydrophobic biological membranes leading to low transfection efficiency[20]. To address those challenges, as well as to improve overall therapy efficiency, many types of gene delivery systems have been created and studied.

There are several important criteria that define a good gene delivery system. It needs to be efficient in terms of cargo packaging and carrying capacity, stable in biological conditions, specifically blood, to protect the nucleic acids from degradation, able to traverse cellular and nuclear membranes, induce endosomal escape and eventually release the cargo. Low cytotoxicity, low immunogenicity and high biodegradability are also crucial for eventual practical applications of the treatment. Finally, target specificity is desired to both decrease potential side effects and improve treatment efficiency, although it is not as vital as other properties described above. To date, no perfect carrier which meets all of those criteria has been discovered or designed. Viral vectors are considered the most efficient, but serious concerns over their safety exist[21], especially considering that integration into the host genome has been detected[22], so development of less immunogenic non-viral alternatives is currently of great interest. Among those, cationic polymer materials are considered one of the most promising options[9] for their relative ease of production and scaling, cost-efficiency and the possibility of versatile modifications to fine-tune their properties.

The key structural feature of cationic polymers is the presence of ionisable cationic groups, commonly amines, which allow polymers to condense and package nucleic acids by forming polyelectrolyte complexes[23,24]. The efficiency and strength of this interaction define carrying capacity and polyplex stability, and are themselves influenced by several extrinsic and intrinsic conditions[25]. Factors related to the molecular structure of the polymer include the number of charged groups in a

molecule[26], charge density, nature of the ionisable group itself[27], backbone structure[28], size and overall hydrophilicity of the compound. In general, stronger cationic properties contribute to better nucleic acid condensation, particle stability and cellular uptake, but also higher cytotoxicity. This tradeoff between efficiency and toxicity is inherent to most cationic polymers.

Numerous materials have been investigated for their potential for gene delivery applications. Poly(L-lysine) (PLL), chitosan, poly(amidoamine) (PAMAM) and poly(ethyleneimine) (PEI) are some of the most studied and commonly used (fig.1).

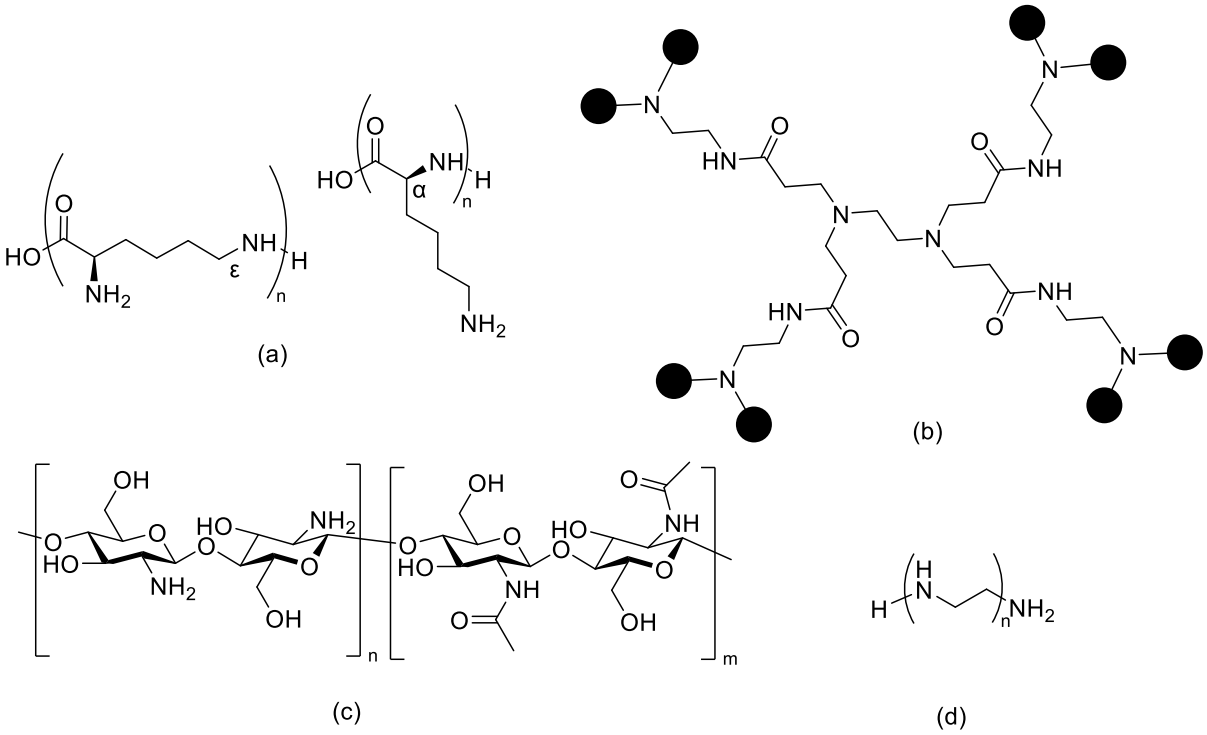

Figure 1. Cationic polymers most frequently explored for gene delivery applications (a) ε- and α-PLL (b) PAMAM (c) chitosan (d) PEI

1.1.1 Poly (L-lysine)

PLL, a polypeptide of one of the proteinogenic amino acids, was the first cationic polymer studied for its gene delivery potential[29]. It interacts very strongly with nucleic acids, condensing them to particles as small as 50 nm in diameter, enabling strong protection and efficient cellular uptake[30]. The amine fragments can be modified, offering the possibility of tuning the carrier properties or introducing ligands for receptor-mediated gene delivery[31]. Successful *in vivo* delivery of DNA cargo to hepatocytes[32,33], liver macrophages[34] and airway epithelium cells[35] has been

reported using glycan or peptide ligands. However, poor endosomal escape capabilities, broad size distribution, which cannot be effectively controlled, and a tendency of PLL-DNA complexes to form aggregates in solution[36] severely limit practical applications of PLL.

1.1.2 Chitosan

Chitosan is a cationic polysaccharide, which can be obtained by partial deacetylation of chitin[37]. It has low toxicity and immunogenicity, good biodegradability[38], forms stable complexes with nucleic acids[39], and is relatively cheap to produce, which makes it an attractive alternative for gene therapy uses. While native chitosan lacks the desired specificity and efficiency for gene delivery, its' amine and hydroxyl groups can be readily modified to yield more promising derivatives. It also exhibits mucous adhesiveness, which opens the possibility of targeted gene delivery, for example, for treatment of mucosal tumors[40,41]. Other potential targets of chitosan-based delivery systems include breast[42] and lung tumors[43]. The key drawback of chitosan-based carriers is their poor solubility at neutral and basic pH[44], which significantly hinders their performance in physiological conditions. Moreover, while chitosan is noticeably more efficient in endosomal escape compared to PLL, it is still not as viable as other alternatives[45]. The mechanism of the endosomal escape will be discussed in detail later in this review.

1.1.3 Poly (amidoamine)

PAMAM is a hyperbranched or dendrimeric polymer, which gives it several unique advantages over linear or branched alternatives. The nature of its' synthesis, which proceeds in distinct steps, allows to tightly control the polymer's molecular weight and size distribution[46,47]. In addition, its symmetrical, almost spherical morphology lends itself well to polyplex formation and nucleic acid condensation[48].Among other dendrimeric polymers PAMAM is the most commercially available, which is also an important factor for potential applications. Unlike PLL and chitosan, it also exhibits rather high transfection efficiency in the absence of endoosmolytic agents due to a relatively high concentration of cationic groups, which promote endosomal escape[49]. PAMAM-based gene carriers have been successfully used for in vivo gene delivery to various cancer cells[50–53]. However, like many other cationic polymers, their efficiency

is hindered by cytotoxicity, which is indisputable for PAMAM. In addition, the shape of the dendrimers limits their maximum size, and, therefore, cargo capacity.

1.1.4 Poly (ethylene imine): gold standard for cationic polymer carriers

Poly(ethylene imine) (fig. 2) was first used in gene therapy studies in 1995[54]. To this day it remains one of the most popular carrier materials and is referred to by many as the golden standard for polymer-based delivery. PEI-based carriers have consistently exhibited high transfection efficiency in brain[55–57], lung[58,59], kidney[60] and tumour[61] tissues in vivo without endoosomolytic agents. In addition, it is commercially available, easy to synthesize and modify.

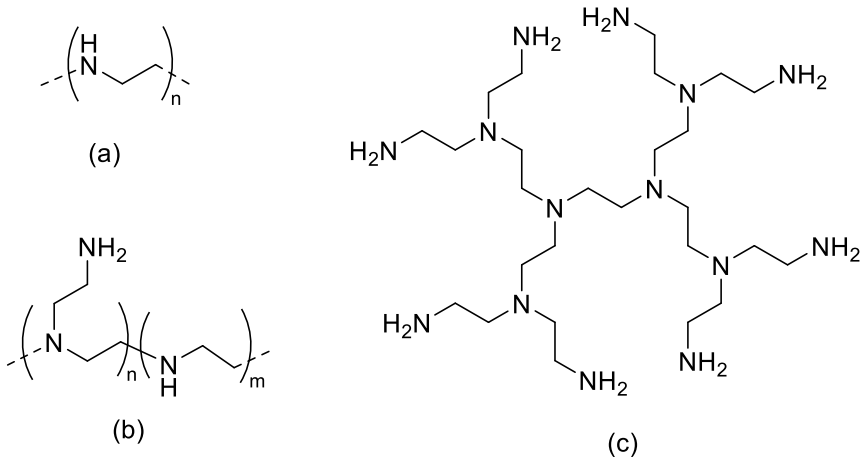

Figure 3. Structure of PEI (a) linear (b) branched (c) pseudo dendrimeric

High transfection efficiency of PEI can be attributed to its simple structure. In it every third atom is a nitrogen, and as many as two thirds of them can be protonated under physiological conditions[62]. This leads to a very high positive charge density, which significantly improves the ability to condense nucleic acids and cross cellular and nuclear membranes. More importantly, however, partial protonation is suggested as the key to PEI's inherent endosomal escape capabilities. According to the widely accepted proton sponge theory[63], unprotonated amine groups of PEI exhibit strong buffering capacity (figure 3). When an endosome forms and matures in the cytosol, its contents are acidified by membrane proteins which pump protons inside it. However, those protons are instead bound by the amine groups of PEI, preventing acidification and prompting more protons to be transported inside. This process is also coupled to transport of chloride anions, which in turn leads to water influx into the endosome to maintain osmolarity. Together, those processes lead to swelling and

bursting of the endosome, releasing its cargo into the cytosol. Recent developments of this hypothesis also describe other mechanisms that contribute to endosomal escape and are induced by protonation, such as polymer swelling and membrane destabilisation[64]. While other polymers, such as chitosan and PAMAM, exhibit the proton sponge effect to a certain extent, it is by far the strongest in PEI due to its high cationic group density.

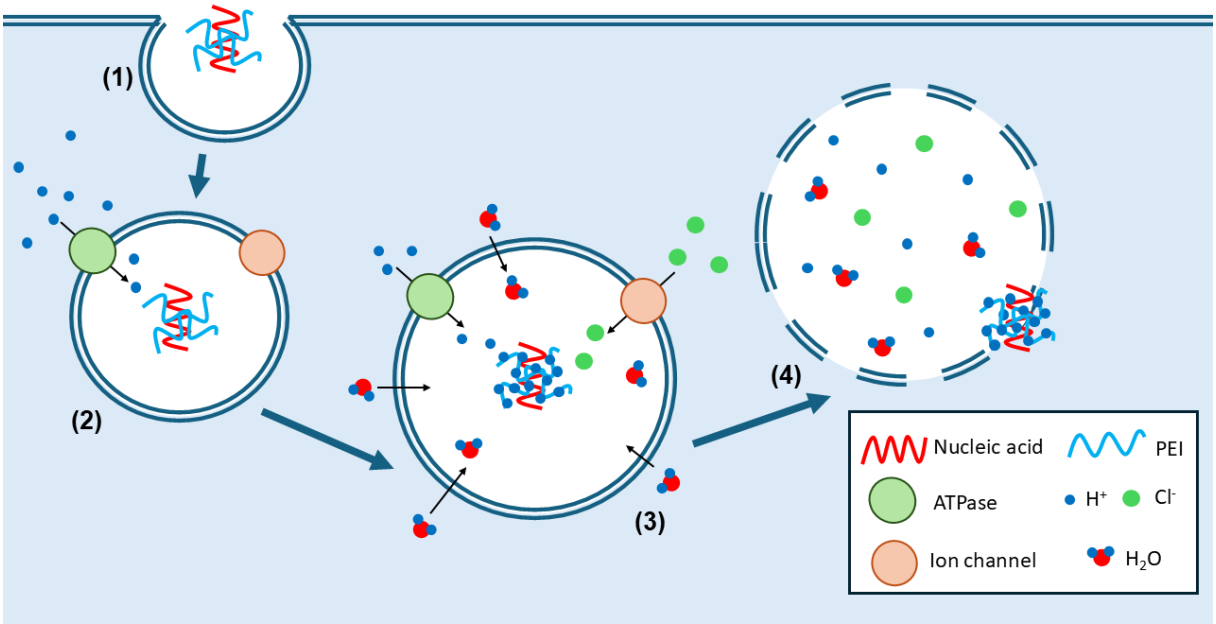

Figure 3. Scheme explaining the key steps in the proton sponge-driven endosomal escape (1) the PEI-nucleic acid complex is taken up by the cell, an endosome is formed (2) ATPase pumps protons from the cytosol to the endosome to acidify it (3) PEI is protonated, absorbing the protons and preventing acidification. More protons are pumped into the endosome. To maintain the net charge, chlorine ions migrate to the endosome as well through ion channels. This creates an osmotic gradient, causing water to also move from cytosol to endosome (4) the endosome bursts from the influx of water. Its' contents, including the PEI-nucleic acid complex, are released into the cytosol

Higher molecular weight PEI has been found to exhibit better carrier properties compared to low molecular weight, since the higher number of ionizable groups per molecule enhances both the polyplex stability and the proton sponge effect[65]. This can be further improved upon by utilizing the branched form of PEI instead of linear (fig. 2b), since primary amine groups can improve the buffering capacity of the polymer. This is believed to be the reason why branched polymers show improved complex stability compared to linear polymers of similar MW[66]. Pseudo dendrimeric polyamines[67] (fig. 2c) are especially notable for that, as they can closely mimic naturally occurring DNA-histone complexes, thus greatly improving polyplex stability. However, unlike their linear counterparts, branched PEI are more difficult

to synthesize with well-controlled molar masses and dispersity, as well as to characterize, which somewhat complicates their practical uses. Moreover, while most studies report higher transfection efficiency of branched PEI compared to linear[68], some suggest the opposite because of such strong interactions hindering cargo release[69]. In addition, high degree of branching has been linked to higher cytotoxicity[70].

However, even in linear form PEI is very cytotoxic due to its cationic nature, which can cause disruption of mitochondrial membranes, leading to apoptosis[71,72]. Moreover, the high influx of free amine moieties caused by PEI can disrupt the cells' homeostasis, also initiating cell death[73–75]. Naturally, as those effects are also linked to the number of ionizable groups, HMW PEI exhibits higher cytotoxicity compared to LMW PEI.

1.2 Approaches to PEI synthesis

1.2.1 Polymerization of aziridines

PEI can be synthesized from the monomer, aziridine. Variations of this method were first described in a number of patents in early 1940s, with a possible mechanism proposed in 1943[76] and further explored and elaborated upon in 1955[77]. The reaction proceeds via cationic ring opening of the aziridine ring, where a Lewis acid, for example, a proton, acts as an initiator, leading to formation of the aziridinium cation. This species is then attacked by another aziridine molecule, turning the cation into a secondary amine fragment, while the charge is transferred to the new terminus, which is also attacked, and the process continues, leading to growth of the polymer backbone (fig. 4). Importantly, proton transfer can happen to a secondary amine group of an already formed chain, which leads to branched structures as opposed to linear chain growth. Thus, PEI synthesized by aziridine polymerization, is very highly branched, with some studies showing a nearly 1:1:1 ratio of primary to secondary to tertiary amines in the structure[78].

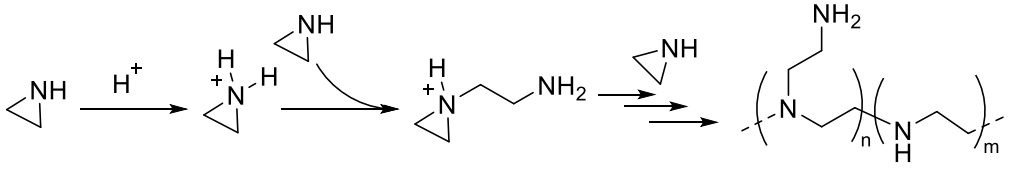

Figure 4. Lewis acid-catalysed ring-opening polymerization of aziridine reaction mechanism

One of the main drawbacks of this synthesis method is the nature of the monomer: aziridine is flammable, toxic and mutagenic, so working with it is dangerous and requires specialized skills and equipment. In addition, since it is difficult to store safely, it is usually synthesized on site from ethanolamine through energy-demanding methods[79]. Currently, this direct polymerization method is mostly used industrially. A promising alternative route which would allow synthesizing branched PEI directly from ethanolamine through dehydrogenative coupling under relatively more mild conditions has been proposed recently[80] (fig.5). However, this reaction still requires a complex manganese-based catalyst, which needs to be prepared before polymerisation.

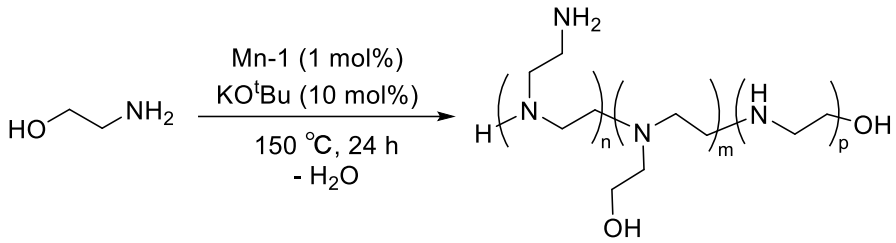

Figure 5. Ethanolamine-based PEI synthesis

Another major drawback of the direct polymerisation method is the fact that it is nearly impossible to control or fully avoid branching. Using N-substituted aziridine derivatives is one of the more straightforward methods to avoid that problem by removing the possibility of proton transfer. This usually does not eliminate branching completely, as tertiary amine groups of polymer chains can still act as nucleophiles, attacking the aziridinium cation, but bulky substituents, such as tert-butyl or 2-tetrahydropyranyl, lower the probability of this process while not hindering the polymerization[81,82]. Moreover, electrophilic substituents, in particular, sulfonyls, lead to a different polymerization mechanism altogether, anionic ring-opening polymerization, where the active end of the growing chain serves as a nucleophile instead of the monomer[83]. The substituent, however, needs to be cleavable under reasonably mild conditions so as not to affect the polymer backbone, which significantly limits the use of this method. In addition, specifically for sulfonyl-substituted aziridines, solubility can also be an issue, leading to only short oligomers instead of longer polymer chains[84]. Finally, like aziridine itself, many of its' derivatives are also toxic and difficult to handle, and need to be prepared before polymerization, which makes this method less time- and resource-efficient. Overall,

while the possibility and benefits of this method for linear PEI synthesis have been demonstrated, its practical applications are very limited compared to alternatives[85].

1.2.2 Hydrolysis of poly(2-oxazolines)

2-oxazolines, which are relatively easy to store and handle, and exhibit significantly lower toxicity, were first proposed as a viable alternatives to aziridines in 1960s [86]. Although traditionally oxazoline polymerization is described as cationic ring-opening polymerization (fig. 6a), it has been shown that in some particular cases it can proceed via two mechanisms, with the covalent species propagating the reaction instead of the ionic species, depending on reaction conditions, nature of the initiator and the monomer itself[87,88]. Both mechanisms, however, lead to formation of a very stable propagating species, which is not subject to chain transfer or termination, giving the polymerization a living character and allowing for good molecular weight control and very pure linear polymer product, which can then be hydrolysed to yield PEI (fig. 6b).

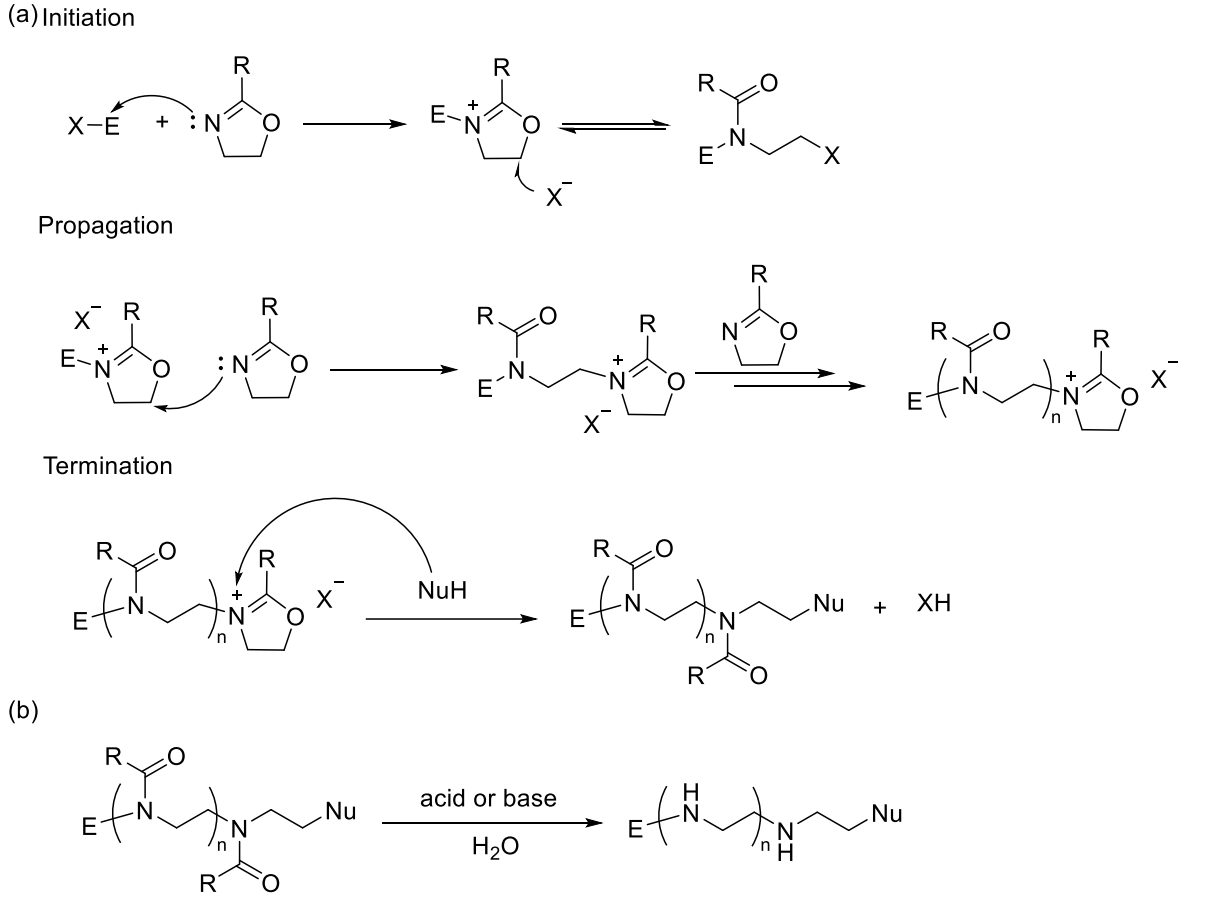

Figure 6. (a) Cationic ring-opening polymerization of 2-oxazolines, where R – substituent in the second position, E – electrophilic initiator group, X – counterion, Nu – nucleophilic terminating group (b) poly(2-oxazoline) hydrolysis to PEI

Basic hydrolysis can be used to synthesize PEI from H-2-oxazoline[89,90] as well as more traditional methyl- and ethyl-substituted monomers, however, partial degradation of the backbone can occur under those conditions[91]. In addition, side chains such as phenyl are reportedly difficult to cleave under basic conditions[92]. Acidic hydrolysis[93,94] is a more favourable alternative, although some backbone degradation may still occur in it.

An important advantage of the oxazoline route is its' tolerance to a considerable variety of functional groups. Restrictions imposed by the mechanism, of course, need to be considered: for example, a nucleophilic moiety in the initiator can lead to undesired early termination. However, even with those limitations, oxazoline polymerization provides a great opportunity to modulate polymer structure and introduce functional groups at the early stage of the synthesis[95]. Multifunctional initiators can be utilized to achieve more complex, yet controllable structures[96], and functional groups including unsaturated bonds[97–100], aromatic structures, amines[101–103], azides[104], carboxylic acids[105–107], etc., can be introduced through either the initiator or the terminating agent. This potential is severely limited for PEI synthesis, as some functional groups cannot survive harsh hydrolysis conditions, however, compared to aziridine-based methods, this approach still allows for significant flexibility.

1.3 PEI post-polymerization modifications

Many modifications of PEI have been shown to significantly decrease cytotoxicity, as well as enhance other properties important for gene delivery. For example, introduction of hydrophobic side groups, such as cholesterol[108] and long hydrocarbon chains[109–111], can enhance transfection efficiency as much as 5-fold, presumably by improving polyplex self-assembly and modulating their interactions with cells[112,113]. Partial acylation with propionic[114] or acetic[115] acid also has a positive influence on transfection efficiency, although this effect decreases above a certain modification degree, suggesting the importance of balance. Among other promising functionalities, succinate[10] and aromatic substituents[116,117] are promising for reducing toxicity and maintaining efficiency. Glucocorticoids[118], amino acids[17] and other polymers, most commonly PEG[119] and pluronic[120], can also be conjugated to PEI to improve its delivery properties.

For linear PEI synthesized through the oxazoline route partial hydrolysis is a possible way of obtaining results similar to modification[12]. The obvious disadvantage of this approach is, of course, the fact that functional groups are limited by the availability of corresponding oxazolines, while introducing modifications to PEI allows for much more variety.

Most reported PEI derivatization strategies utilize either alkylation or acylation of amine groups. There are several methods for either approach, based in non-polymer amine chemistry: reductive alkylation[92,121], nucleophilic substitution, epoxide ring-opening[122], Michael-type addition[123], reactions with isocyanides[119] or carboxylic esters[124,125]. Together, those methods allow access to a large variety of modifications. However, each approach on its own has noticeable limitations on functionalities which can be introduced, as well as other disadvantages, such as need for catalyst use or difficulty of synthesizing or obtaining the required reagents. Because of that, while small libraries of PEI-derivatives have been synthesized and studied using classical methods[126], until recently a quick, high-throughput, easily scalable and diverse approach to PEI modification has not been described.

1.3.1 Split-Ugi multicomponent reaction as a method of PEI modification

The Ugi reaction, named after Ivar Karl Ugi who first described it[127], is a multicomponent process, in which a carbonyl compound, an amine, an isocyanide and a carboxylic acid consecutively react to form a functionalized bis-amide (fig. 7). It is rather simple to perform, very atom-efficient, can be carried out in accessible solvents such as water[128] or ethanol[129], requires very mild conditions, and allows for a great variety of functional groups, which makes it very attractive for synthesis of large libraries of versatile compounds.

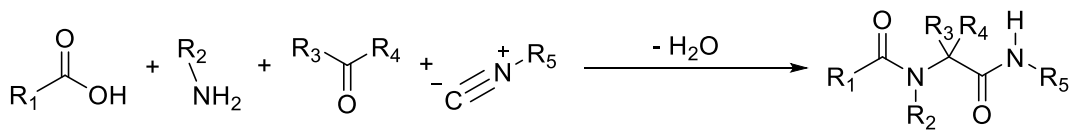

Figure 7. Classical Ugi four component reaction

Moreover, classical Ugi products are often referred to as peptoids or peptomers for their similarities to short peptides, and have attracted significant interest for their biological and pharmacological properties[130,131]. However, while this is one of the reasons for the significance of the Ugi reaction, its applications are far beyond only

the synthesis of peptidomimetics. In polymer chemistry specifically classical Ugi has been used for monomer synthesis[132,133], polycondensation[134,135] and post-polymerisation modifications, for example, synthesis of a block copolymer from two separate chains[136]. Many variations of the reaction have also been developed, which makes it even more versatile for a variety of uses.

According to the most widely accepted mechanism of the classical Ugi reaction (fig. 8), first, the carbonyl and the amine produce an imide, losing one equivalent of water in the process. The structure is then protonated by the acid and reacts with the isocyanide and then the carboxylate leading to formation of the intermediate product, which then turns into the final product through a Mumm rearrangement[137].

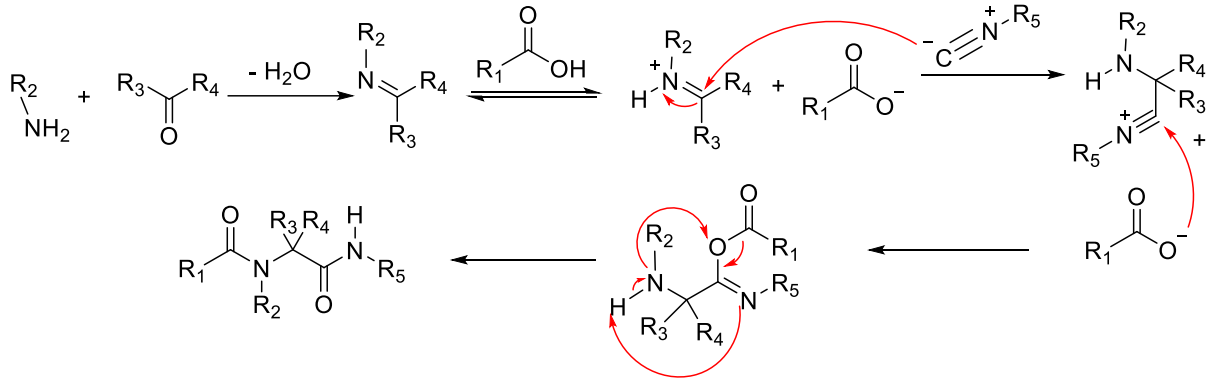

Figure 8. Mechanism of the Ugi reaction

This mechanism shows that, for the reaction to proceed, a primary amine has to be used, since its protonation is the key to allowing the final rearrangement. For that reason, while the classical Ugi reaction can be used for some post-polymerisation modifications, it is severely limited in applications to non-primary amines. However, in 2006, an alternative approach was described by Giovenzana et al[138], which bypasses this limitation (fig. 9). In this reaction, a bifunctional secondary amine is used to compensate for the lack of protonation: one amine group forms the imide, and the second one participates in the rearrangement. This approach, titled the Split-Ugi reaction, yields products very similar to the classical Ugi reaction under similarly mild conditions, while significantly expanding its applicability and flexibility even further[139].

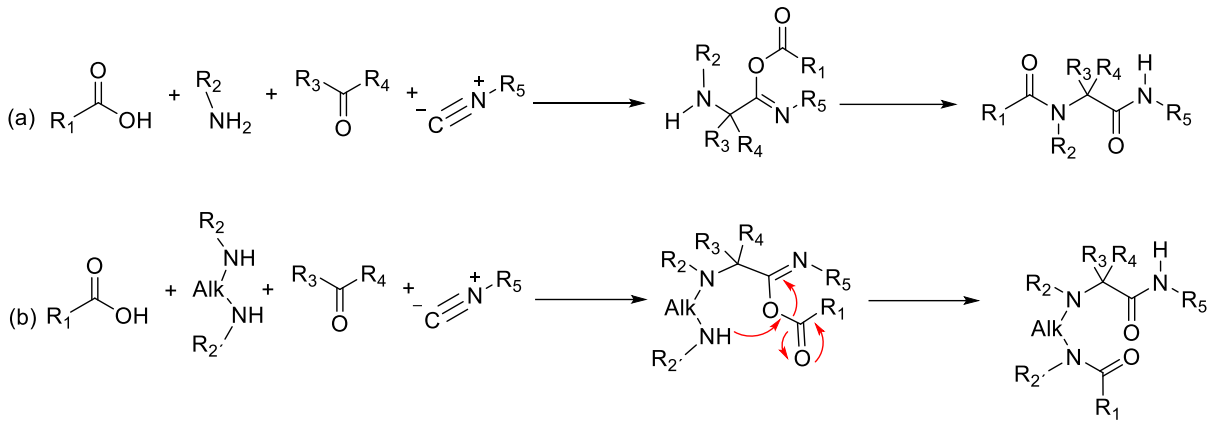

Figure 9. The mechanism of the final rearrangement in classical Ugi reaction (a) compared to the split-Ugi (b)

Recently, Vlasova et all[140] explored the potential of the split-Ugi reaction for post-polymerization modification of linear PEI. In this study, a library of nearly 150 polymers was created, and transfection experiments *in vitro* and *in vivo* were carried out to determine their delivery efficiency. A large variety of substituents, as well as different molecular weights, structures and modification densities were compared, and a lead polymer was identified: 4-arm PEI_{17} modified with decanal, dodecyl isocyanide and acetic acid with target modification density of 100%. Lipopolymer-lipid nanoparticles formulated with this compound showed significant transfection efficiency increase compared to commercially available alternatives. Successful gene delivery to the lungs through multiple routes of administration was achieved, and the expression of cargo was efficiently induced with minimal cytotoxicity. As such, the remarkable potential of the split-Ugi modification strategy was demonstrated, and the need for further studies on the topic was established.

1.4 Bioreducibility in gene delivery

The redox environment of the cell or tissue is defined by the ratio of interconvertible oxidized and reduced forms of certain compounds, also known as redox couples[141]. Cells rely on it to facilitate movement of electrons and support the most vital cellular functions, such as catabolic and anabolic processes, cell signalling, control of reactive oxygen species levels, etc. One of the most abundant redox couples is the glutathione system: a tripeptide which contains a thiol group in the reduced state (GSH) and a disulfide bond in the oxidised state (GSSG)[142] (fig. 10).

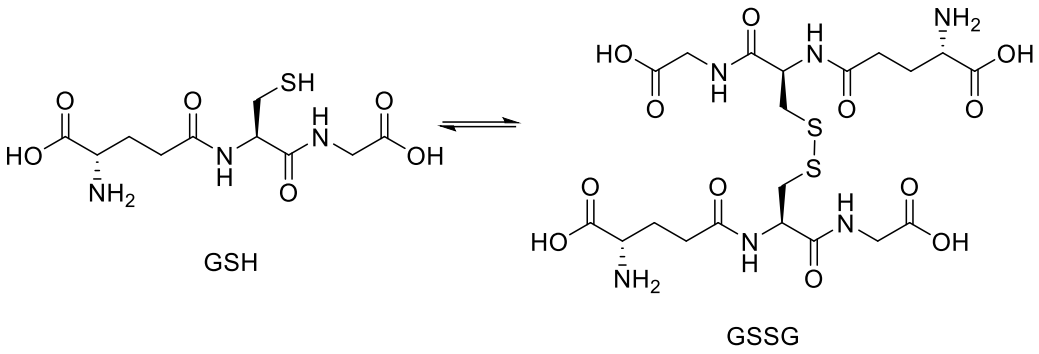

Figure 10. Glutathione in its reduced (GSH) and oxidized (GSSG) forms

GSH is a strong reducing agent and antioxidant, which protects cells against oxidative stress by neutralizing reactive oxygen species and reducing non-native disulfide bonds, which can sometimes form in proteins under normal conditions[143,144], through the thiol-disulfide exchange reaction. Its' concentrations in the intracellular space can be 50-100 times higher than in the extracellular matrix depending on the stage of the cell cycle[145–147]. This creates a strong reducing environment inside the cells, favouring reduction of S-S bonds to thiols. In addition, the difference between intracellular and extracellular redox environments can be even stronger in tissues affected by diseases such as breast cancer[148], lung cancer[149], bone marrow[150], colon[151] larynx and mouth cancer[152], as those cells are subject to stronger oxidative stress, and, therefore, need higher GSH levels to survive. All those factors can be exploited for drug- and gene delivery applications to design a carrier with high stability in plasma and ECM, targeted cargo release on site, low accumulation in cells and quick degradation to prevent toxicity by introducing disulfide bonds into polymer backbone.

For PEI this potential was first investigated in 2001 by Gosselin et all[153], who cross-linked LMW PEI using a disulphide-containing linker DTPB. They demonstrated that cross-linked polymers exhibited high transfection efficiency similar to unmodified HMW polymers, but significantly lower cytotoxicity even at high N/P ratios. Degradation of disulphide-linked PEI in GSH solution, as well as the cytosol of living cells was visualized several years later by Lee et al[15]. In the same year the transfection efficiency of such polymers was confirmed to be comparable to or higher than of many commercially available transfection reagents[154]. Many other studies have been carried out since clearly demonstrating the advantages of bioreducible PEI, both

branched and linear, over regular PEI, including successful *in vivo* gene transfection experiments[155,156].

There are two main approaches to introducing disulphide bonds into polymers. Cross-linking polymer chains with disulphide-containing linkers, such as cysteamine[157], cysteine[158], DSP, DTPB[153], DSDMA[159], etc. is the more common and widely studied method. The other alternative is prethiolation: thiol groups are introduced directly into the polymer and then oxidized to form disulphide bonds. Prethiolation can be done as a post-polymerization modification with ring opening of methylthiirane[160,161], or during the polymerization process by utilizing protected thiol terminating groups, like xanthate[162]. The latter approach is especially useful, since the protective group can be cleaved one-pot with the following oxidation, so the process is more time- and resource-efficient. Since linear PEI is usually synthesized through the oxazoline route, where reaction conditions tolerate a large variety of initiators and terminating reagents, xanthate-based prethiolation can be applied.

2 Motivation and objectives

It has been established that modifications of PEI significantly influence its' properties, including transfection efficiency and cytotoxicity, and can noticeably improve its' potential for gene delivery applications. The split-Ugi based post-polymerization modification approach has allowed researchers to synthesize a diverse library of PEI derivatives and analyse the influence of various hydrophobic modifications on their properties. In this project, we will continue and expand upon their research by combining the novel split-Ugi approach with other modifications, specifically, bioreducible disulphide bonds to explore their combined effects.

We aim to explore and optimize synthesis of bioreducible hydrophobically-modified PEI compounds using the split-Ugi derivatization strategy. To achieve this goal, we will use a well-established cationic ring-opening polymerization reaction to synthesize POx as the starting polymer. We will then carry out post-polymerization modifications to obtain HMW PEI containing disulphide bonds. Finally, we will use the split-Ugi strategy to introduce hydrophobic side groups into the bioreducible PEI. The products will be studied by various analytical methods, including 1H-NMR spectroscopy, MALDI-TOF mass spectrometry and size exclusion chromatography to accurately describe their structure and chemical composition, and confirm the outcome of the synthesis. In addition, we plan to optimize the conditions of the one-pot thiol deprotection and oxidation step to achieve HMW disulfide-linked polymers more efficiently. For this, an array of experiments using different solvents and reagents will be carried out, and the molar mass, MW distribution and composition of the resulting polymers will be compared using size-exclusion chromatography.

3 Materials and Methods

Materials

DCM, chloroform, diethyl ether, DMF, Mg_2SO_4, acetic acid and 30% hydrogen peroxide solution were obtained from ThermoFischer Scientific. Potassium ethyl xanthate, *α, α*'-dibromo-p-xylene, succinic anhydride, DCTB and decanal were obtained from Tokyo Chemical Industry. Ethylene diamine was obtained from Sigma Aldrich. All chemicals listed above were used as received.

Deuterated chloroform was obtained from EurIsotope. Deuterated methanol was obtained from VWR Chemicals.

Acetonitrile (VWR Chemicals, HPLC LC-MS grade) and 2-ethyl-2-oxazoline (Tokyo Chemical Industry) were dried over CaH_2 and purified by distillation before use. Potassium ethyl xanthate (Tokyo Chemical Industry) was dried in vacuum overnight before use. PEG 2K monomethyl ether (Tokyo Chemical Industry) was dissolved in toluene and evaporated to dryness on rotary evaporator to remove water residues before use.

3M HCl solution was prepared by dissolving 1 ml of concentrated HCl (Sigma-Aldrich, 37%) in 3 ml of distilled water. 0.5 M HCl solution was prepared by mixing 1M HCl solution (50 mL, VWR chemicals) with distilled water (50 mL). 4M NaOH solution was prepared by dissolving NaOH pellets (640 g, ThermoFisher Scientific, analytical grade) in distilled water (4 mL). Na TFA 1 g/L solution was prepared by dissolving Na TFA (5 mg, Sigma-Aldrich) in MeOH (5 mL, ThermoFischer Scientific, HPLC grade).

NMR measurements

NMR spectra were recorded using Bruker Ultrashield 500 MHz Plus system at 25 °C and analyzed using Brucker TopSpin software.

MALDI-TOF measurements

MALDI-ToF-MS was performed on a Shimadzu Axima Performance instrument in or positive-reflector mode. DCTB (100 mg mL^{-1} in ACN) was used as the matrix. NaTFA salt was used as the ionization agent (1 mg mL^{-1} in MeOH). Matrix, polymer (10 mg mL^{-1}), and salt solutions were mixed in a 1:1:0.5 volume ratio and then 2 μL of the

mixture was deposited onto a ground steel target plate before insertion into the ion source chamber. The instrument was calibrated against a PEG 2K monomethyl ether standard prepared under the same conditions with DCTB matrix

SEC measurements

Poly (ethyl oxazoline)s were analyzed by GPC using the Agilent 1260 Infinity II chromatography system with Stryagels HR2, HR4 and HT5 columns and Agilent 1260 infinity RI detector was used for the SEC studies. DMF + 0.1% LiBr was used as an eluent. The flow rate was 0.8 mL/min. The column was thermostated at 40 ^{0}C.

Poly (ethylene imine)s were analyzed using the system consisting of Waters 515 HPLC pump, Biotech DEGASi GPC Degasser, Waters 717 plus Autosampler and Waters 2410 Differential Refractometer together with Waters Styragel HR 2, HT 3, and HT 4 7,8x300 mm and guard column. HFIP was used as an eluent. The flow rate was 0.8 ml/min. The column was thermostated at 30 ^{0}C.

Ugi-modified polymers were analyzed using the same system and parameters as PEIs. $CHCl_3$:IPA:TEA mixture (94:4:2) was used as an eluent.

UV-vis measurements

UV-vis spectra were measured using JASCO V-750 spectrophotometer, with D2/WI as the light source. Absorbance was screened between 600 and 200 nm, at 1 nm intervals. Quartz cuvettes were used for all measurements.

For xanthate group determination, polymer samples were dissolved in MeOH to final sample concentration of 0.063 mmol/L.

Ellman's assay for thiol group determination

DTNB (Ellman's reagent, 11.9 mg, 0.03 mmol) was dissolved in phosphate buffer (0.1 M, pH = 7, 3 ml). **P4** (5 mg, ~2.3 μmol) was dissolved in distilled H_2O (1 ml). 50 μl of polymer solution, 50 μl of DTNB solution, 450 μl of distilled water and 500 μl of the phosphate buffer were then mixed and left for 5 minutes at room temperature to incubate. The solution was then diluted 10 times with distilled water and analysed by UV-vis using water as a baseline solvent.

Synthesis of xanthate-terminated two-arm poly (ethyl oxazoline) P2

In a heat-dried Schlenk flask equipped with a magnetic stirring bar, EtOx (10.2 mL, 10 g, 100.9 mmol) were dissolved in dry MeCN (30 mL) under inert atmosphere. The solution was degassed using three freeze-pump-thaw cycles. After degassing, *α, α´*-dibromo-p-xylene (1.3 g, 5.05 mmol) were added. The reaction mixture was heated to 80 ^{0}C in an oil bath and left overnight. After 22 hours, when 1H-NMR showed that polymerization was complete, the reaction mixture was cooled to room temperature. Potassium ethyl xanthate (8.1 g, 50.45 mmol) was added in portions. Dry MeCN (20 mL) was added to the mixture. The reaction was left to terminate overnight at room temperature. Then the mixture was diluted with CH_2Cl_2, filtered, and the solvent was removed using a rotary evaporator. The remaining yellow solid was dissolved in water, transferred to a dialysis membrane (regenerated cellulose, MWCO = 1KD) and dialyzed against water, exchanging solvent 3 times over 3 days. The solution was freeze-dried to yield **P2** as an off-white powdery solid. Yield - 3.2 g (32%).

Synthesis of disulphide-containing poly (oxazoline) P4

In a heat-dried Schlenk tube equipped with a magnetic stirring bar **P2** (1 g, 0,36 mmol) was dissolved in 10 ml of THF under inert atmosphere. The solution was degassed by bubbling argon through it for 1 minute. Ethylene diamine (240 mg, 3.99 mmol, 11 Eq) was added to the mixture, and the reaction was left to proceed overnight. After 20 hours the reaction mixture was transferred to a round-bottom flask equipped with a stirring bar, 30% H_2O_2 (45 L, 1,44 mmol, 4 Eq) was added, and the reaction was left overnight once more. After 20 hours the product was precipitated once from diethyl ether and centrifuged at 5000 rpm for 10 minutes. The precipitate was dissolved in water, transferred to a dialysis membrane (regenerated cellulose, MWCO = 1KD) and dialyzed against water, exchanging solvent 2 times over 1 day. The solution was freeze-dried to yield the product **P4** as a white powder. Yield - 0.7 g (74.8%).

Synthesis of disulphide-containing poly (ethylene imine) P5

In a dry round-bottom flask equipped with a stirring bar **P4** (600 mg) was dissolved in 9 ml of 3M HCl. The solution was heated to 100 °C in an oil bath and refluxed overnight. After 24 hours the reaction mixture was cooled to room temperature, and 4M solution of NaOH was slowly added to it over an ice bath until solution pH 10

(measured at regular intervals with pH paper). The resulting precipitate was washed 5 times with water, centrifuged at 5000 rpm for 10 minutes between the washes, and dried under vacuum to yield product **P5** as a light yellow solid. Yield – 226.5 (78.9%).

Synthesis of carboxyl-terminated PEG 2K P7

Dried PEG monomethyl ether (10 g, 5 mmol) was dissolved in 20 ml of toluene in a heat-dried round-bottom flask equipped with a stirring bar. Succinic anhydride (1.001 g, 10 mmol, 2 eq) were then added under stirring. The reaction mixture was heated to 120 C on an oil bath and refluxed for 4 hours. After that time the reaction mixture was cooled down to room temperature, and the solvent was removed by rotary evaporation. The residue was dissolved in DCM, transferred into a separating funnel, washed three times with 0.5 M HCl and once with concentrated NaCl solution. The organic phase was dried over $MgSO_4$, and the solvent was then removed by rotary evaporation to yield product **7** as a white solid, which was then dried in vacuum. Yield – 8.5 g (80.6%).

Synthesis of Ugi-modified polymers P6 and P8

The reactions were run targeting 100% modification density, assuming the formation of the split-Ugi product. One repeat unit of PEI was considered as 1 eq, and the amounts of other reagents were calculated based on this: 0.5 eq of aldehyde, 0.5 eq of isocyanide and 1 eq of acid.

P5 was dissolved in EtOH/H_2O 9:1 mixture to give 10 mg/ml concentration. The solution was heated to 50 C under stirring on the hot plate and the aldehyde was added while still under heating. After 10 minutes, acetic acid and isocyanide were added, the solution was heated for 4 hours and left overnight at room temperature. The reaction mixture was then diluted with isopropanol, transferred to a dialysis membrane (regenerated cellulose, MWCO = 1KD), dialyzed against isopropanol once, isopropanol/water 1:1 once and distilled water twice, exchanging the solvent every 24 hours. The solution was then freeze-dried to yield product **6** as orange oil-like liquids.

Product **8** was synthesized using the same protocol, with carboxyl-functionalized PEG compound **7** in place of acetic acid. It was obtained as a white powdery substance.

4 Results and discussion

4.1 Synthesis plan

Based on the literature data, a plan of synthesis of compounds 5 and 6 was developed (fig. 10.1). Also, a plan to synthesize and incorporate PEG 2K-containing acidic fragment was proposed (fig. 10.2 and 10.3).

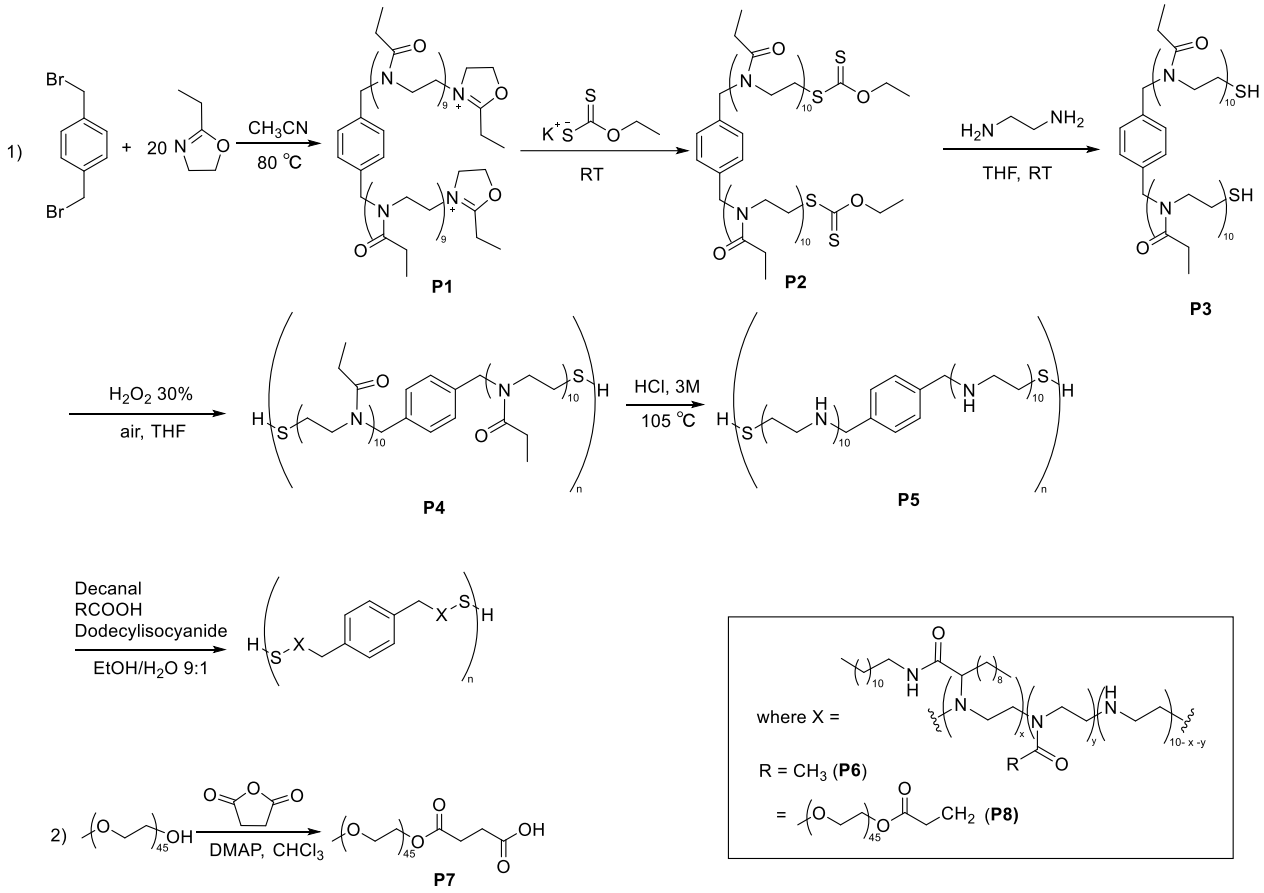

Figure 10. Planned synthesis and reduction of compounds **P6** and **P8**

4.2 Synthesis of two-arm xanthate-terminated poly(2-ethyl-2-oxazoline) P2

Two-arm poly (ethyl oxazoline) **P1** with target DP = 20 (10 per arm) was synthesized by cationic ring-opening polymerization. Initially, *α, α´*-dibromo-meta-xylene was used as a difunctional initiator, following the successful protocol of the reference material. However, later experiments showed no difference in performance for the more affordable para-isomer. So, for further synthesis *α, α´*-dibromo-para-xylene initiator was used.

Reaction progress was monitored by 1H-NMR (fig. 11ab), which confirmed formation of the desired polymer. In the spectrum measured 20 hours after the start of the

reaction, peaks *c* and *d* of the monomer at 4.22 ppm and 3.81 ppm respectively have disappeared, instead forming a single tall peak *c* at 3.23-3.77 ppm, which corresponds to the polymer backbone. The signal of side chain protons *b* of the initiator at 4.48 ppm also shifted to 4.49-4.75 ppm and significantly changed in shape, which correlates well with debromination and subsequent polymer chain growth. Finally, changes in signal of the ethyl pendant from 2.28 ppm and 1.18 ppm to 2.23-2.53 and 1.13 respectively are also characteristic for poly (2-ethyl-2-oxazoline).

This living polymerization was then terminated by addition of potassium ethyl xanthate, successful incorporation of which was also confirmed by NMR (fig. 11c) In the spectra of the final product **P2** a new peak appeared at 1.33 ppm, corresponding to the methyl fragment of the terminating group. In addition, the signal of the methylene group of the terminating fragment appeared in the 4.49-4.75 ppm region, combining with the initiator residue signals.

The identity of **P2** was also confirmed by MALDI-TOF mass spectrometry (fig. 12). The observed ion mass is 2548.46 Daltons, which is exceptionally close to the theoretically calculated value of 2548.51 for the sodium-cationized polymer ion with DP = 22. It is important to note, however, that while the discrepancy between expected and measured degree of polymerization is insignificant for MALDI-TOF,

analysis of NMR spectra shows a much more noticeable deviation, with observed DP between 21 and 26, depending on the batch.

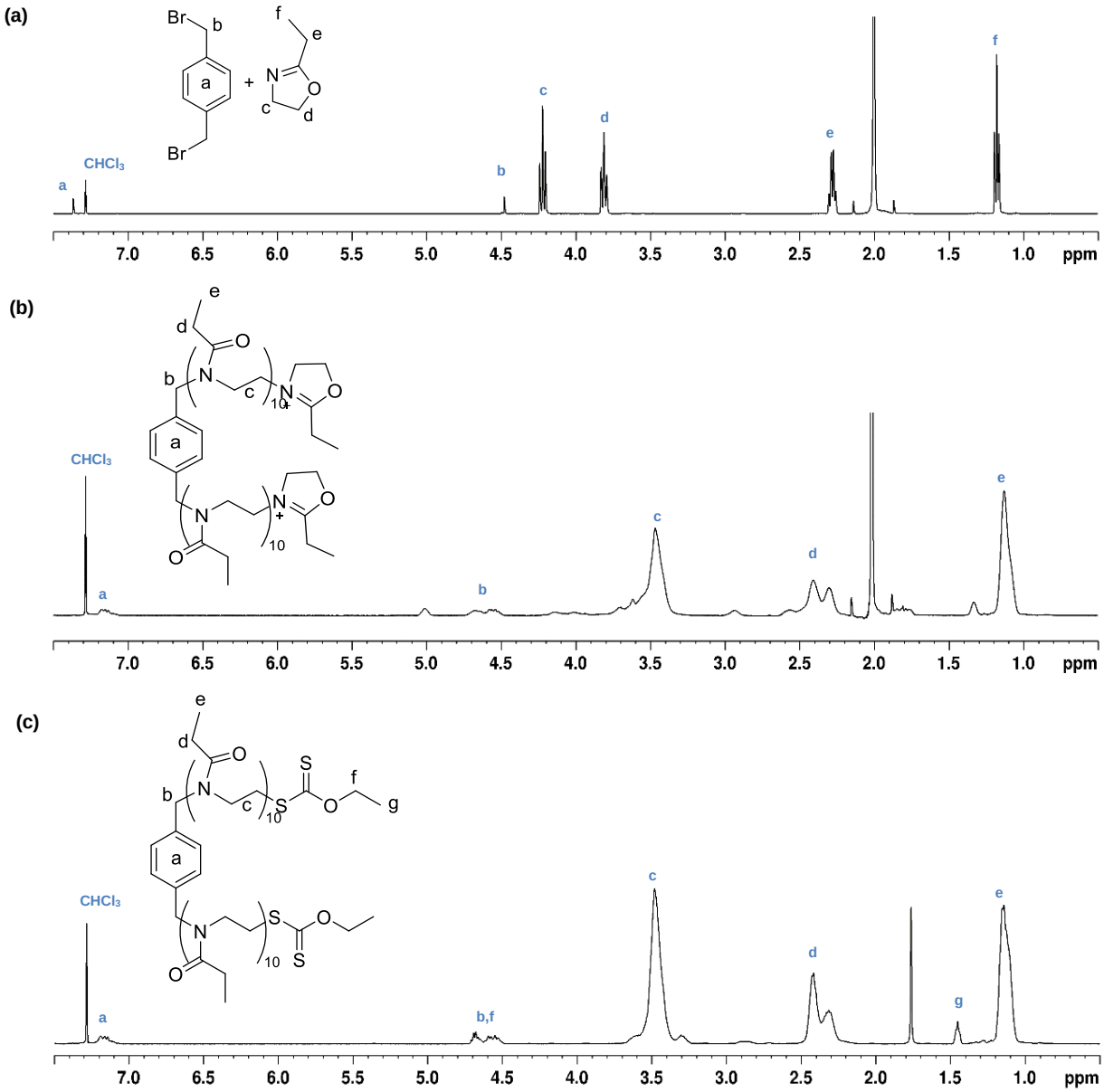

Figure 11. 1H-NMR ($CDCl_3$, 500 MHz, 25°C) spectra of (a) reaction mixture at the start of polymerization (b) reaction mixture 20 hours after the start of polymerization and (c) polymer **P2** after termination and separation

Table 1. Batches of **P2** synthesized for this project.

Name	Purification method	Yield (%)	DP MALDI	DP NMR	M_n NMR (kDa)	M_n SEC (kDa)	M_p SEC (kDa)	PDI SEC
P2a	Washing	59	22	26	2.9	4.3	5.3	1.42
P2b	Dialysis	25	23	24	2.7	4.4	4.3	1.27
P2c	Dialysis	32	20	23	2.6	3.8	3.8	1.26
P2d	Dialysis	23	22	21	2.4	3.9	3.7	1.31
P2e	Dialysis	30	21	23	2.6	3.6	3.0	1.28

Notably, gel permeation chromatography data showed the presence of an unknown second species with higher hydrodynamic radius compared to **P2** (fig. 13b). This could hypothetically be explained by the presence of chain-transfer products (fig 13a), which can be formed during cationic polymerization in the presence of water and proton species. This hypothesis can explain why the second species cannot be detected by NMR or MALDI-TOF, as those polymers only differ structurally, but not chemically. It can also explain why NMR spectra show a higher DP than expected.

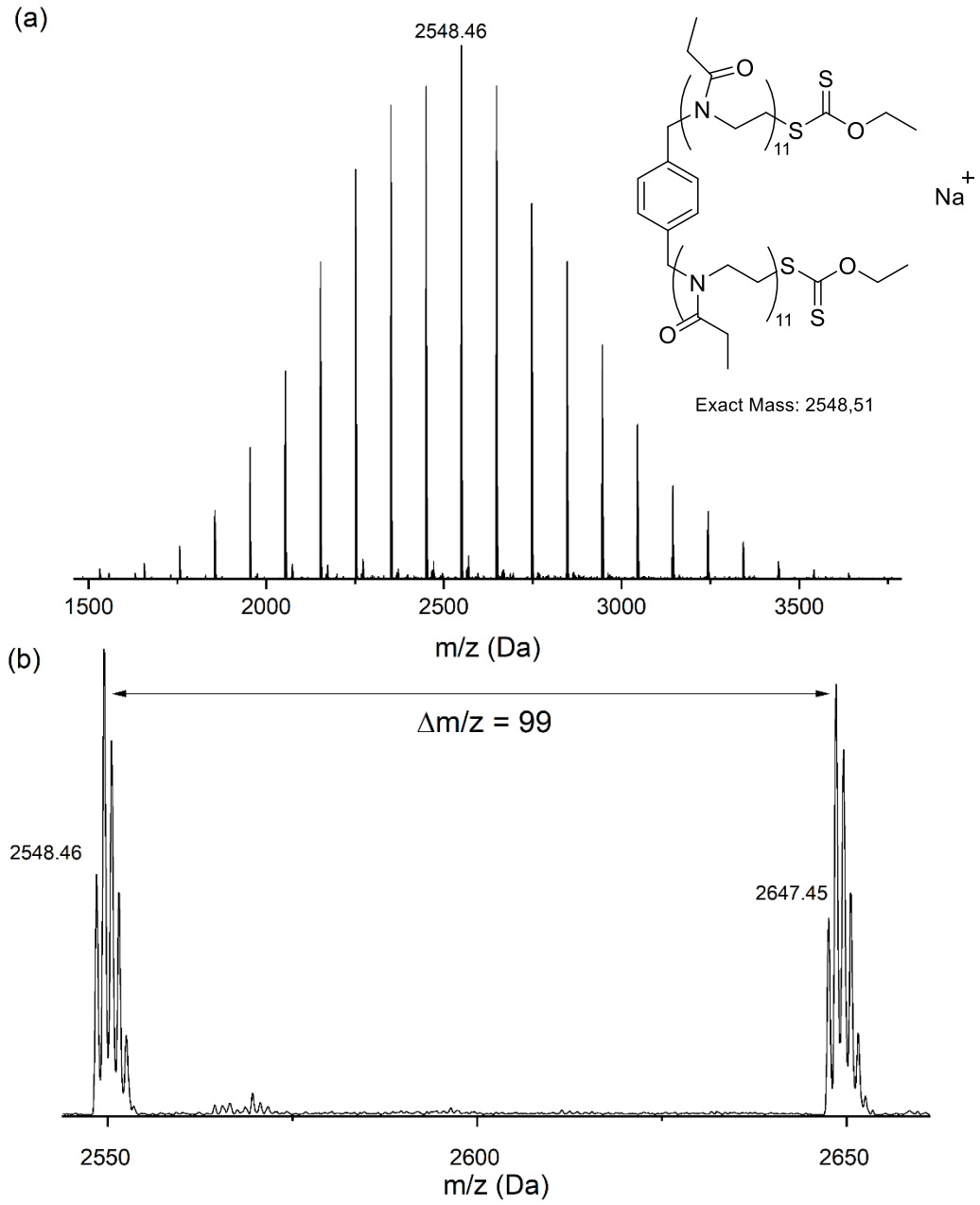

Figure 12. (a) MALDI-TOF mass spectra of **P2** obtained using DCTB as matrix, NaTFA as ionisation agent, in reflectron mode, with laser intensity = 45 (b) close-up of the peaks with highest intensity. Isotope distribution and Δm/z are in accordance with expected data for **P2**

Interestingly, the presence of this second species varied between different batches of **P2**, and we were unable to identify the reasons for this during the scope of the project. However, since it was unlikely that the chain transfer product would interfere noticeably with the further synthesis, we decided to use all successful batches, regardless of the presence of the second peak.

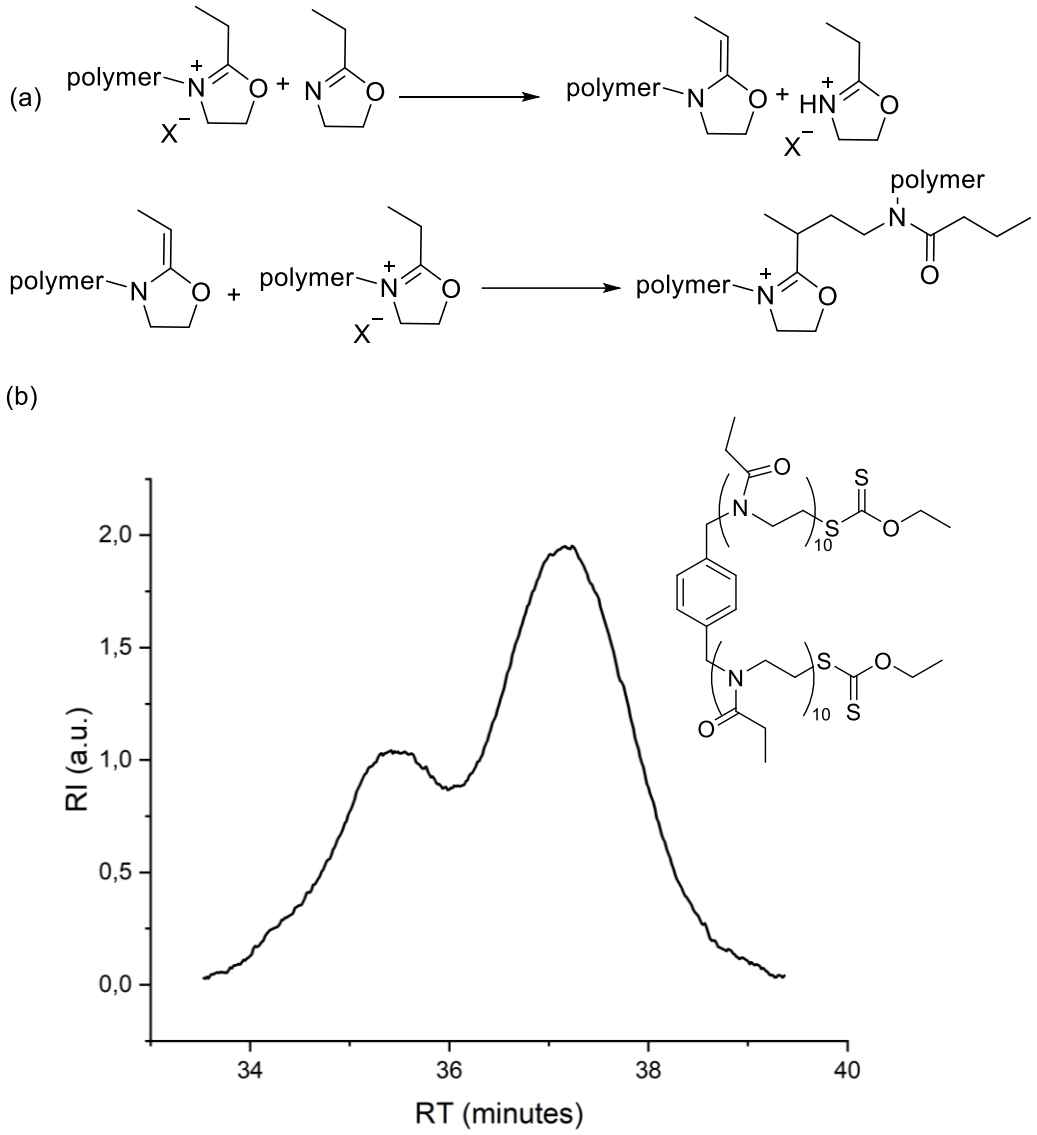

Figure 13. (a) mechanism of chain transfer, which can lead to a by-product with higher MW (b) SEC trace of **P2e** (DMF), which has the highest amount of the chain-transfer by-product. Figures for all other batches are presented in the Appendix

Another major problem we have run into during this synthesis was the purification of P2 after termination. Initially, the product was extracted out of the reaction mixture by washing with saturated NaCl solution and consecutive precipitation from diethyl ether, which lead to a yield of approximately 60%. We hypothesized that, since **P2** is water-soluble, a significant portion of it may be lost during the washing and decided

to explore an alternative purification method. Hydrolysis through a regenerated cellulose membrane (MWCO = 1 kDa) against water was tested, as the solubility of **P2** would not interfere with it, and even be useful instead. This method has supposedly allowed to get rid of more residual xanthate, as the resulting product was less yellow in colour and did not have such a strong smell compared to batches obtained by washing and precipitation. However, it has also led to an even lower yield of 30%. A probable cause could be the long hydrolysis time, during which more polymer molecules statistically can adapt the conformation required to permeate the membrane. If so, it should be possible to prevent this by exchanging water more frequently to achieve similar purity over a shorter time, for example 24 hours as opposed to 3 days. This hypothesis should be tested in further studies.

In conclusion, two-arm xanthate-terminated poly(2-ethyl-2-oxazoline) **P2** was successfully synthesized for further experiments.

4.3 Synthesis of poly(2-ethyl-2-oxazoline) disulfide P4

Next, deprotection of the xanthate groups of **P2** to form a thiol functionalized intermediate **P3,** and its subsequent oxidation to disulfide-linked oligomer **P4** was performed. The identity of all **P4** samples was confirmed by 1H-NMR spectroscopy (figure 19). Characteristic signals of the xanthate group's ethyl pendant fragment at 1.3 ppm and 4.49-4.75 ppm have disappeared, while backbone signals did not change. However, a small peak close to the main backbone signal at 3.27-3.72 is present, which corresponds to protons of the monomer unit bound to the disulphide fragment (*f*). This confirms that the xanthate groups have been removed from the polymer, but sulphur is still present at the terminus.

UV-vis data also shows that the absorbance peak of the xanthate, which is clearly visible for P2, is not present in P4 (fig.15). This also confirms the successful deprotection of thiols.

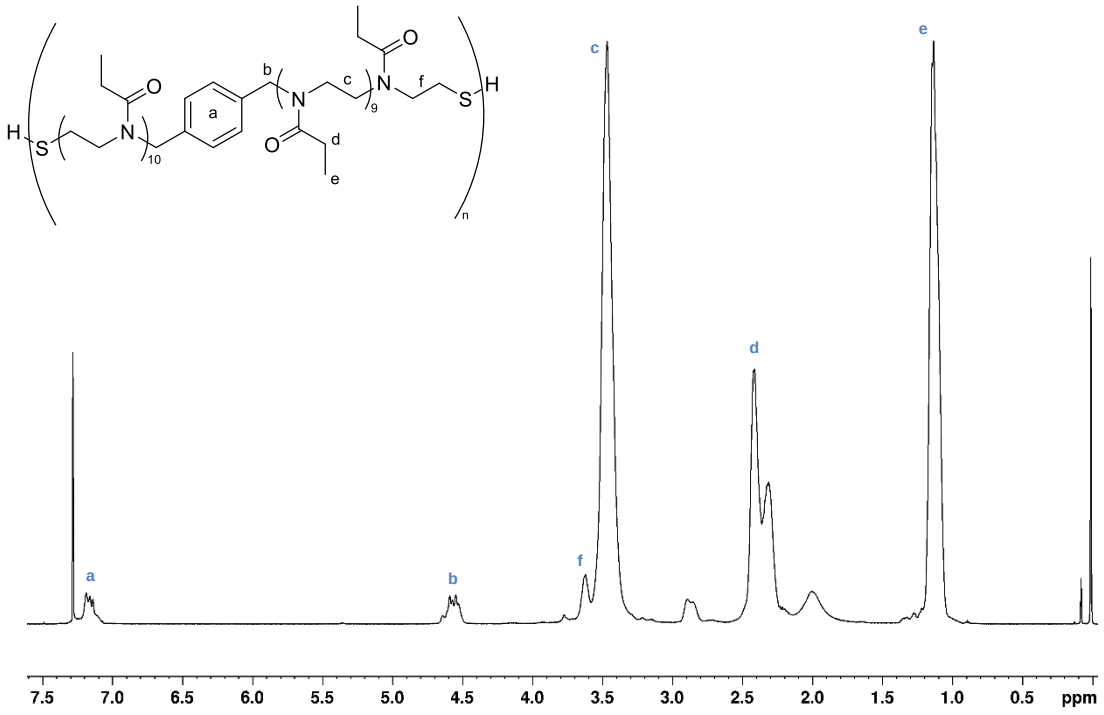

Figure 14. 1-H NMR ($CDCl_3$, 500 MHz, 25°C) of **P4**

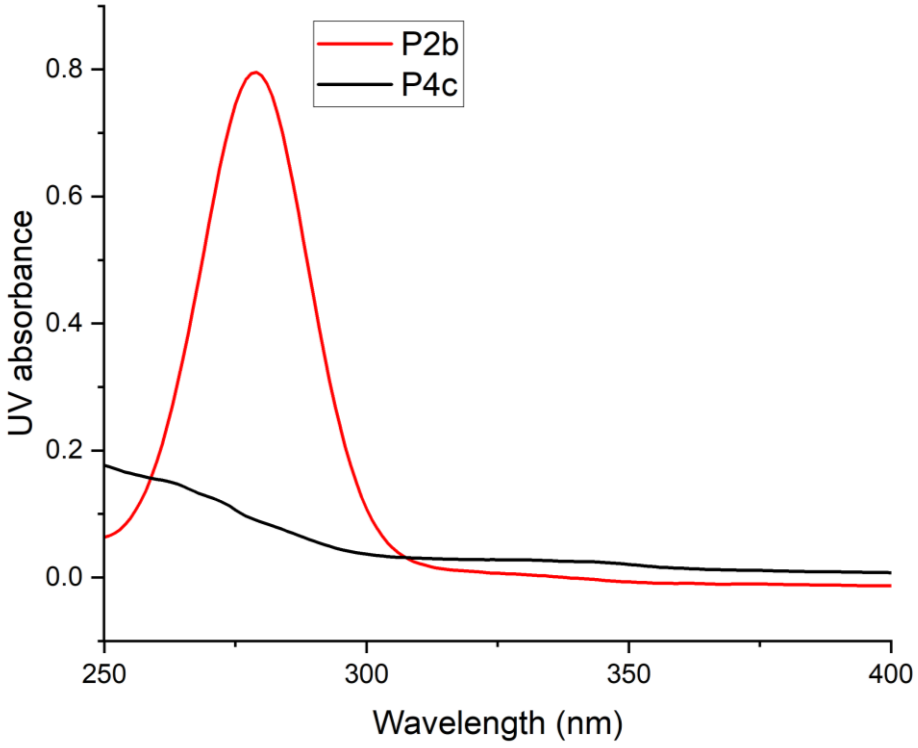

Figure 15. Close-up UV-vis spectra of **P2b** and **P4c**. The absorbance peak at 275 nm corresponds to the xanthate fragment

To optimize synthesis of **P4** number of important factors had to be considered to promote the oligomerization reaction as opposed to formation of a cyclic by-product.

Firstly, high concentrations of the starting polymer (100 mg/ml) are required so that intermolecular interactions of thiol groups are preferred over intramolecular interactions leading to cyclization. The nature of the solvent is also important, as it defines polymer conformation and, therefore, the proximity of chain ends to each other. Secondly, a large excess of the cleaving amine agent (10 eq) is beneficial, as more molecules are deprotected simultaneously and can interact with each other. Finally, premature oxidation during the deprotection step needs to be avoided, as it increases the possibility of reaction between two thiol groups of the same chain. All those factors were considered and screened for this reaction (table 2).

Table 2. Study and optimization of conditions for the one-pot thiol deprotection and oxidation. All yields were fairly similar and were not considered for optimization purposes. For all MeCN reactions dry solvent was used. In reactions **P4i-P4l** argon was bubbled through reaction mixture for 1 minute before addition of the amine to degas the solution. In reactions **P4e-P4h** air was bubbled through the reaction mixture for 1 minute after the thiol deprotection step was complete. In reaction **P4m** the reaction mixture was precipitated from Et_2O immediately after deprotection, with no oxidation.

Name	P2	Solvent	Degassing /drying	Air	Oxidation	M_n SEC kDa	M_p SEC kDa	PDI SEC
P4a	P2b	THF	-	-	+	7.2	6.9	1.31
P4b	P2b	MeCN	+	-	+	6.1	3.4	1.67
P4c	P2b	$CHCl_3$	-	-	+	4.9	3.3	1.55
P4d	P2c	MeCN	+	-	+	4.1	3.0	1.44
P4e	P2c	MeCN	+	+	+	3.3	2.8	1.29
P4f	P2c	MeCN	+	+	+	4.5	3.0	1.52
P4g	P2c	THF	-	+	+	4.3	3.0	1.72
P4h	P2d	THF	-	+	+	3.1	2.8	1.23
P4i	P2d	THF	+	-	+	7.9	19.1	2.06
P4j	P4i	THF	+	-	+	9.4	25.8	2.42
P4k	P2d	THF	+	-	+	5.7	2.9	2.35
P4l	P2e	THF	+	-	+	6.1	2.6	3.14
P4m	P2d	THF	+	-	-	3.2	2.9	1.26

We hypothesized that using acetonitrile or chloroform as a reaction medium as opposed to the standard THF could be beneficial, as THF is considered a rather poor solvent for poly (oxazolines), which may contribute to cyclic by-product formation. To test this hypothesis, we carried out the same reaction in three different solvents and compared the SEC traces of the resulting polymers **P4a**, **P4b** and **P4c** (fig. 16). Interestingly, while the reaction in acetonitrile yielded the polymer with the highest MW, judging by the early elution time, the product obtained in THF contained the lowest amount of the LMW by-product, which was significant for the other two solvents. This suggests that, contrary to our hypothesis, THF may be a better solvent for this polymer than $CHCl_3$ and MeCN. It could be explained by the presence of the

aromatic fragment in the polymer structure: in a polymer with rather short arms the influence of the benzene ring may be significant enough to change the solubility of **P2** and **P3**, making THF a better solvent than it would be for other poly (oxazolines). However, we did not study this solubility phenomenon in detail, as it was beyond the scope of our project.

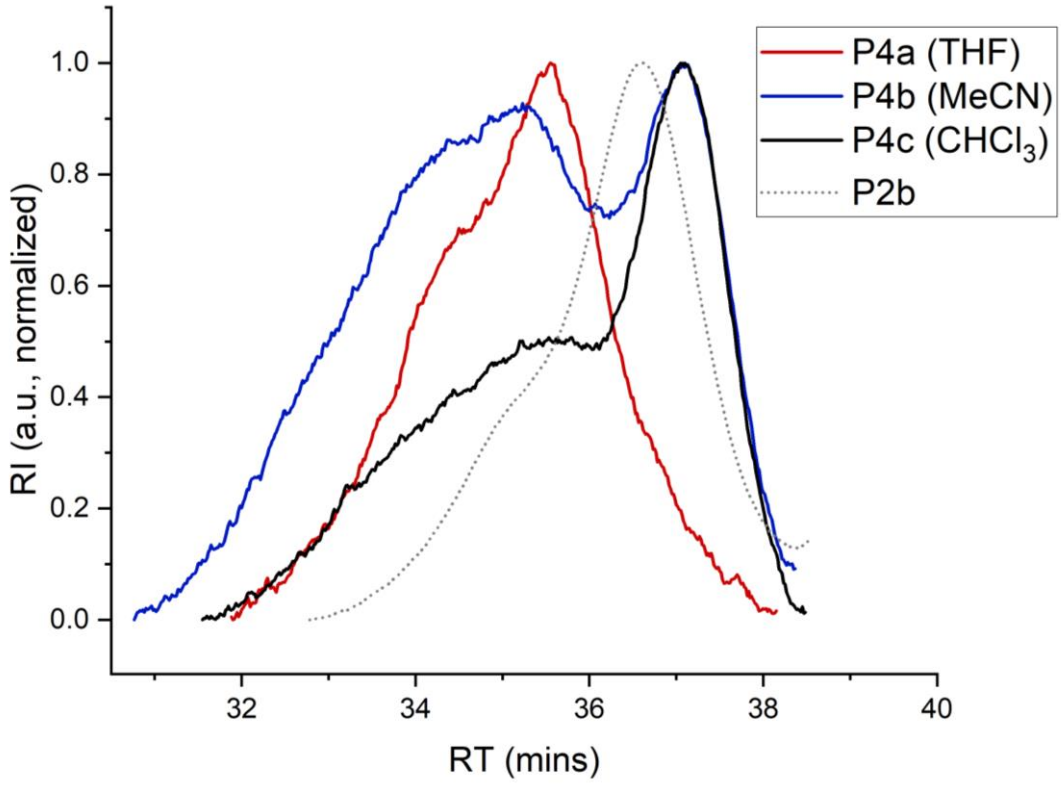

Figure 16. SEC traces (DMF) of polymers **P4** obtained in THF, MeCN and $CHCl_3$. All reaction were performed using **P2b** as a starting product

For THF and MeCN the influence of the oxidation intensity was also studied. For that, compressed air was bubbled through the reaction mixture after the addition of the oxidant for 1 minute to presumably saturate the solution with oxygen; after that the reaction proceeded as normal. In THF some oligomer was still formed, but not as efficiently as under standard oxidation conditions (fig. 17a), while in MeCN only the LMW by-product was present (fig.17b). This suggested that air bubbling is not an efficient way of enhancing oxidation intensity for this reaction, as it reduces condensation efficiency and prevents formation of the desired product rather than promote it. A potential explanation for this is precipitation of **P3** from the solution induced by the bubbling, which prevents molecular chain ends from interacting. It was observed that after bubbling the reaction mixture became cloudy, which can also

be explained by such precipitation. It is, however, interesting, that even after the polymer re-dissolved, reaction still did not occur.

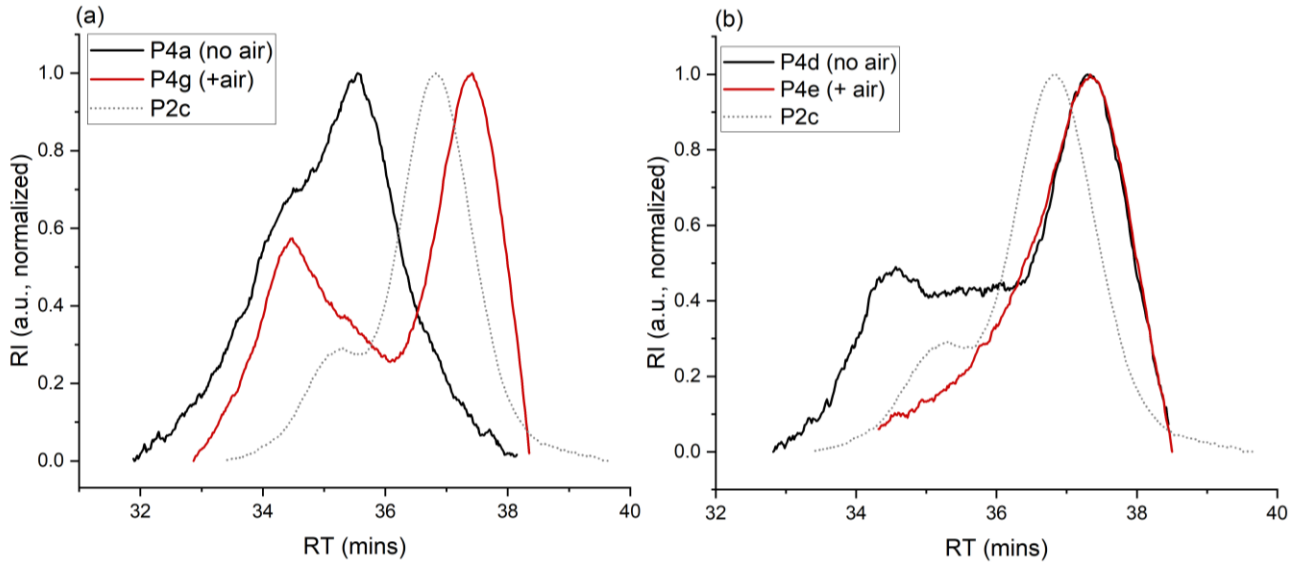

Figure 17. SEC traces (DMF) of polymers obtained under excessive air oxidation compared to the standard protocol (a) in THF (b) in MeCN. Starting material used for all reaction is **P2c,** except for **P4a**, which was synthesized from **P2b**

Interestingly, in experiments, where the reaction mixture (in THF) was precipitated immediately after thiol deprotection, with no oxidation applied at all, the same LMW species was also observed as the sole product (fig. 18a). This strongly suggested that this peak corresponded to the thiol-terminated unreacted polymer **P3** rather than its' cyclic disulphide counterpart, which can sometimes form as a by-product in such reactions. However, identifying either of them by SEC alone is nearly impossible, as they have a fairly similar retention time, especially for polymers with lower DPs. So Ellman's assay was used to identify the presence of free thiols or disulphide bonds in this LMW product (fig. 18b). For **P4m**, which was precipitated directly after deprotection, UV-vis data showed absorbance at 412 nm and no visible absorbance at 320 nm. This suggests that the polymer is mostly present in the solution in its' thiol form, with rather low, if any, disulphide concentration. For **P3e**, which was oxidized both by H_2O_2 and by intensive air bubbling, a small shoulder peak was present around 320 nm, but the peak at 412 still dominated. This data clearly identifies the LMW by-product as unreacted **P4**, with negligible amounts of disulphide-containing species present.

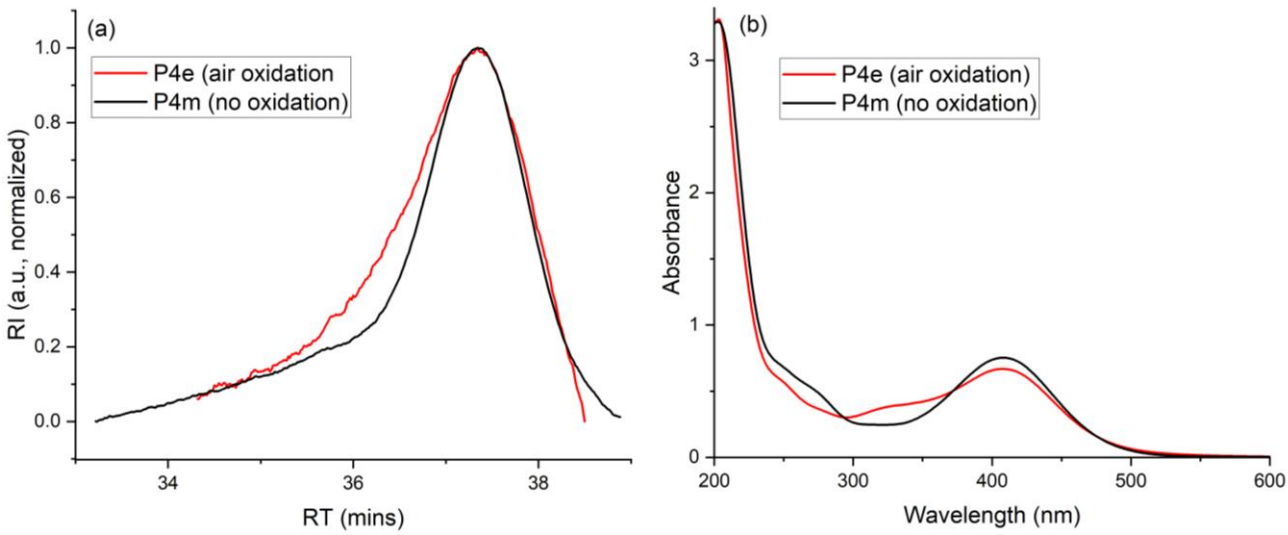

Figure 18. (a) SEC traces (DMF) of P4e and P4m (b) UV-vis spectra of P4e and P4m

We attempted a simple degassing by bubbling argon into the reaction mixture for 1 minute under inert atmosphere before the addition of amine. The resulting product contained a higher amount of the LMW by-product when compared to the polymer obtained in the non-degassed solvent, but also the target polymer exhibited higher MW (fig. 19). Further reactions confirmed this trend, although noticeable differences were observed in amount of the LMW by-product and molecular weight distribution of the target compound synthesized identical conditions. This irreproducibility may be attributed to the crude nature of our chosen degassing method. In further studies, a more robust approach, such as the freeze-pump-thaw method, should be explored as well to achieve optimal and reproducible oligomerization.

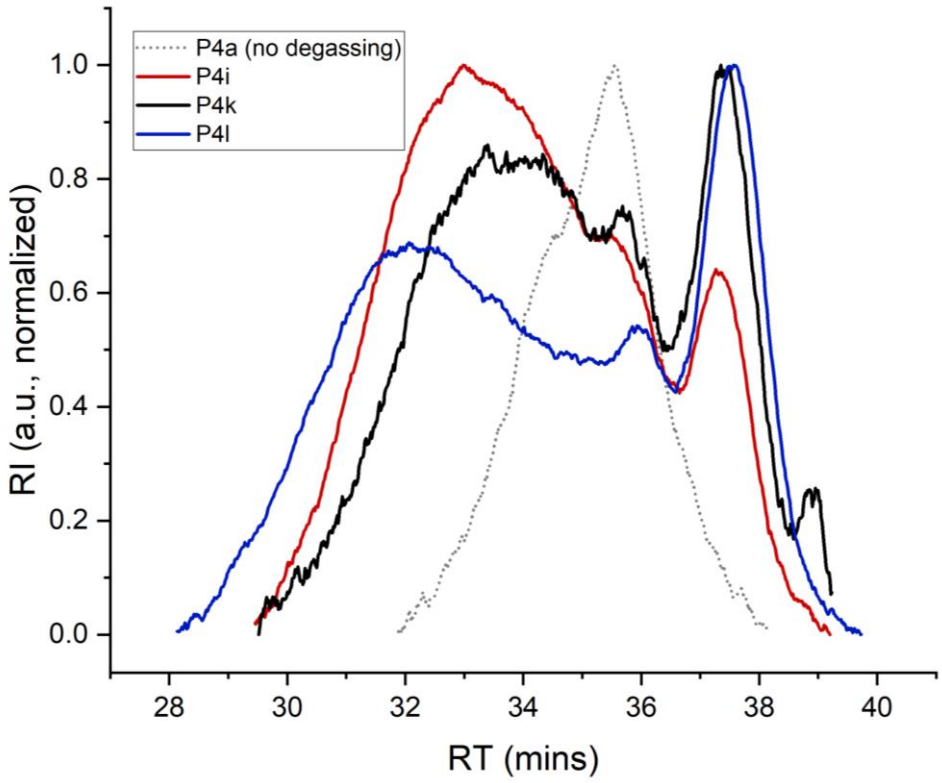

Figure 19. SEC traces (DMF) of polymers synthesized in THF with and without degassing. Synthesis conditions for **P4i**, **P4k** and **P4l** were identical

Finally, we tested the possibility of increasing the reaction efficiency by repeating the oxidation reaction on the product **P4i**. Upon this second oxidation reaction, GPC data (fig. 20) showed a noticeable decrease in the amount of the LMW by-product, as well as a shift in molecular mass of the product, which means that the oligomerisation progressed further. Importantly, this data demonstrates that the LMW by-product is not inactive and can participate in oligomerisation. This suggests that it is possible to achieve such results in a single reaction, and the conditions require further optimisation to do so. For example, longer reaction times or higher concentrations of the oxidant can be explored.

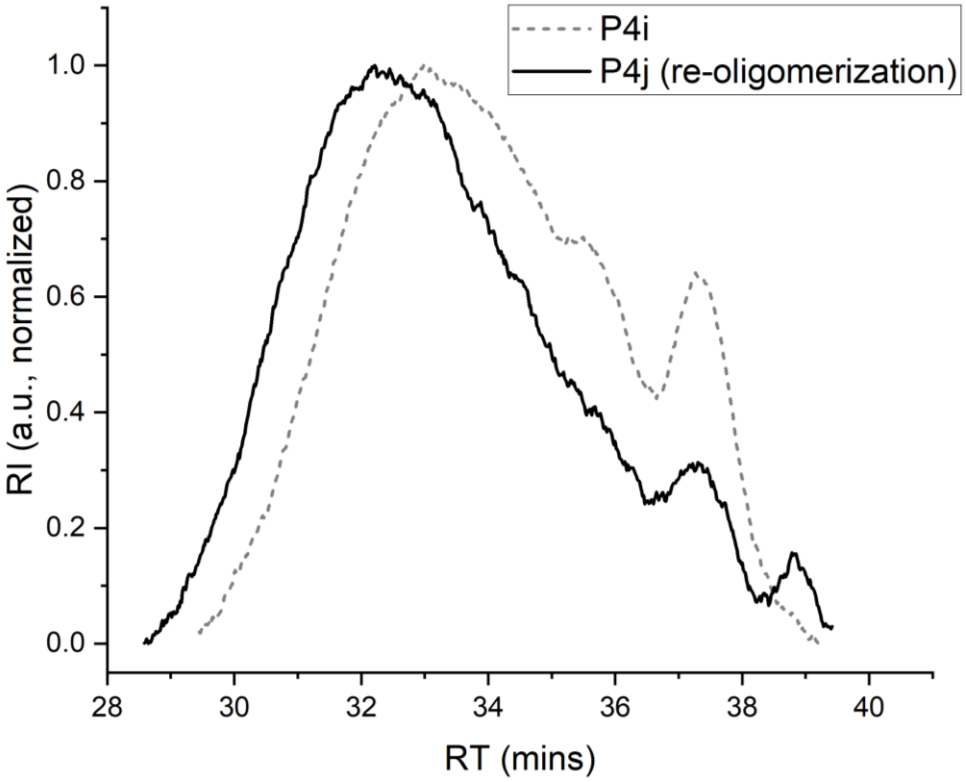

Figure 20. SEC traces (DMF) of **P4i** before and after (**P4j**) a second oligomerization reaction

4.4 Synthesis of poly (ethylene imine) P5

P5 was obtained by hydrolysis of **P4** in 3M hydrochloric acid. To confirm the identity of the product, 1H-NMR in deuterated methanol was used, as the resulting polymers showed poor solubility in $CDCl_3$, which is unexpected for PEI. However, the spectrum observed corresponded very well to the target product (fig. 21). The prominent backbone peak at 2.26 ppm, as well as initiator group signals at 3.78 ppm and 7.45 ppm are present, while signals of the ethyl pendant of EtOx have completely disappeared. The spectrum also visualizes the presence of trace amounts of propionic acid, which has been cleaved off the polymer during the hydrolysis: it is represented by the triplet at 1.11 ppm and the quadruplet at 2.18 ppm. Integration of those signals showed that there are less than 0.5 equivalents of propionic acid present for 20 monomer units of PEI, which is acceptable for the following Ugi reaction.

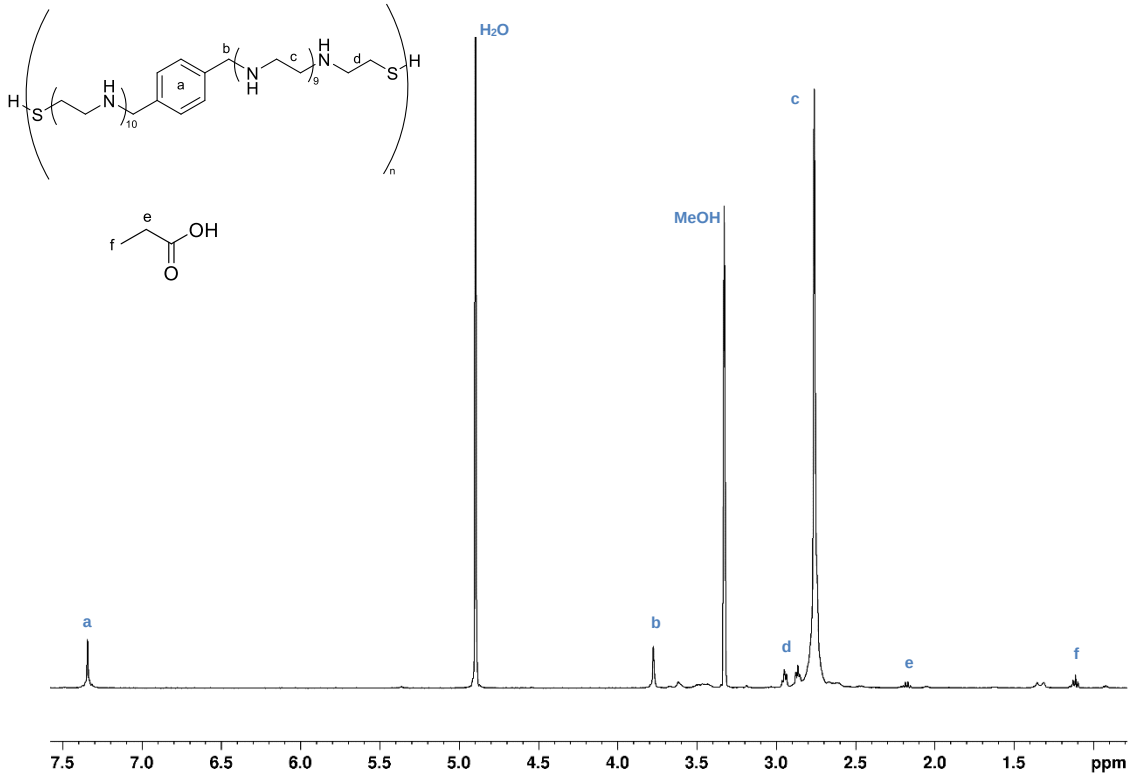

Figure 21. 1H-NMR spectra (MeOD, 500 MHz, 25°C) of PEI **P5**. Traces of propionic acid are also labeled

Due to the poor solubility of PEI in DMF, an alternative system based on HFIP was used to analyse **P5** by GPC (fig.22). Retention time of **P5** is significantly lower compared to its precursor, which is expected, as unprotected cationic groups of PEI

interact better with the polar solvent compared to POx, therefore leading to higher hydrodynamic radii and faster movement on the column.

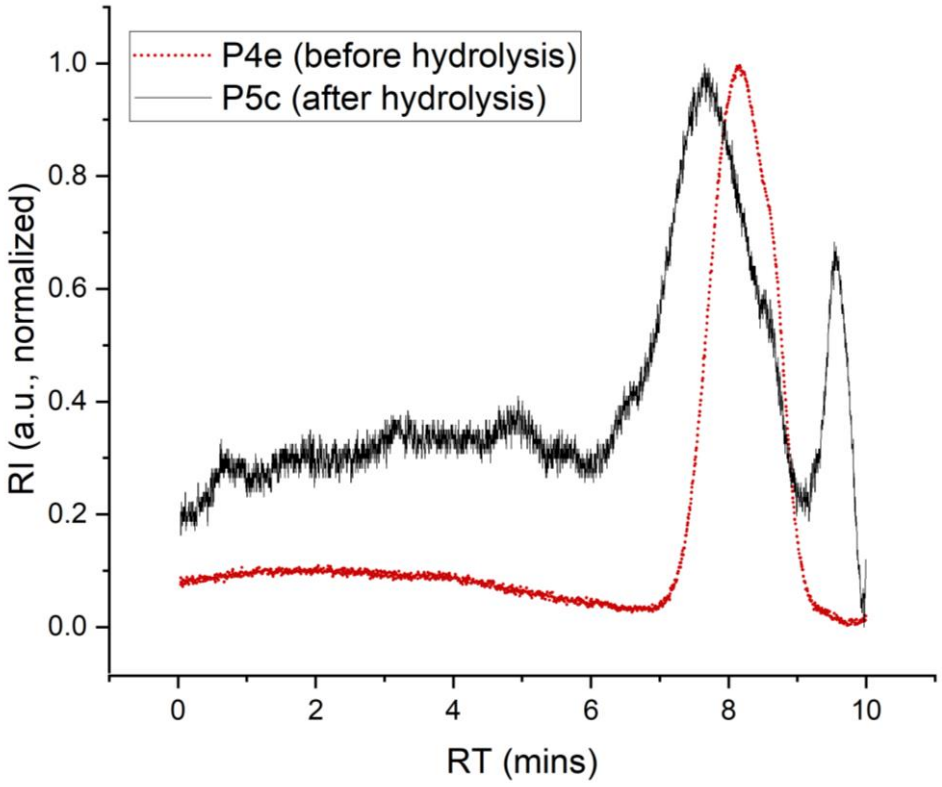

Figure 22. SEC traces (HFIP system) of **P5c** and its precursor **P4e**

It is important to mention that only two out of six batches of **P5** (table 3) showed good enough solubility for GPC to be carried out. Notably, those batches were synthesized from **P4** with the highest content of unreacted LMW dithiol compared to HMW oligomers, which could be and explanation for those unexpected results. It is possible that the extremely high cationic group density of HMW **P5** makes it difficult to dissolve it even in HFIP. If that is the case, this phenomenon can be regarded as proof that no significant backbone degradation has occurred during hydrolysis.

Table 3. Solubility of different **P5** batches in HFIP (5mg/ml)

Name	Starting polymer	Solubility in HFIP
P5a	P4c	-
P5b	P4d	+
P5c	P4e	+
P5d	P4i	-
P5e	P4j	-
P5f	P4m	-

Another method which could be used to analyse the molecular weight of the **P4** is DOSY-NMR, since our PEI product can be dissolved in MeOD. Using this approach, diffusion coefficient of polymer molecules can be identified, and samples synthesized from polymer 3 with both low and high presence of LMW unreacted dithiol can be compared to demonstrate difference or lack thereof. Time limitations of this project did not allow us to carry out this experiment.

In conclusion, HMW PEI disulfide **P5** was successfully synthesized.

4.5 Synthesis of PEG-acid P7

P7 was then synthesized by esterification of commercially available PEG monomethyl ether with succinic anhydride. Initially, we planned to carry the reaction out in chloroform at 50 °C over 24 hours, using DMAP as a basic catalyst, as it is a well-known and established method in literature. However, 1H-NMR spectra of the resulting product showed that under those conditions full conversion is not achieved (fig. 23). This was determined by comparing the integrals of signals corresponding to the methyl group and the succinic acid group. If full conversion is reached, the relation between those integrals should be 3:4. This was, however, not the case for our product.

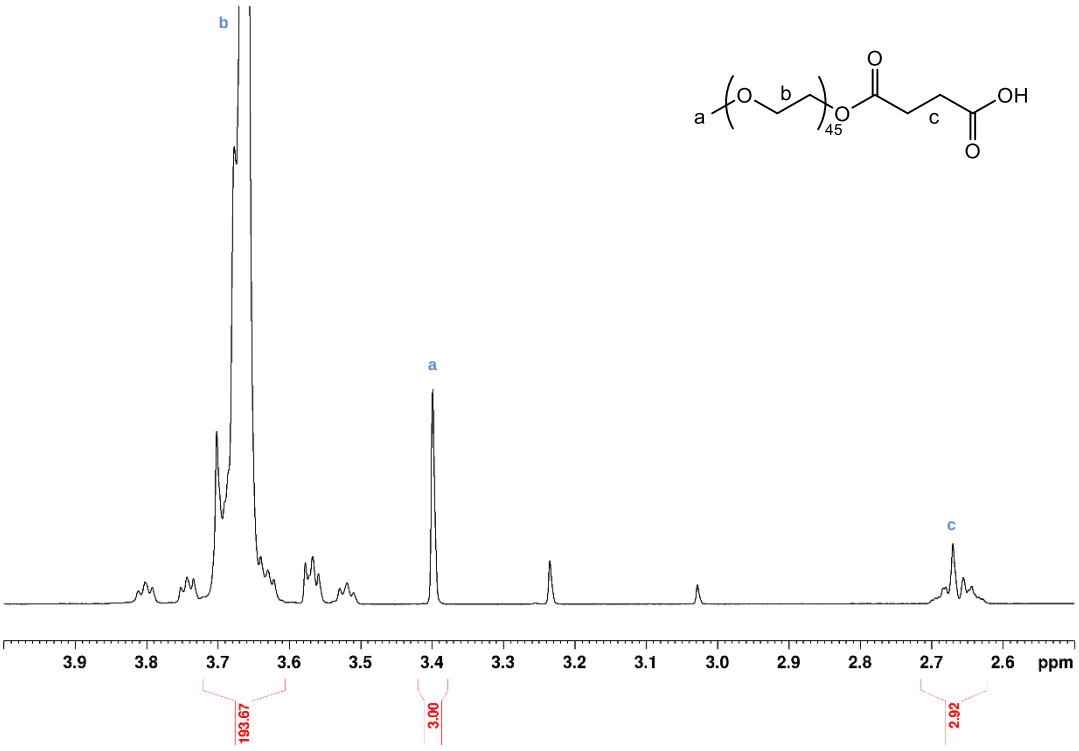

Figure 23. 1H-NMR spectra ($CDCl_3$, 500 MHz, 25°C) of **P7** synthesized following the protocol described above

Moreover, even if the reaction was allowed to carry out for three days, full conversion still was not reached, though it has overall improved (figure 24a). The remaining unfunctionalized PEG monomethyl ether, however, remained reactive, and after re-subjecting the resulting polymer mixture to the same conditions for the second time, we were able to achieve the desired result (fig. 24b).

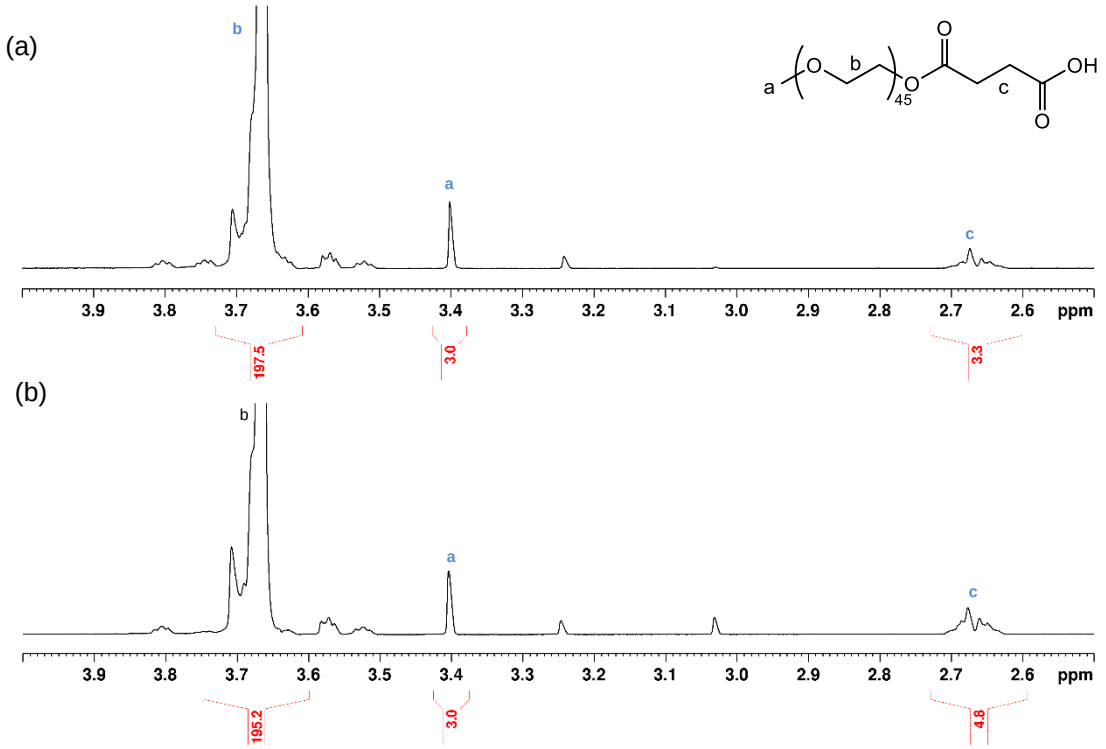

Figure 24. 1H-NMR spectra ($CDCl_3$, 500 MHz, 25°C) of product **P7** obtained with (a) longer reaction times and (b) re-subjecting under-reacted polymer to a second reaction

We hypothesized that the reason behind this issue is the poor solubility of succinic anhydride in chloroform, which makes it unavailable for reaction with the polymer. Solubility can be increased at higher temperatures. However, since the boiling point of chloroform is only 67 °C, the possible increase in temperature might be too low to impact the solubility significantly. For this reason, we opted to use an alternative solvent with a high boiling point. Several articles describe esterification with succinic anhydride in boiling toluene, which occurs at 110 °C under refluxing. Not only does this protocol use high temperature, which can contribute to better dissolution of reagents and more efficient conversion, but it is also more time and resource-efficient compared to the one used previously. The reaction is said to occur over 2-3 hours as opposed to overnight, and does not require the use of DMAP, as the acidity of the anhydride itself is enough to catalyse the process under those conditions.

The reaction was carried out according to the protocol, with reaction time increased to 4 hours to ensure the best possible conversion while retaining the time efficiency. 1H-NMR (figure 25a) showed that after 4 hours the conversion was better compared to the previous protocol, but, regrettably, full functionalisation still has not been achieved. After re-subjecting the resulting polymer to the same conditions, we were again able to complete the reaction (fig. 25b). While it is still more time-efficient compared to the chloroform-based method, this approach also does not allow to reach full conversion with a single reaction. It is possible that the claimed reaction times are too low, as many articles that use a similar no-DMAP approach report refluxing for 16-18 hours instead of 2-3. However, since our previous attempts have yielded enough PEG-COOH **7** for further experiments, we did not test this theory further in the scope of this research.

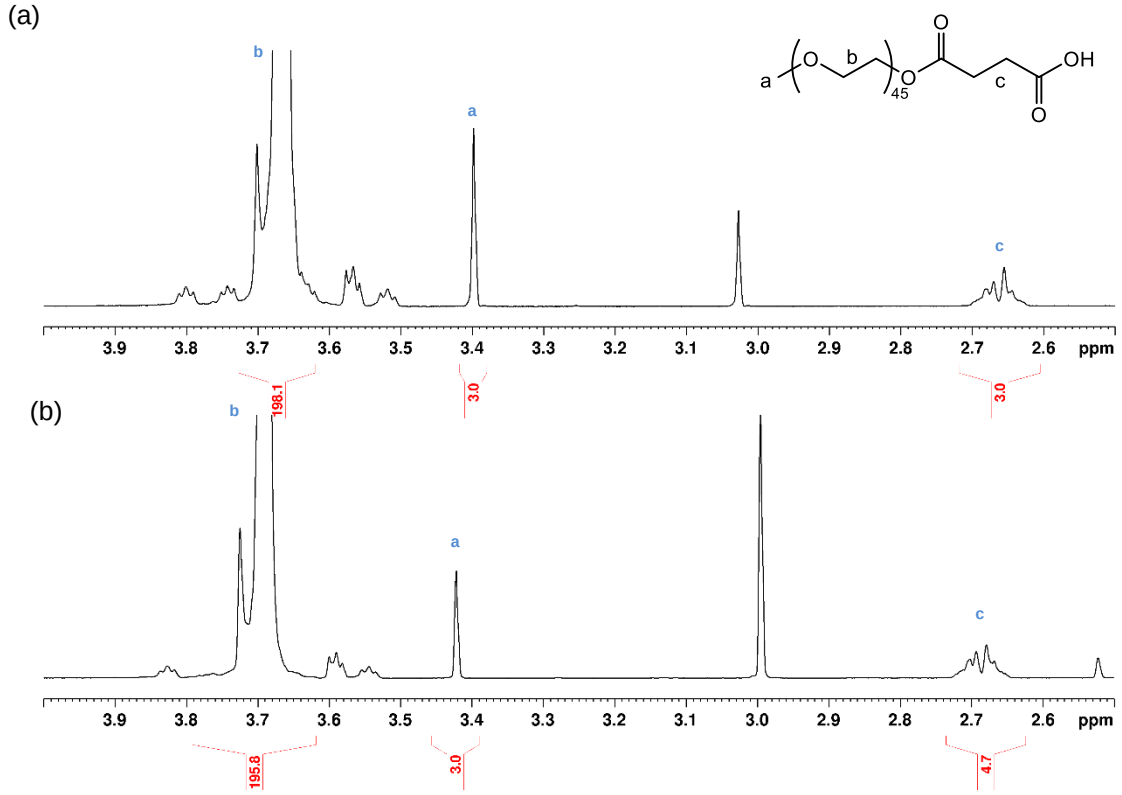

Figure 25. 1H-NMR spectra ($CDCl_3$, 500 MHz, 25°C) of **P7** obtained by (a) the standard protocol (b) re-subjecting the resulting polymer to the same conditions.

The identity of the polymer was also confirmed by MALDI-TOF mass spectroscopy (fig. 26). The difference between the peaks, m/z, is 44, which is to be expected for a PEG derivative. The peak with the highest intensity corresponds to m/z = 2069.75, which does not align with the predicted m/z for the target product (2048.16).

However, it is important to note that polymer 7 contains a carboxylic acid fragment, which can become ionized under MALDI-TOF conditions and appear as a sodium salt. Taking that into account, we can see that predicted m/z for sodium-cationized salt of compound 7 (2070.14) is within reasonable error compared to experimental values obtained. Thus, we can still identify the target product with reasonable certainty.

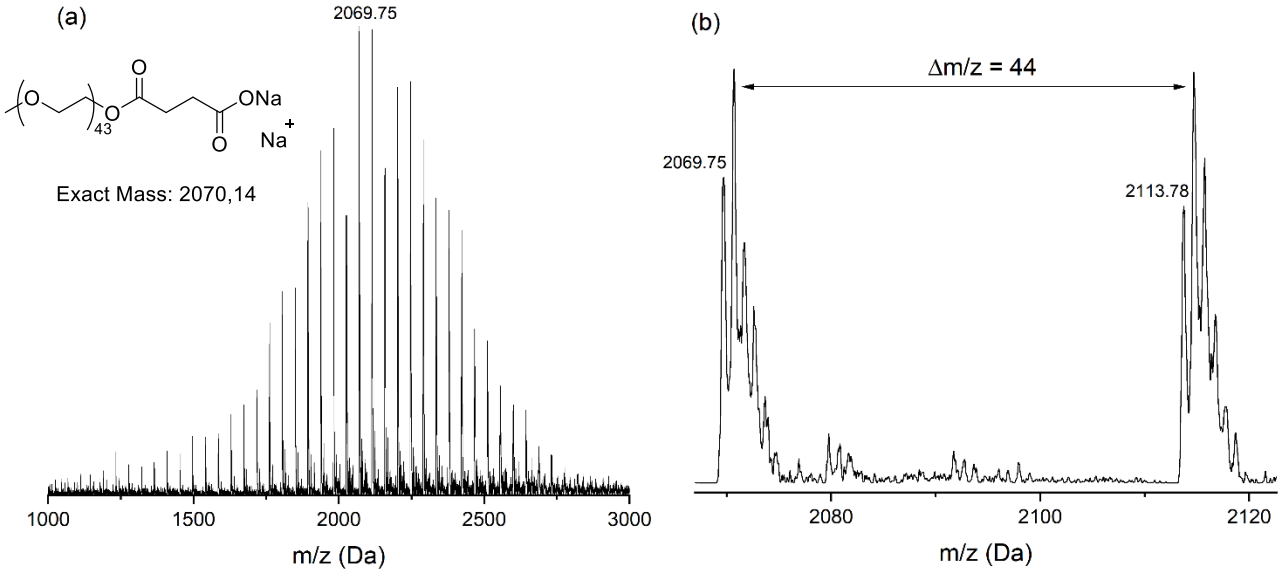

Figure 26. (a) MALDI-TOF spectrum of **P7** obtained using DCTB as matrix, NaTFA as ionisation agent, in reflecton mode, with laser intensity = 45 (b) close-up of the peaks with highest intensity. Isotope distribution and Δm/z are in accordance with expected data for **P7**

In conclusion, we have successfully synthesized PEG-COOH **P7**.

4.6 Synthesis of hydrophobically modified PEI P6 and P8 using the split-Ugi reaction

Finally, we have applied the protocol developed by Vlasova et al to synthesize Ugi-modified polymers **P6** and **P8**. Seeing as the article by Vlasova et al demonstrated that the combination of decanal, acetic acid and dodecyl isocyanide lead to polymers with the best gene delivery performance, those reagents have been chosen for our initial experiments. 100% modification density was aimed for.

Identity of the final product was confirmed by 1H-NMR (figure 27). Since the split-Ugi reaction introduces many modifications, some of which carry rather long hydrophobic chains, the backbone signals of the PEI are not as prominent anymore. Two distinct peaks can be found at 0.93 ppm and 1.32 ppm, corresponding to the long hydrocarbon fragments introduced into the polymer. The acetate group signal is

also visible at 2.11-2.48 ppm. All other signals are also present in accordance with the literature data.

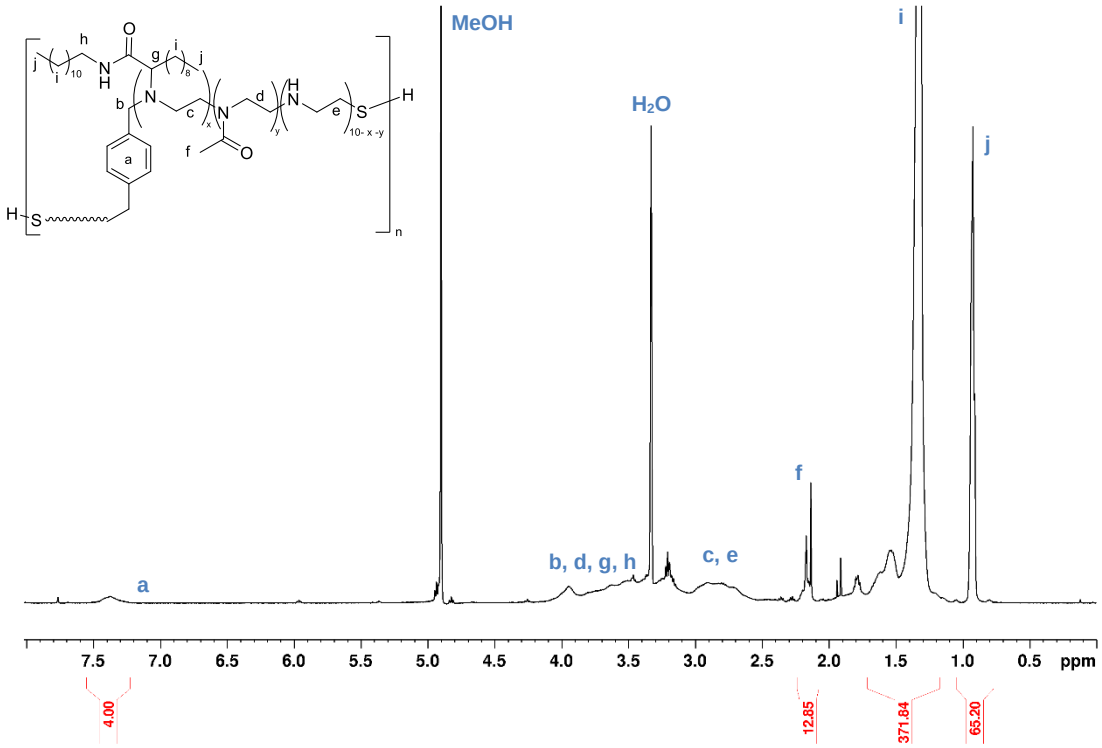

Figure 27. 1H-NMR spectrum (MeOD, 500 MHz, 25°C) of polymer **P6**

The modification density of the resulting polymer was then calculated by comparing the integrals of the newly introduced functional groups to the signal of the aromatic protons of the initiator fragment at 7.23-7.55 ppm. It was shown that on average 30% of PEI units carry the bis-amide functionality and 30% carry the acetate functionality, which is similar to what has been reported by Vlasova et al (approximately 40% and 30% respectively). This confirms that the reaction has performed as expected.

Polymer **P6** was also analysed by GPC, using $CHCl_3$:IPA:TEA 94:4:2 as an eluent (fig. 27). Two distinct species are clearly present, with one eluting at approximately 24.5 ml, and the other at 27.9 ml. It can be reasonably assumed that the species which elutes later has a smaller hydrodynamic radius and, therefore, corresponds to the non-oligomeric PEI, which was present in all batches of the initial polymer. This can also be indirectly confirmed by comparing GPC traces of batches of polymer **P6** with those of **P4** from which the PEI was synthesized (table 4). If **P4** had low amounts of unreacted POx-SH, the intensity of the second peak for the corresponding Ugi-modified polymer **P6** is also low. The difference between the relative quantities of the

HMW and LMW species for polymers 4 and 8 can be explained by a possible loss of some of the LMW species during the long dialysis process for polymer **P6**.

Table 4. All batches of P6 synthesized and described. Unfortunately, for undetermined reasons the program was not able to calculate MW data for P6a

Name	Starting polymer	M_n SEC (kDa)	M_p SEC (kDa)	PDI SEC
P6a	P5b	-	-	-
P6b	P5f	4.2	4.3	1.43
P6c	P5d	4.0	4.5	1.33
P6d	P5f	3.8	3.3	1.55

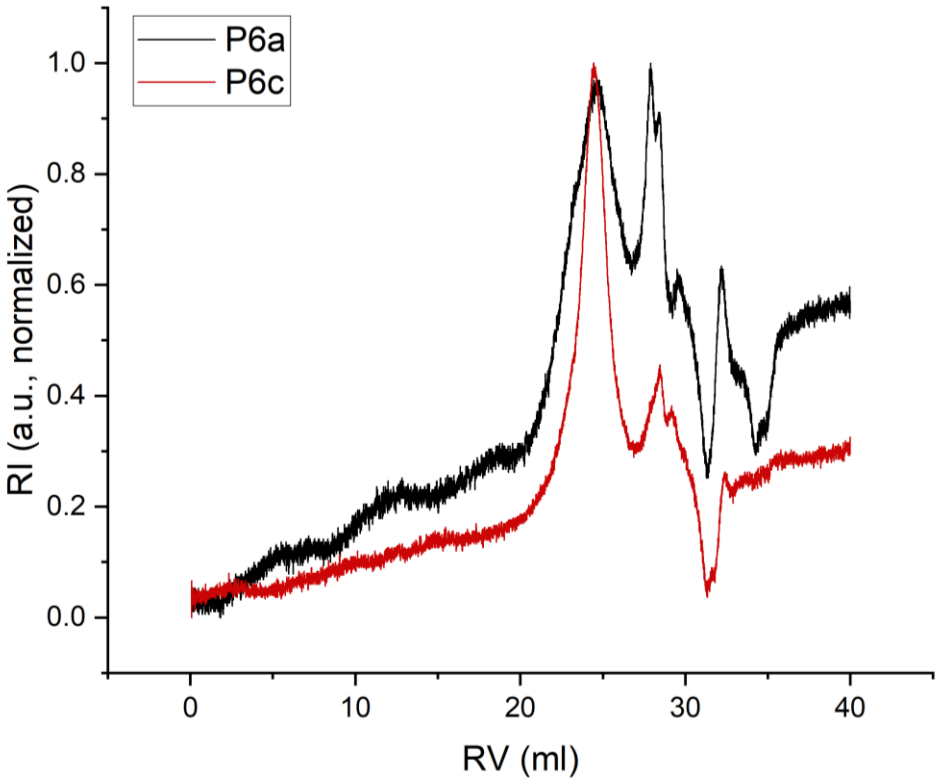

Figure 27. SEC traces ($CHCl_3$:IPA:TEA) of **P6a** with the highest amount of the LMW by-product and **P6c**, with the lowest amount of the LMW by-product

Polymer **P8** was also synthesized using the same approach as for **P6**, except PEG-COOH 7 was used as an acid. 1H-NMR showed that the reaction has been completed successfully, as signals of the PEG-COO fragment, as well as the characteristic signals of the Ugi product as visible (fig. 28). However, since the PEG peak is so prominent, it is nearly impossible to determine the modification degree accurately like it was done for **P6**, as the signal of the aromatic initiator fragment is of too low intensity. However, by comparing the integrals of peaks corresponding to the methyl fragment

of PEG-COO and the methyl fragments of the Ugi product pendant, we can estimate that for every one Ugi fragment there are two PEG-COO fragments present. This is in accordance with the amounts of reagents used in the reaction in which an excess of 2 equivalents of the PEG-COOH were used, indicating the unreacted reagent is not removed in the purification process. It could be hypothesized that, since PEG-COOH is itself a large molecule, the dialysis did not get rid of all of it, and it interferes with the spectrum, making it more difficult to analyse. In that case, using a membrane with higher MWCO should solve this problem, as PEG-modified PEI should be bigger than individual PEG-COOH molecules. This issue is not reported by Vlasova et al, however, despite the fact that they have also synthesized PEG-modified Ugi product and used the exact same purification method.

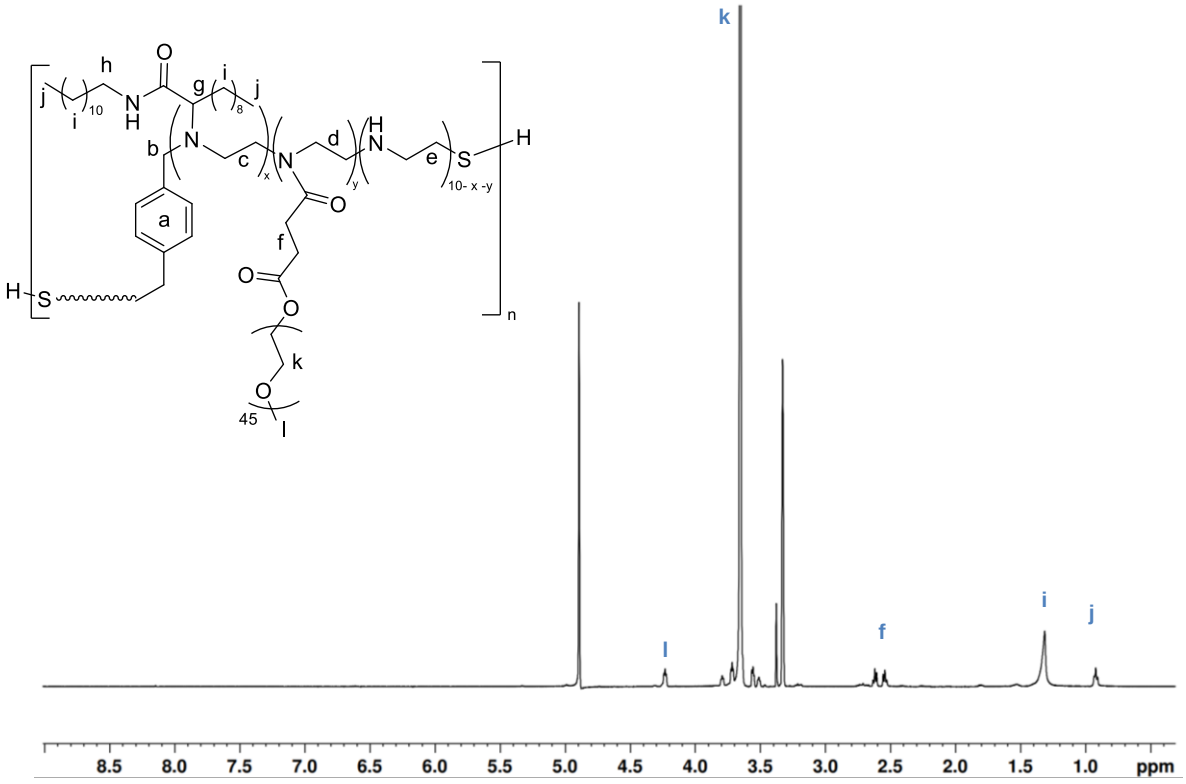

Figure 28. 1-H NMR spectrum (MeOD, 500 MHz, 25°C) of polymer **P9**

Moreover, analysis of polymer **P8** by GPC (fig. 29) shows only one polymer species present, with slightly lower retention time compared to polymer **P6**, which suggests that this peak corresponds to the PEG-modified PEI. Interestingly, the difference in retention times, and, therefore, hydrodynamic radii, is rather small between polymers **P6** and **P8**, which is unexpected considering the additional bulk of PEG

fragments. Moreover, the LMW peak is not visible at all for polymer **P8**, despite its presence in polymer **P6** synthesized from the same starting material.

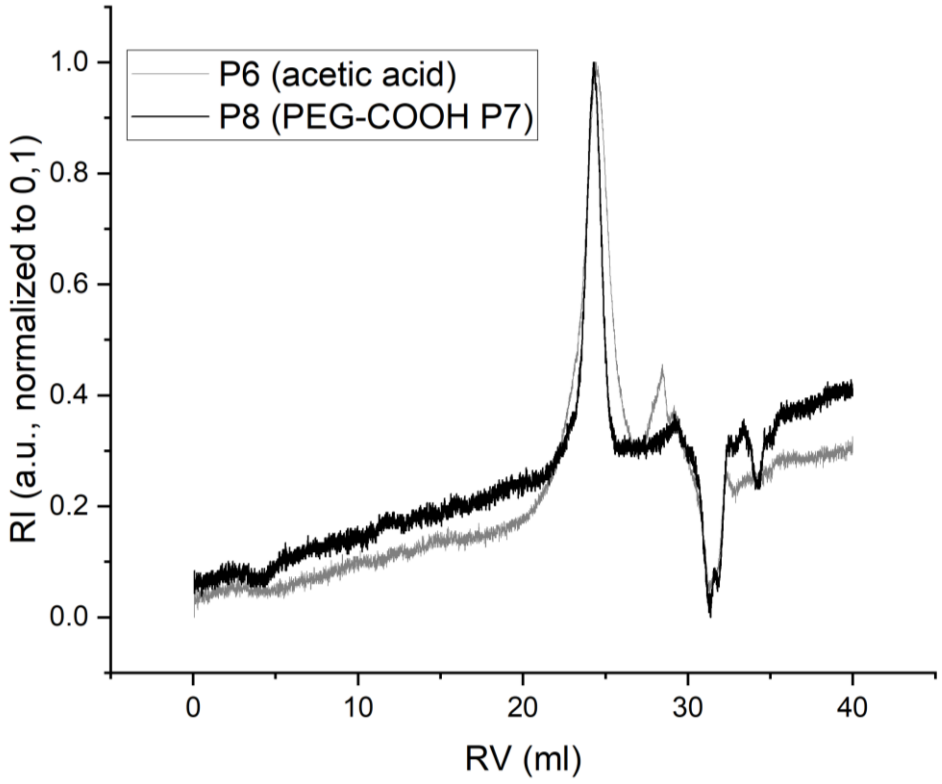

Figure 29. SEC traces ($CHCl_3$:IPA:TEA) of **P8** compared to **P6** synthesized from the same starting material.

Overall, the split-Ugi modifications of bioreducible PEI have been successfully completed.

5 Conclusions and outlook

Linear PEI disulphide compounds were successfully synthesized by hydrolysis of POx obtained by cationic ring-opening polymerization. We have also demonstrated that such compounds can be modified by the split-Ugi type reaction to obtain potentially bioreducible PEI derivatives with hydrophobic moieties, with potentially lower cytotoxicity and higher gene delivery efficiency compared to standard PEI.

In addition, we have optimized conditions for the one-pot thiol deprotection and condensation, which is used to synthesize HMW polymers with bioreducible disulphide bonds. It was shown that using THF as a solvent leads to the most consistent results and high molecular weights. The importance of degassing the reaction mixture for the deprotection step was also highlighted. However, the desired reproducibility was not achieved, and further studies are required to look into other potential contributing factors.

In the future, we are planning to advance this project by synthesizing a library of hydrophobically modified PEI containing disulphide fragments. Physical, chemical and biological properties of those polymers will then be described and compared to standard PEI and non-bioreducible polymers to explore the influence of hydrophobic modifications and bioreducible bonds and potentially identify new promising candidate for gene therapy development and clinical applications.

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Appendices

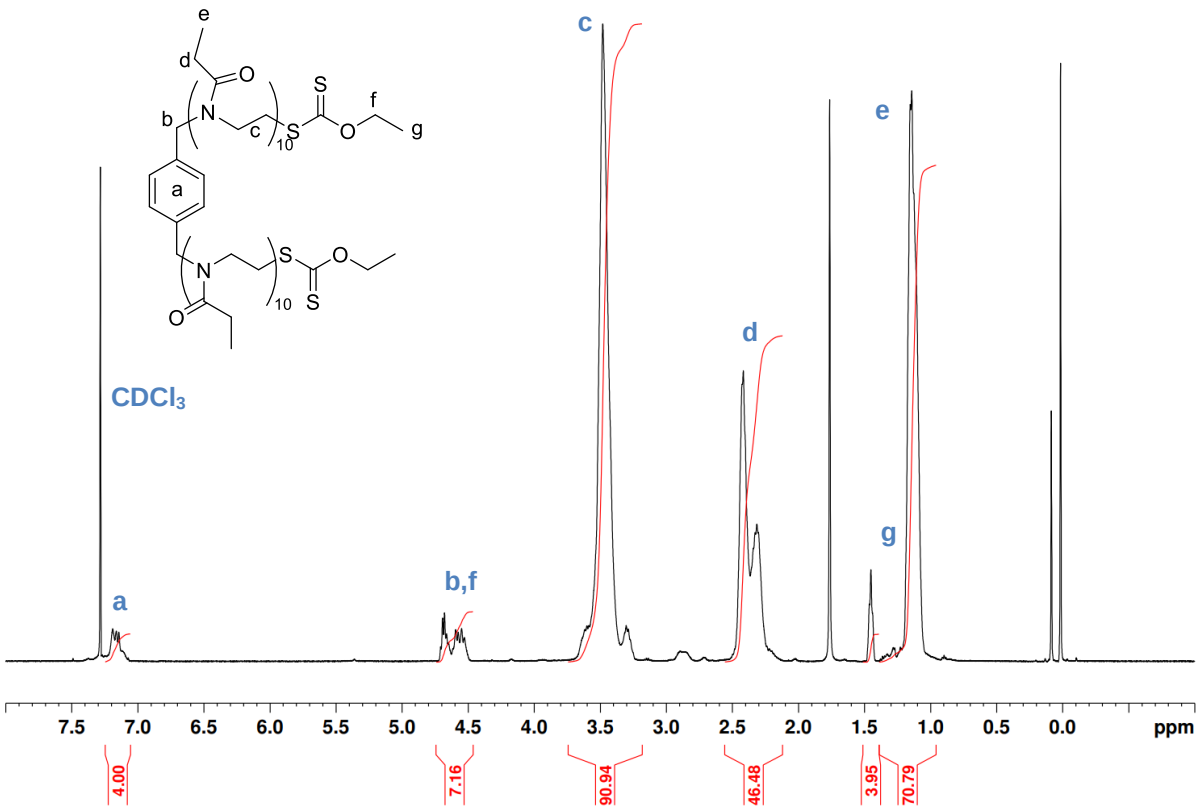

Figure S1. 1H-NMR ($CDCl_3$, 500 MHz, 25°C) of **P2**

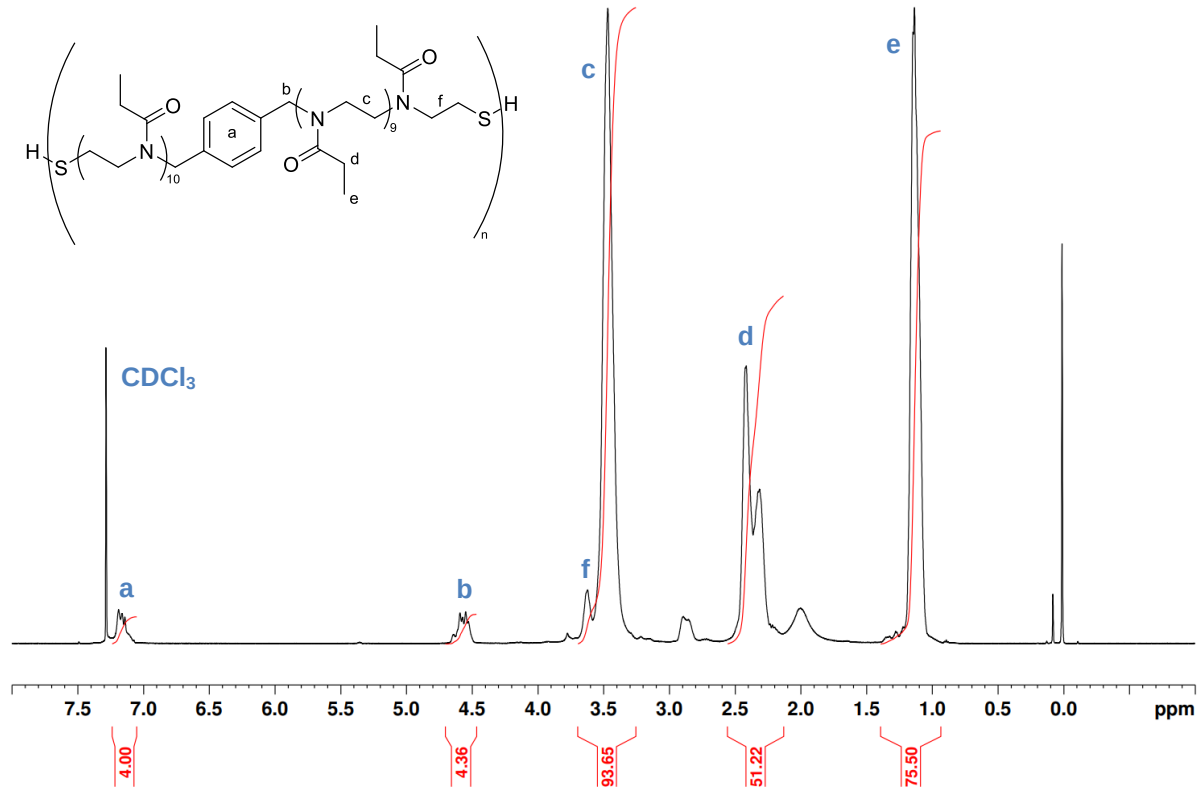

Figure S2. 1H-NMR ($CDCl_3$, 500 MHz, 25°C) of **P4**

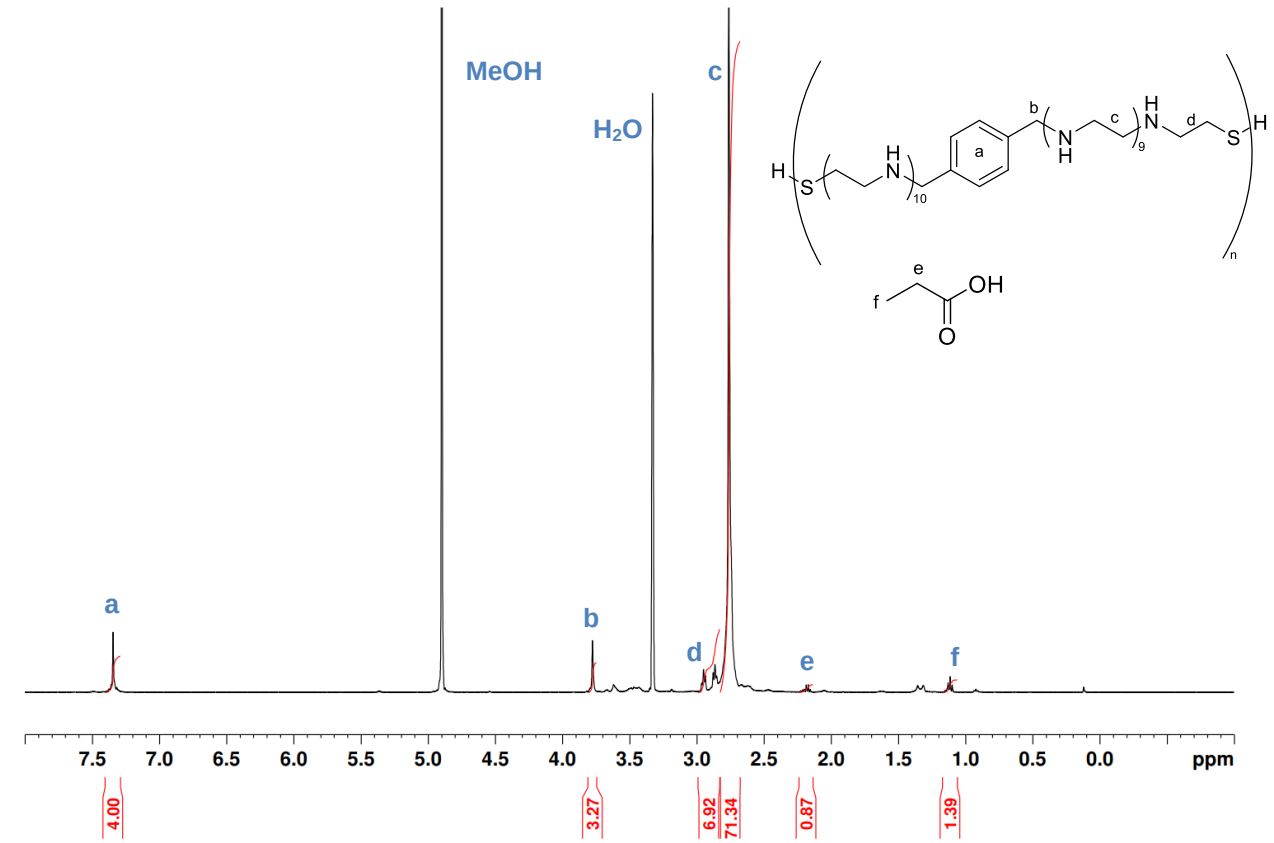

Figure S3. 1H-NMR ($CDCl_3$, 500 MHz, 25°C) of **P5**

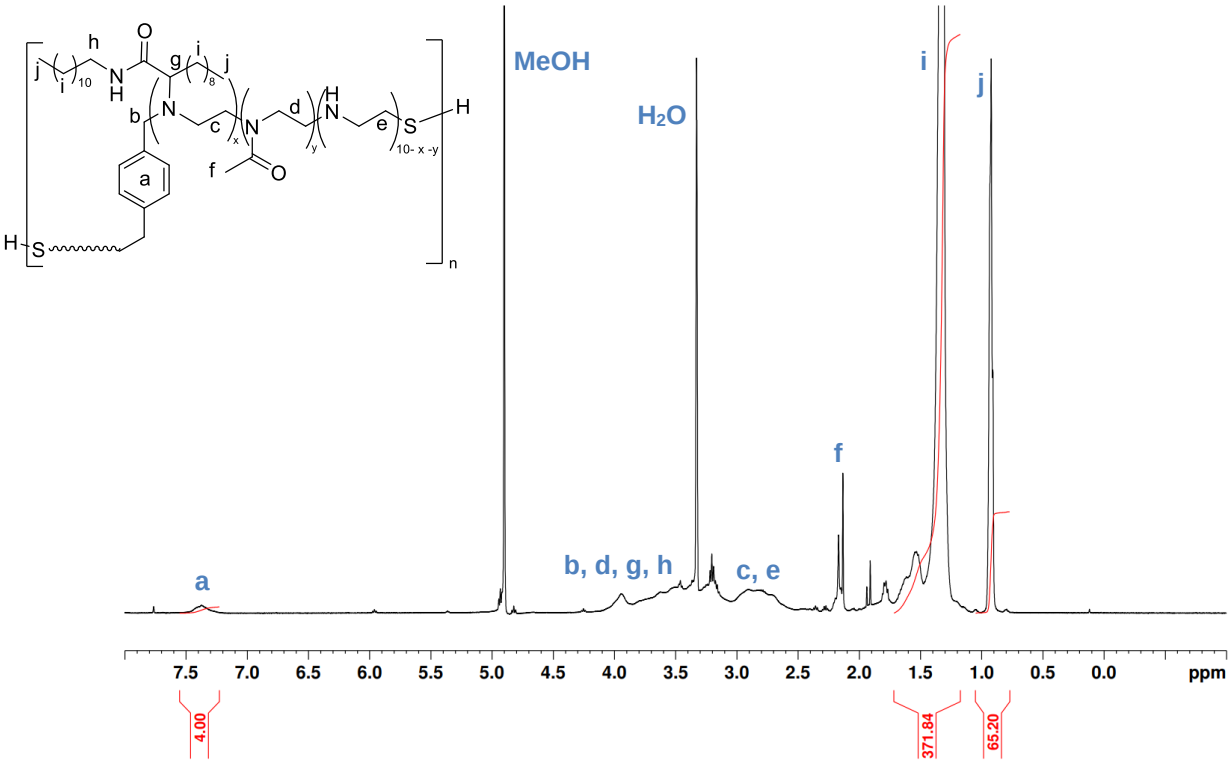

Figure S4. 1H-NMR (MeOD, 500 MHz, 25°C) of **P6**

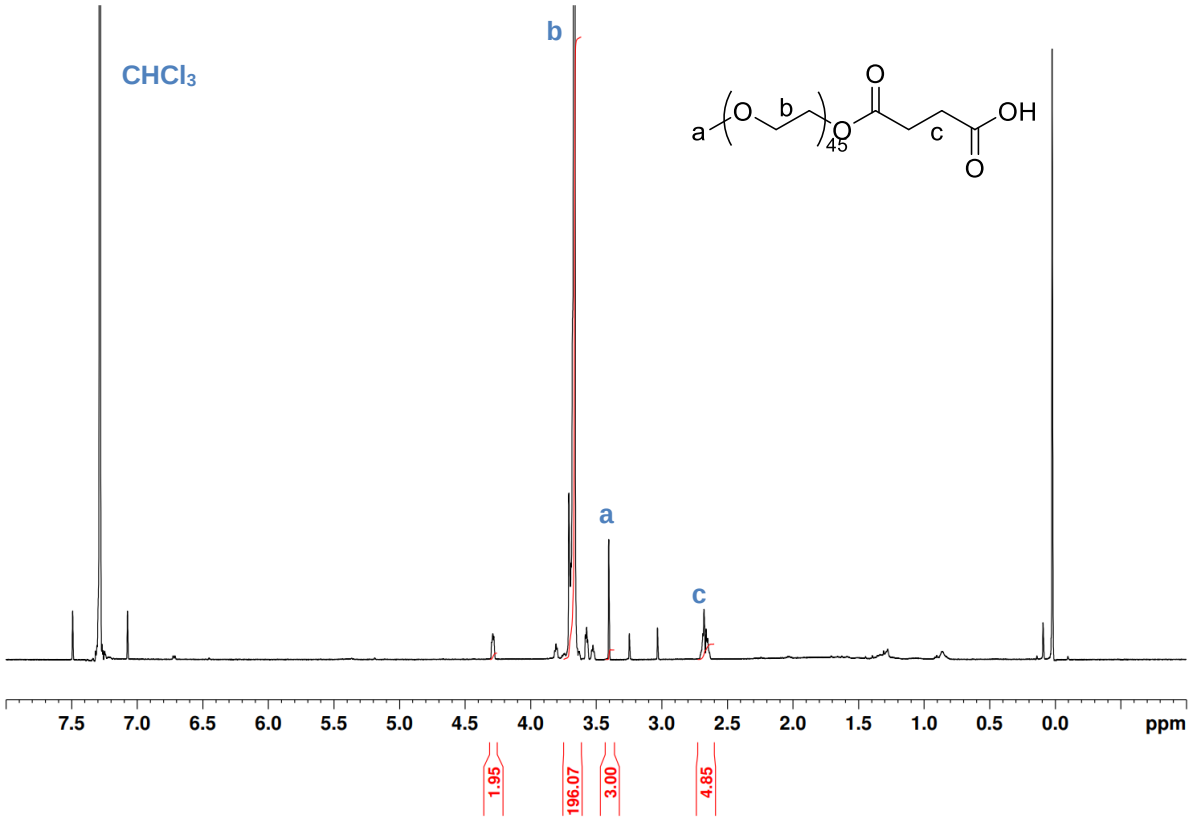

Figure S5. 1H-NMR ($CDCl_3$, 500 MHz, 25°C) of **P7**

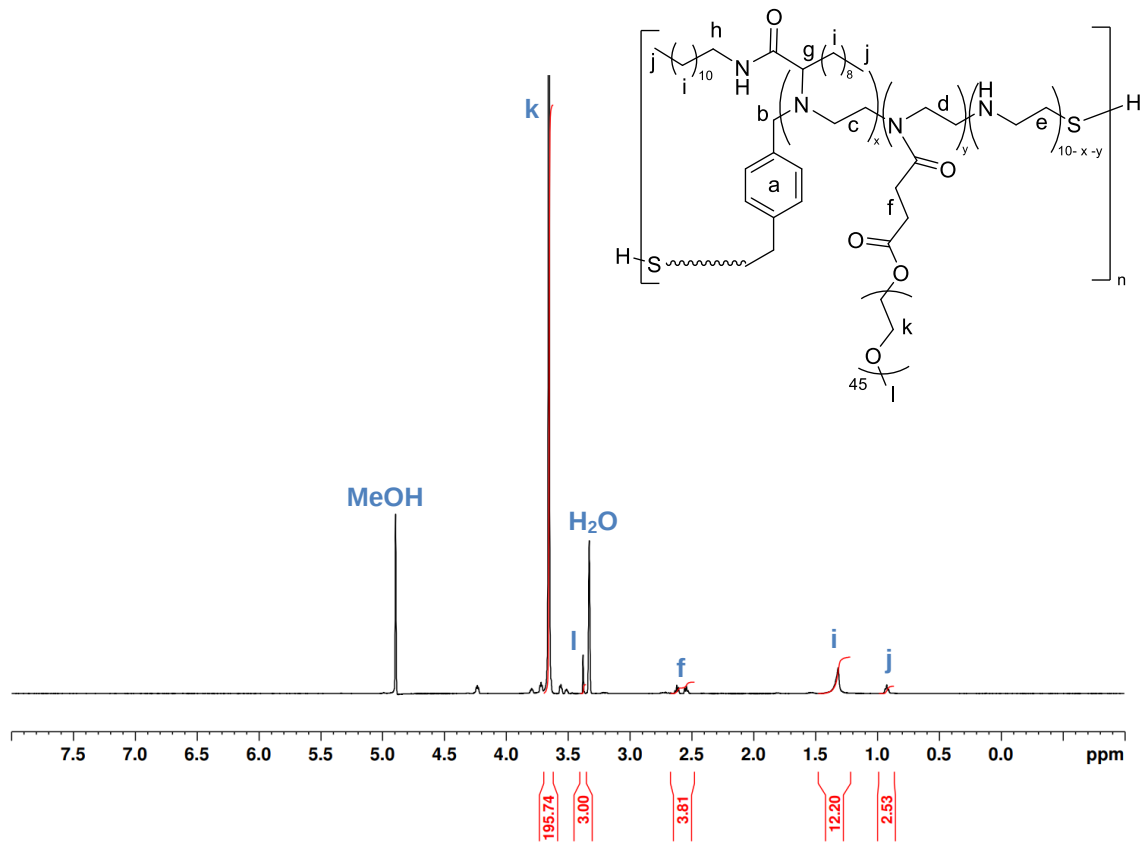

Figure S6. 1H-NMR (MeOD, 500 MHz, 25°C) of **P8**

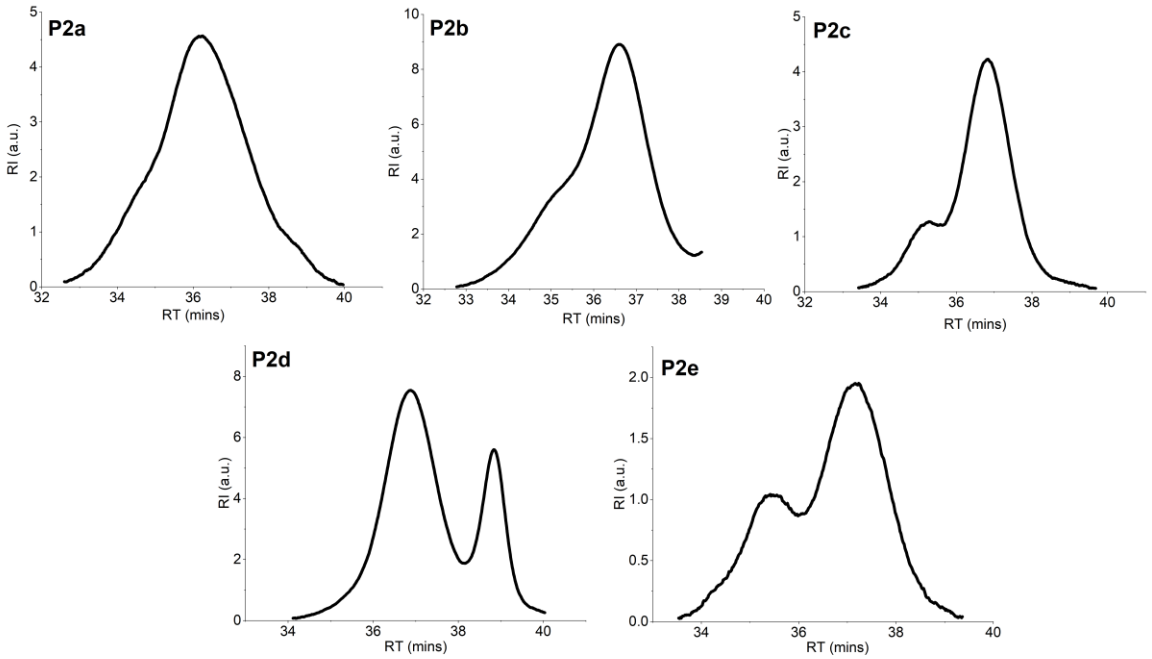

Figure S7. GPC traces (DMF) of of **P2**

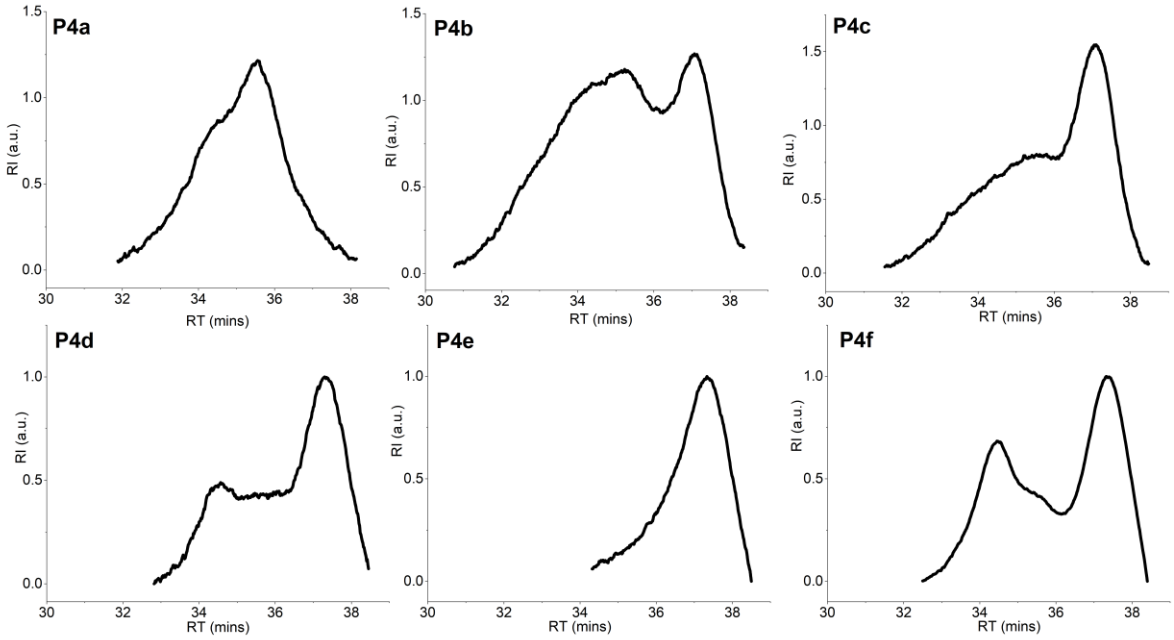

Figure S8. SEC traces (DMF) of **P4a**-**P4f**

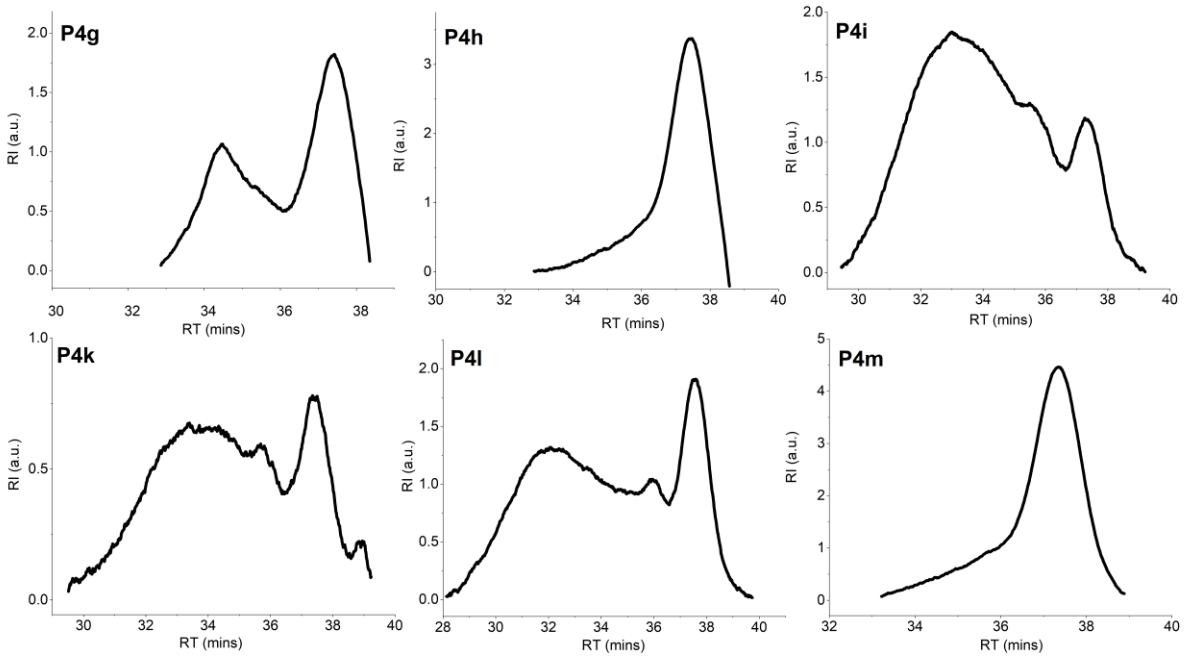

Figure S9. SEC traces (DMF) of **P4g**-**P4i** and **P4k**-**P4m**

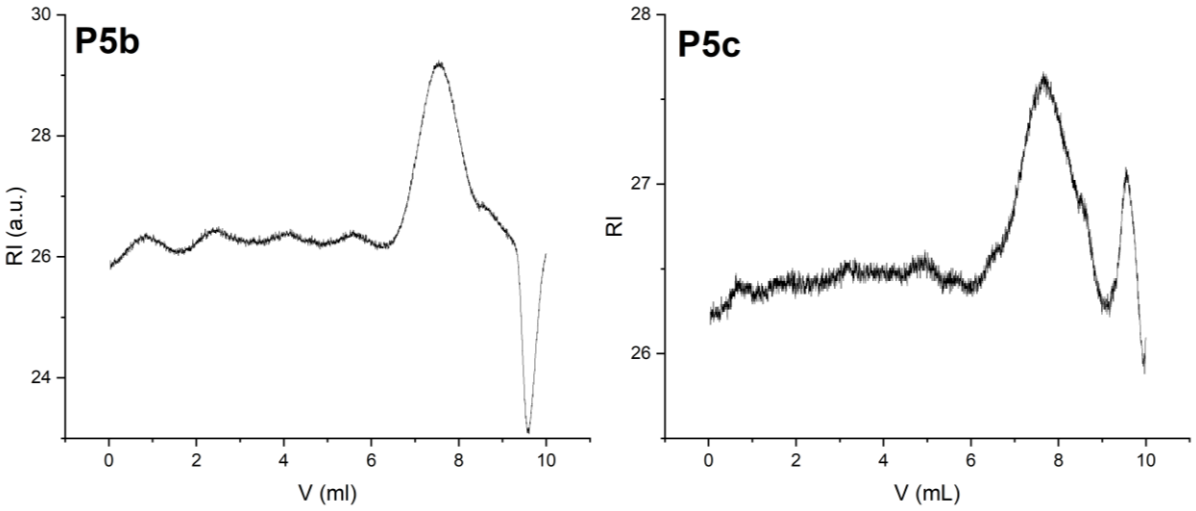

Figure S10. SEC traces (HFIP) of **P5b** and **P5c**

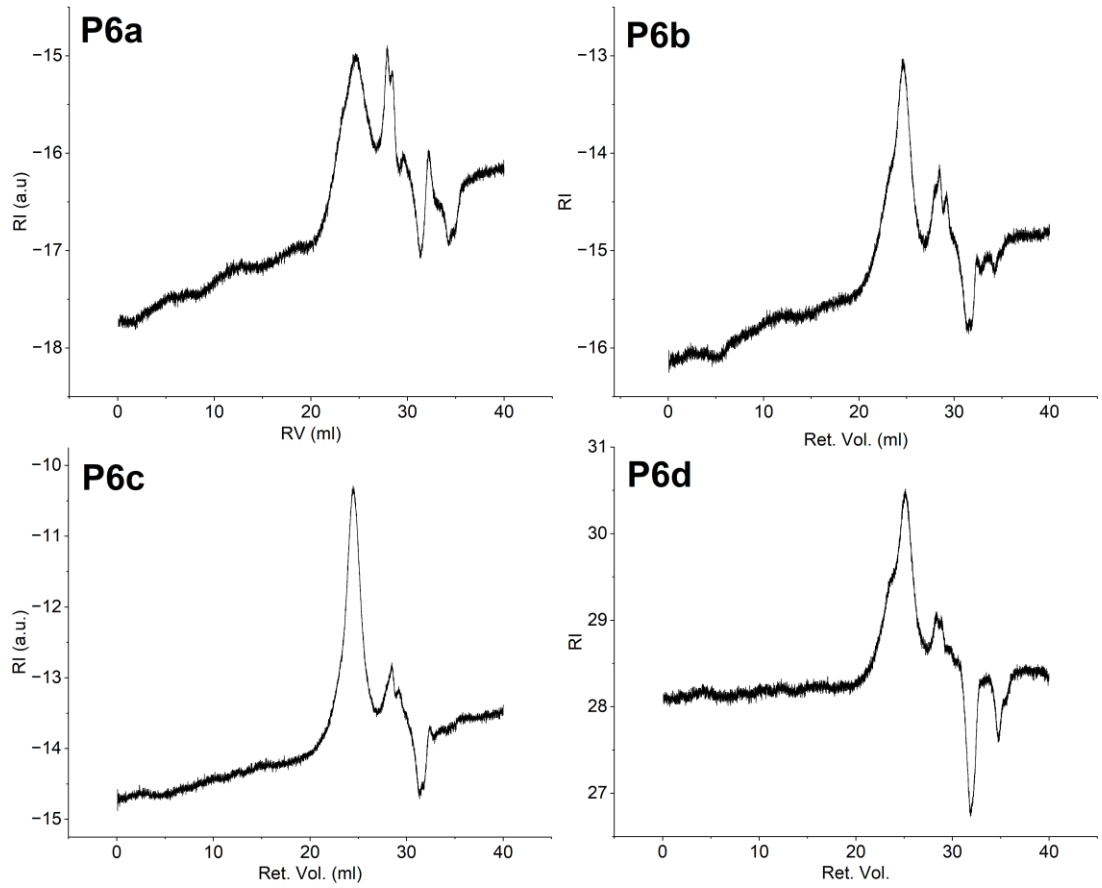

Figure S11. SEC traces ($CHCl_3$:IPA:TEA) of **P6**

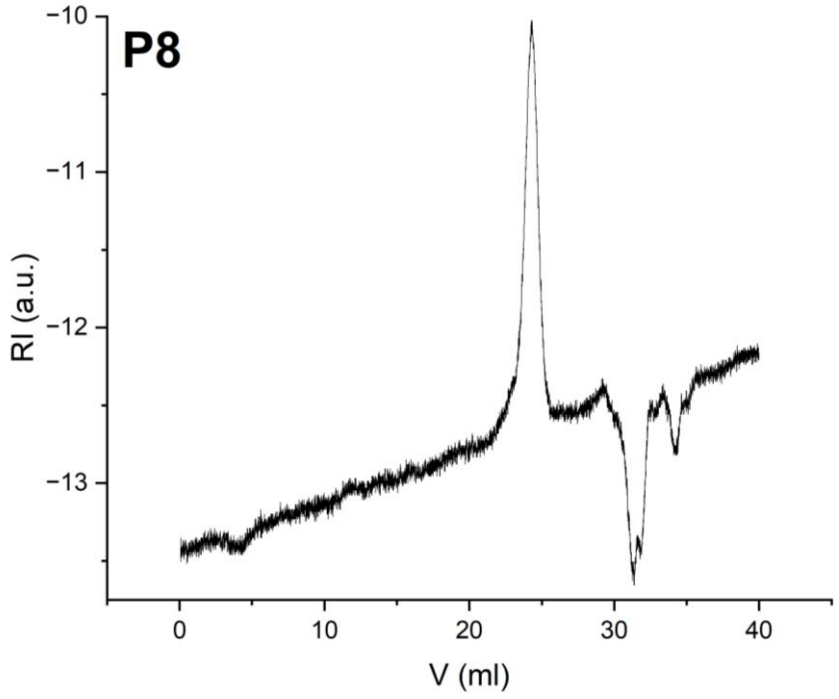

Figure S12. SEC traces ($CHCl_3$:IPA:TEA) of **P8**