Exploring the cytotoxicity of polycations

Synthesis and PEGylation of polycations

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Abstract

In the delivery of genetic material in vitro, such as mRNA-vaccines, non-viral vectors are used as the transporting system. To enable the full potential of the vectors, a positive charge on the surface is necessary, which has been proven to have cytotoxic effects. The aim is to, with polyethylene glycol with a tosylate group (mPEG-tosylate), coat the positive charge and simultaneously keep the necessary charge for the desired application. In this thesis, the cytotoxic effect of a synthesized polycation and polycations coated with mPEG-tosylate are investigated. A three step synthesis was made and tested with multiple methods to confirm the occurrence of each step and the last step was tested for its cytotoxic effect. To coat a hyperbranched polyester mPEG-tosylate was used, this was also tested with several analysis methods, and the cytotoxic effects were evaluated. The results confirm that all 3 steps of the synthesis did occur and the cytotoxic effect of the polycation was visible. It also confirms that the coating with PEG was successful and the cytotoxic effect decreased. These results suggest that further studies can be necessary, in case of what the degree of PEGylation versus the necessary charge of the polycation ratio is.

Contents

List of Figures

List of Tables

Nomenclature

^{1}H-NMR	Proton nuclear magnetic resonance
Compound 1	BoltornTM H40 Regular
Compound 5	Amine Functional BoltornTM H40
Compound 7	Amine Functional BoltornTM H20
d6-DMSO	Deuterated Dimethylsulfoxide
DCM	Dichloromethane
DLS	Dynamic Light Scattering
DMF	Dimethylformamide
EtOAc	Ethyl Acetate
LC-MS	Liquid Chromatography–Mass Spectrometry
mCPBA	meta-Chloroperoxybenzoic acid
MeOH	Methanol
mPEG-Tosylate 2k	Linear monofunctional PEG with a tosyl group, Mn = 2000
SEC	Size-exclusion chromatography
tBTMG	2-tert-Butyl-1,1,3,3-tetramethylguanidine
TLC	Thin Layer Chromatography

1 Introduction

During the spread of SARS-CoV-2 throughout the world focus has been directed at nanoparticles (NPs) and their applications in biomedicine (*1*, *2*). This has resulted in NPs and mRNA vaccines have been used all over the globe. The size and properties of the NPs administered are crucial to ensure the NP uptake by cells via endocytosis (*1*, *3*). Their size enables interaction with targets of similar size and facilitates the NPs passage through membranes, such as lung blood vessel junction and the blood brain barrier. When modifying NPs properties, such as its hydrophilicity, they are enable to increase their surface stability, binding to various drugs, reduce their precipitation and aggregation (*1*). When NPs are utilized within the biomedical field they are a part of the "non-viral vectors" (*4*). This refers to a system of transporting genetic material into a target cell, such as the mRNA within a vaccine. Non-viral vectors with polycations as surface groups are common for this application, due to their non pathogenic and non immunogenic properties (*5*, *6*). When non viral vectors are applied in gene therapy and in the delivery of mRNA, facilitated endocytosis, endosomal escape and protection of oligonucleotides from enzymatic degradation are key to ensure a successful outcome, as illustrated in Figure 1. These functions are independent of the materials or technologies used when the vectors are constructed. The delivery of mRNAs main difference from gene therapy is that the mRNA does not enter the cell nucleus or interact with the DNA (*7*). The delivery is temporary and alterations are not inherited by a daughter cell. This means that the mRNA delivery has no long-lasting effects on the individual, which is ideal for vaccines and is why it is preferred in this implementation.

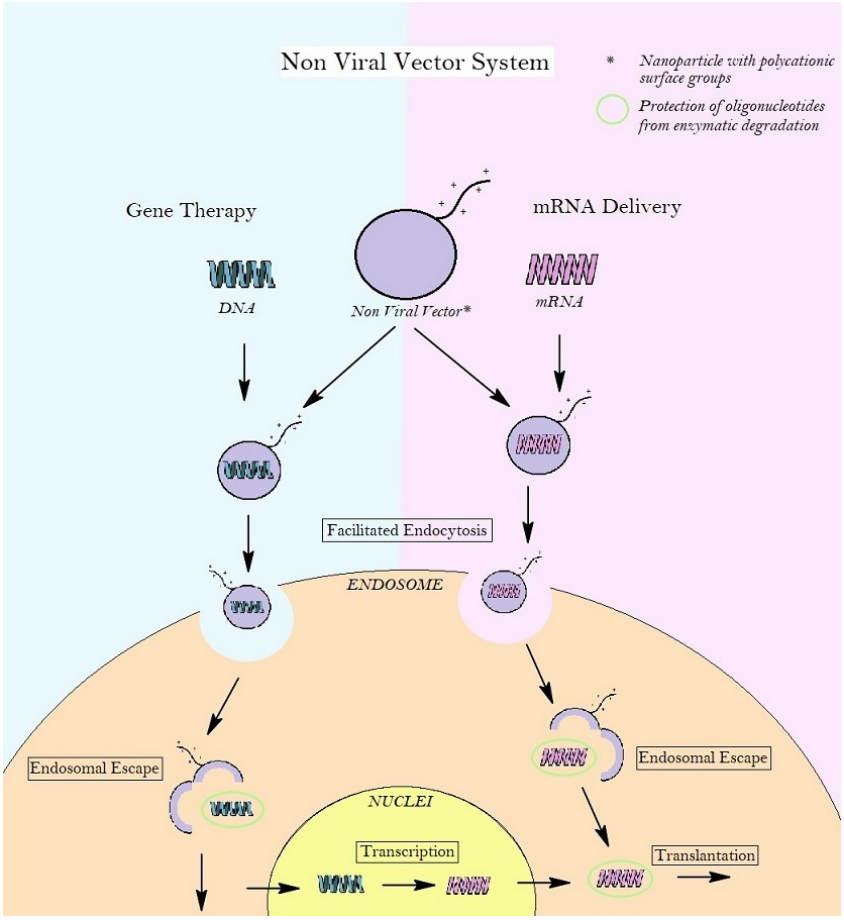


Figure 1: Difference between gene delivery and mRNA delivery by a non-viral vector system.

A non-viral vector consisting of lipids is referred to as a solid-lipid NP. These are customizable to behave in different ways depending on surrounding pH and interactions with environmental particles. Depending on the needed application, active groups bonded to the surface of non-viral vectors can be altered. When these groups are ionizable lipids that gain a positive charge at a low pH, they present with a cationic functionality. These ionizable lipids are used when manufacturing solid-lipid NPs for delivery of mRNA (*3*, *8*). The positive charge is needed due to its enabling of RNA complexation, which allows the RNA to be stored within the NPs and the RNA remains protected when administered into the body (*3*). The surface charge of ionizable lipids does not remain positive when administered in vitro. This is because under physiological pH and high ionic strength within the bloodstream (0.15 mol/I), electrostatic interactions between NP's with counterions neutralize the positive charge (*8*). The now uncharged lipid NP's promptly aggregate via hydrophobic forces in aqueous conditions in the blood. When the NP's have a neutral charge and aggregate the desirable cell uptake decreases.

On the other hand, there are NPs with surface groups that keep their polycationic functionality within the body. Their application is therefore not hindered by aggregation. There is evidence indicating that polycationic NPs facilitate pore opening in cell membranes, which allows transfection of the cells. This is called poration and is schematically shown in Figure 2. The charge of the polycation is therefore a key factor in the initial cellular uptake and the charge also enables endosomal escape, which allows release of the cargo into the cytoplasm (*3*, *5*). A problem that arises with a kept positive charge is that it has been shown to be cytotoxic (*3*, *9*). The cytotoxicity of polycations are related to their pKa and their molecular weight (Mw). Higher Mw and more cationic material is more cytotoxic in vivo, by weakening the membrane in the cell or impending cellular energy production pathways (*3*, *5*). Consequently, a problem faced within the field has been how to minimize aggregation thus utilizing the NPs full potential and simultaneously lower the cytotoxicity of administered NPs (*9*).

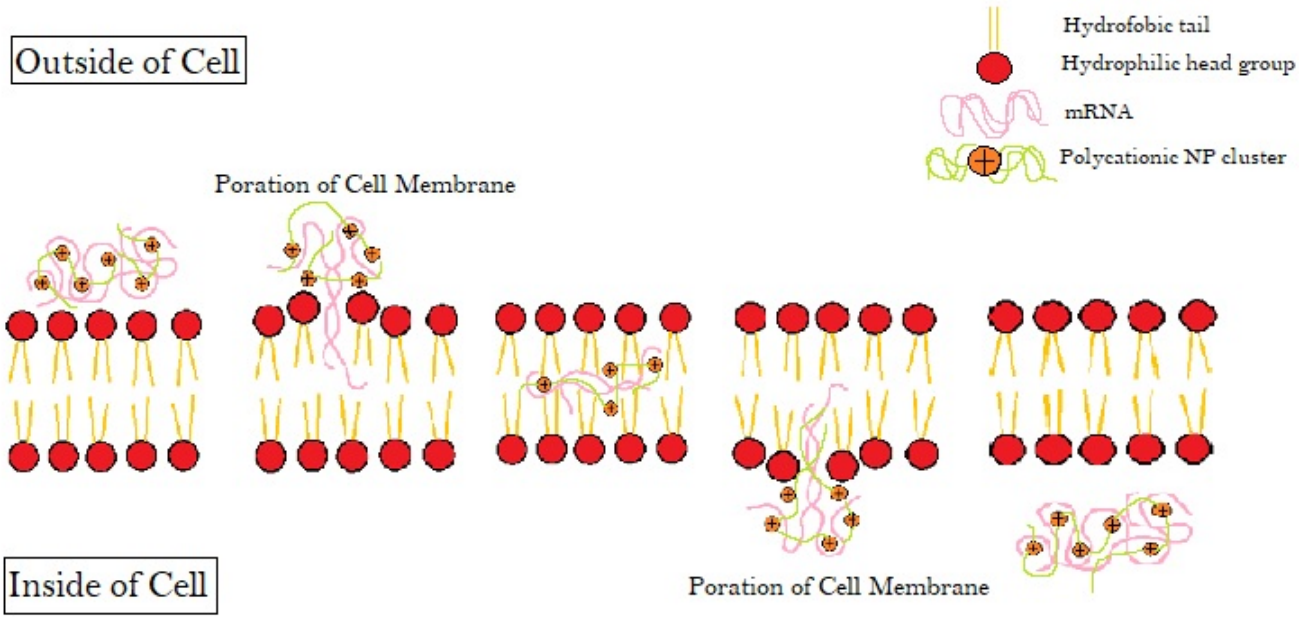


Figure 2: Simplified illustration of how polycationic compounds utlilizes poration to enter a cell.

To combat this, polyethylene glycol (PEG) is used to coat NPs with surface groups that keep their positive charge in the body (*8*). PEG is a widely used polymer in the drug delivery field, due to its classification as Generally Regarded as Safe (GRAS) and its history of safe use in the human body. Modifications on the PEG-polymer chains are crucial for the application of interest, due to the characteristics of the different derivatives (*10*). The derivatives can differ between surface groups and number of repeating units of the polymer e.g., which determine the use of the PEG. Coating the surface of NPs with PEG is called "PEGylation", and is a commonly used method for lowering the cytotoxic effects and thereby improving the efficiency of gene and drug delivery to target cells (*8*). PEG is an inert, hydrophilic polymer which resists interactions with components of the blood stream. When the long chains are attached to the NP, they constitute a hydrophilic outer layer with a large volume that obstructs NP's interactions with the surrounding environment, which is shown in Figure 3. The hydrophilic surface shield that PEGylation administers can decrease the NPs immunogenicity and improve systemic circulation time inside the body, by reducing aggregation within the bloodstream.The factors that have an impact on the circulation time of the coated NPs are PEG's molecular weight, surface density and the properties of the nanoparticle-core.

Prolonging circulation time provides a higher probability of circulating NPs to encounter the targets of interest. It is generally assumed that PEGylation will decrease desirable cell uptake by obstructing poration of the cell membrane. The assumption originates from that cationic parts of the NPs, as earlier mentioned, are the main enablers of poration (*9*). When the cationic surface groups are coated with PEG, their interaction with the cell membrane is reduced. Within the biomedical field research is continuously conducted to decrease the cytotoxic effects of charged NPs and increase the persistency inside the human body, without affecting the activity profile necessary to enable sufficient cellular uptake.

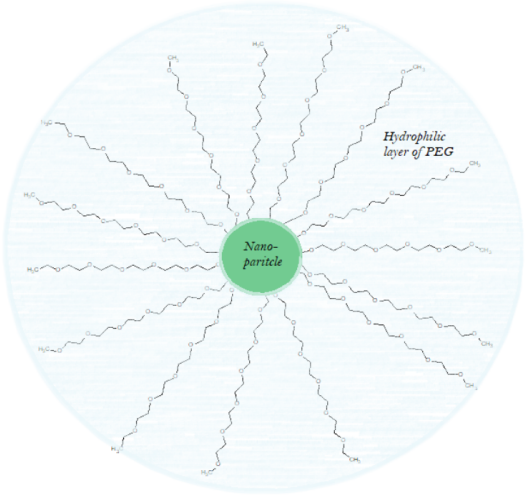


Figure 3: Illustration of a nanoparticle and it's hydrophilic layer when PEGylated.

1.1 Aim

In this thesis the cytotoxicity of polycations and PEGylated polycations were investigated. A synthesis of a polycation (three step synthesis) was made, using the hyperbranched polyester Boltorn™ Regular H40 as starting material (Compound **1**, seen in 3D in Figure 4 and in 2D with end groups after each step of the synthesis in Figure 5a). Subsequently, a PEGylation of a hyperbranched polyester with primary amine surface groups (Amine Functional Boltorn™ H40, seen in 2D in Figure 5b) was performed. The aim of the synthesis was to synthesize an epoxied that in the following step can accept an amine bond. By doing so a polycation was synthesized and the cytotoxicity in comparison to Compound **1** was evaluated. Conditions and molar equivalent of mPEG in relation to Compound **5** were analyzed and evaluated. Cytotoxicity of the starting materials and the products in both the synthesis and PEGylation were evaluated and discussed, as was the reproducibility of the methods for further studies. The laboratory work in this report were performed at Spago Nanomedical in Lund. The limitations of the thesis are seen in the following section (1.2).

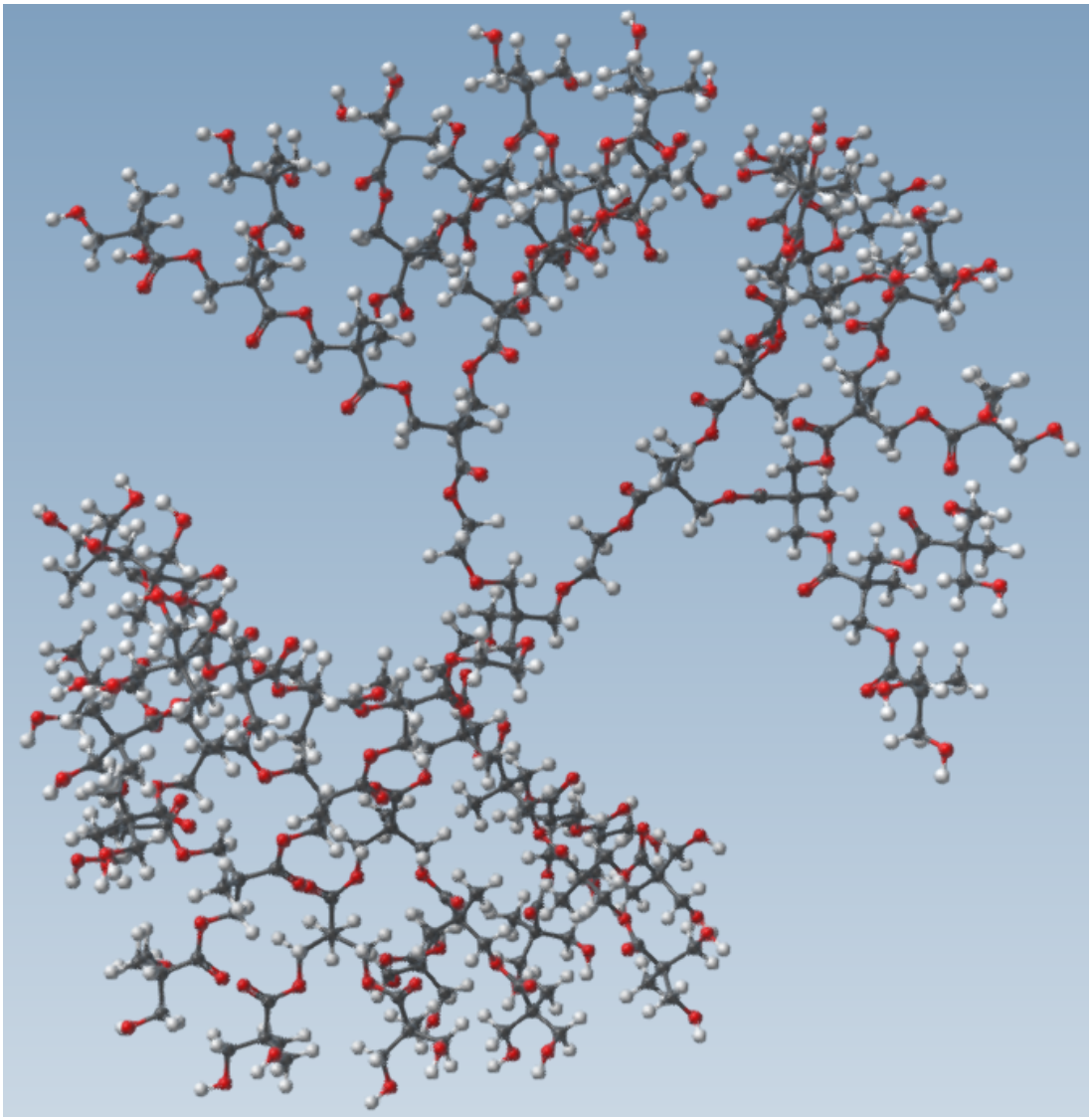
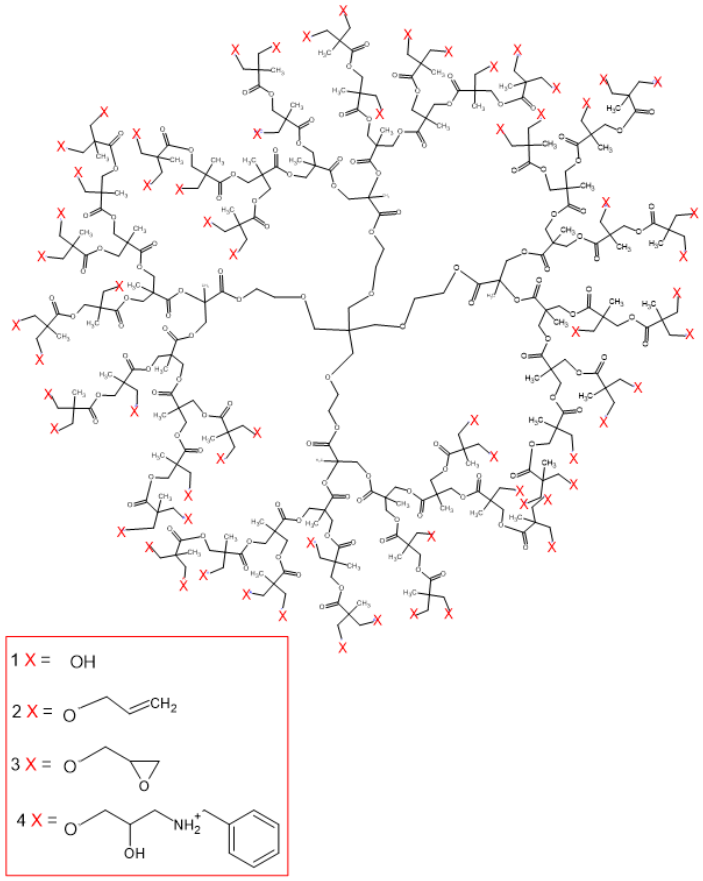
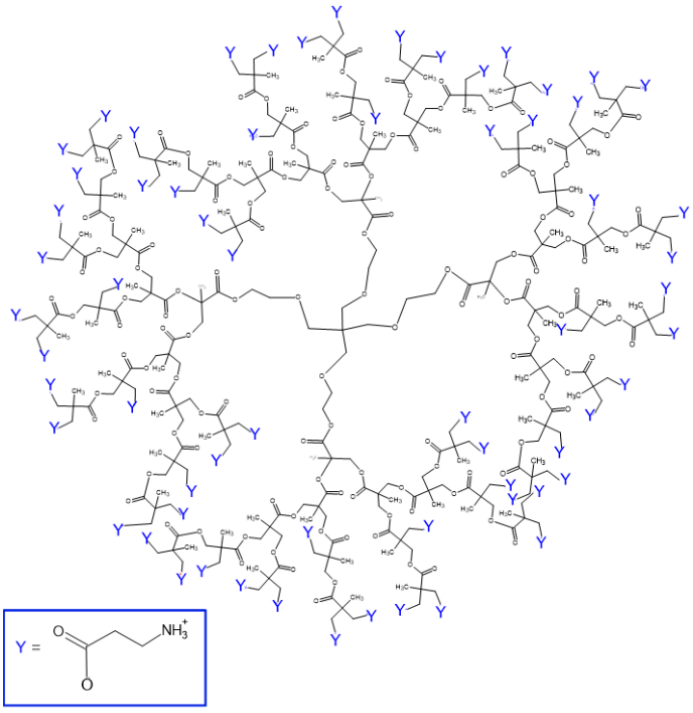


Figure 4: 3D illustration of Compound **1**. Atoms: red - oxygen, dark grey - carbon, light grey - hydrogen.



(a) Compound **1**, with illustrations (bottom left) of end groups alterations throughout the three step synthesis.



(b) Compound **5**

Figure 5: Idealized structures of starting materials in 2D. The series of products are dispersed with a distribution of molecular weights. Top, Compound **1**, with illustrations (left bottom) of end groups alterations throughout the three step synthesis. Subfigures: (a) and (b).

1.2 Delimitations

In the synthesis of a polycation the writers did not compare any reaction parameters between different reaction groups. This was due to the limited size of this project and that some parameters, such as reaction time, were not relevant to the aim. When determinations regarding purity of the product from step three were made, the assumption was that the majority of unreacted material was removed by the final wash.

In the PEGylation of a polycation the writers briefly compared the solvent and temperature used. No impact of the reaction time was taken into account, this was due to a shift of timeframe when the reactions took place. Determinations regarding purity of the product was made with the assumption that the sample preparation removed the majority of contaminants.

The ^{1}H-NMR spectra was interpreted by the writers with the guidance from their supervisor. Conclusions drawn from the spectra are limited to: 1. The confirmation that the skeleton of the starting material remains. 2. Indicating that an alteration of the starting material has taken place by looking for any movement of peaks within the spectra in relation to the starting material or in relation to a previous step (previous step is only applicable to the synthesis). 3. Support the need for LC-MS analysis by combining the two previous statements.

When using LC-MS; the method used, the chemical conformation of the expected degradation products from both the starting material and the products was founded on tests done at the company before the project started. The degradation of the starting material in LC-MS has been made and confirmed at Spago Nanomedical before and, using their expertise, the spectra from all analyzed products were interpreted.

The cytotoxicity was analyzed by the biological laboratory at Spago Nanomedical, the information given from them is the base of the conclusion. As previously stated, the purity of samples was based on assumptions, these assumptions were deemed necessary due to the limited size of the thesis. They also open up for further, more thorough studies within the field as seen in 5.

2 Results and discussion

In this thesis two experiments took place, the first one is referred to as the synthesis, in which a polycation is synthesized. The second experiment is a modification of an existing polycation with mPEG-tosylate, which is referred to as the PEGylation. All three steps of the synthesis were analyzed by ^{1}H-NMR and LC-MS. The third and last step (diagnostic reaction) were in addition to those, analyzed by SEC and on a cell culture. This was to determine the molecular weights and the cytotoxicity of the products. The PEGylation yielded many products and all reaction products were not analyzed with every instrument. This was due to both environmental reasons and the limiting time frame of the project. Due to the complex nature of both the synthesis and the PEGylation and with limited sources within the field trial and error was deemed necessary. The limited sources are not on the reactions themselves but rather the reactions of the rare and specific compounds used. By assuming that alterations are bound to the end groups and are not affecting the skeleton of the starting material, the approach was to follow procedures found in *Fieser and Fieser's Reagents for Organic Synthesis, Volume 2*. Detailed descriptions regarding time frame and solvent usage are found in Appendix A.1 and A.2. The methods of the analytical instruments and general equipment used are found in Appendix B, the limitations of the analytical instruments are described in Appendix C and the calculations are presented in Appendix D.

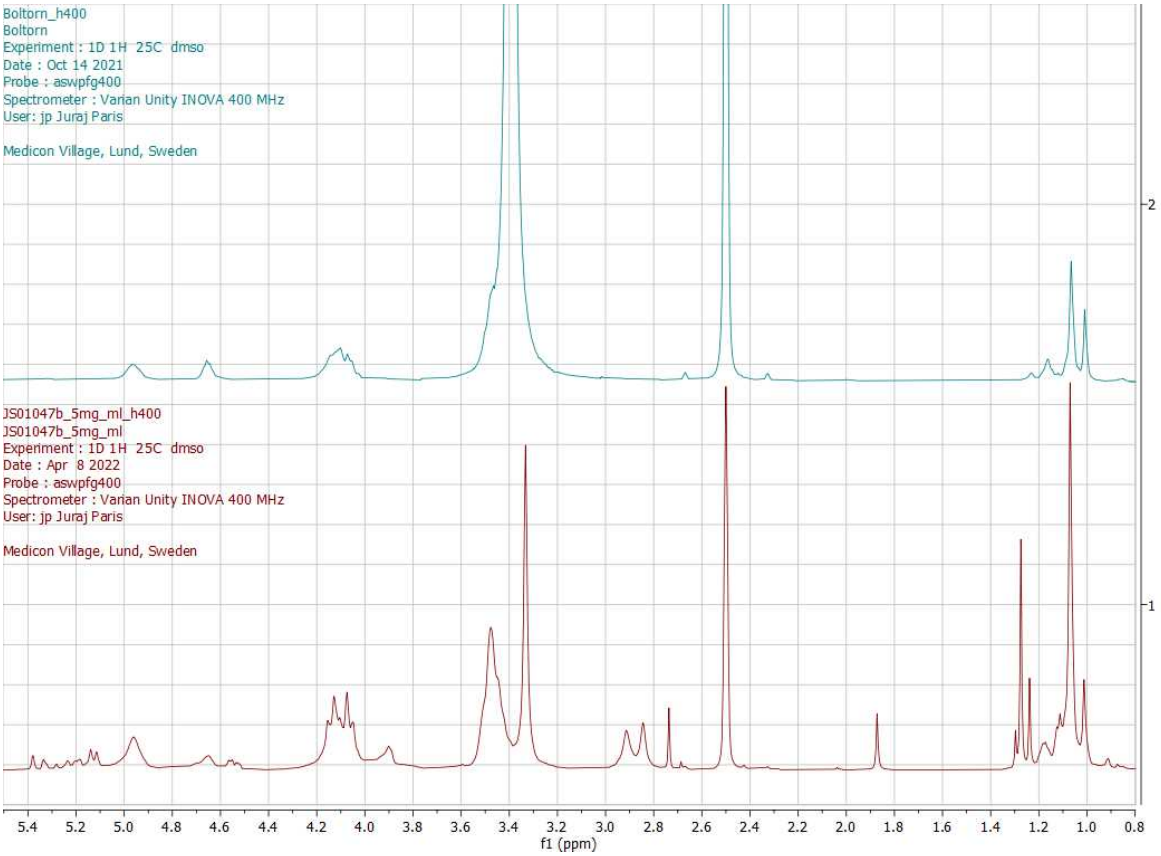
2.1 Synthesis of a polycation

2.1.1 Allylation

By an S_N1-reaction with allyl bromide the hydroxyl-functional groups of compound **1** were allylated by the reaction with allyl bromide in the presence of a non-nucleophilic base. This step is referred to as Step 1 and the reaction is shown in Figure 27.

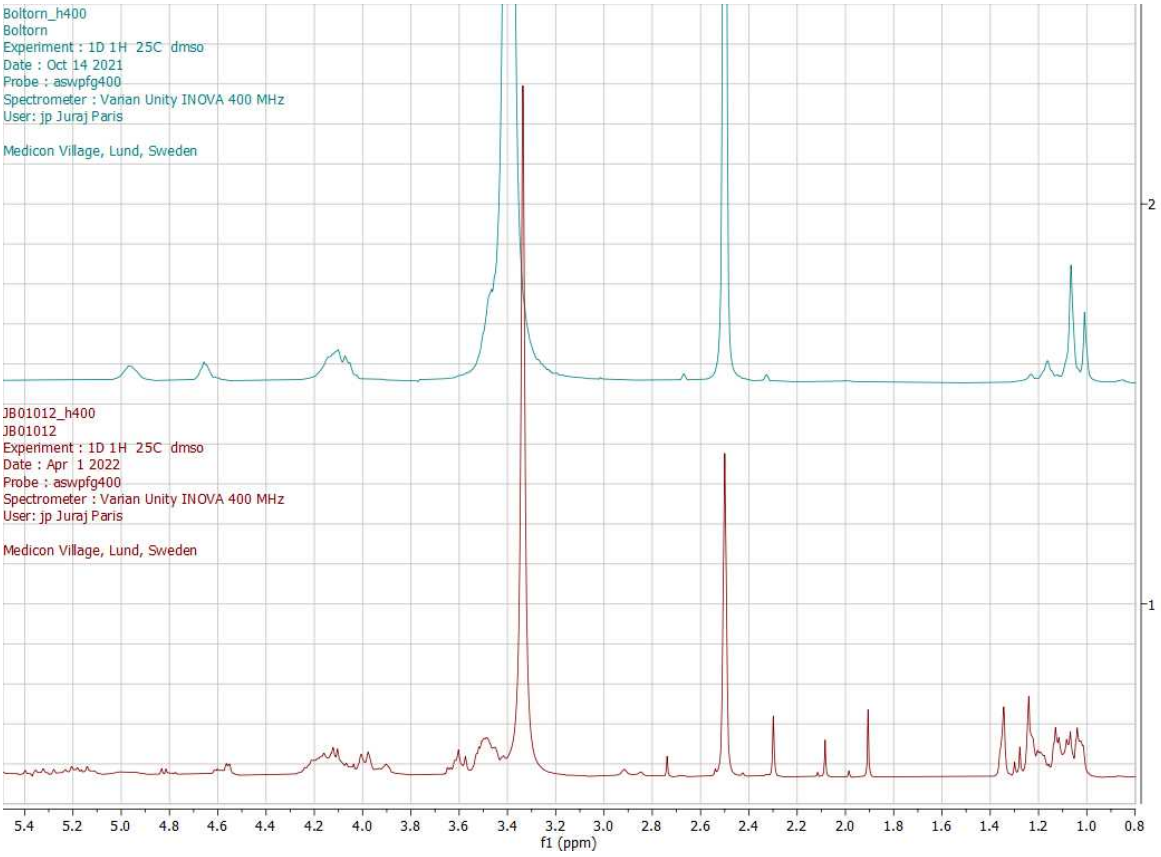
^{1}H-NMR

The usage of ^{1}H-NMR was founded in the fast data acquisition and the high precision of the technique. The ^{1}H-NMR (400 MHz) spectra seen in Figure 6 of compound **2** from reactions A1, B1 and C1 indicated allylation in the expected region (3.6-5.4 ppm). The relevant peaks are a multiplet at 1-1.4, a multiplet/broad at 4-4.2 and an addition of a group of overlapping multiples between 5.1-5.4. A movement of peaks in relation to the reference (compound **1**) further confirms a change of the structure. Broad peaks present on the ^{1}H-NMR are typical for big molecules; this shows that the starting material still is present and not degraded. This is because the hydrogen atoms within a molecule with similar resonance frequencies can present as broad peaks. But determination of the structures of the products are limited due to the high molecular weight. To gain a precise idea of the molecule's structure the decision was made to collect LC-MS data.

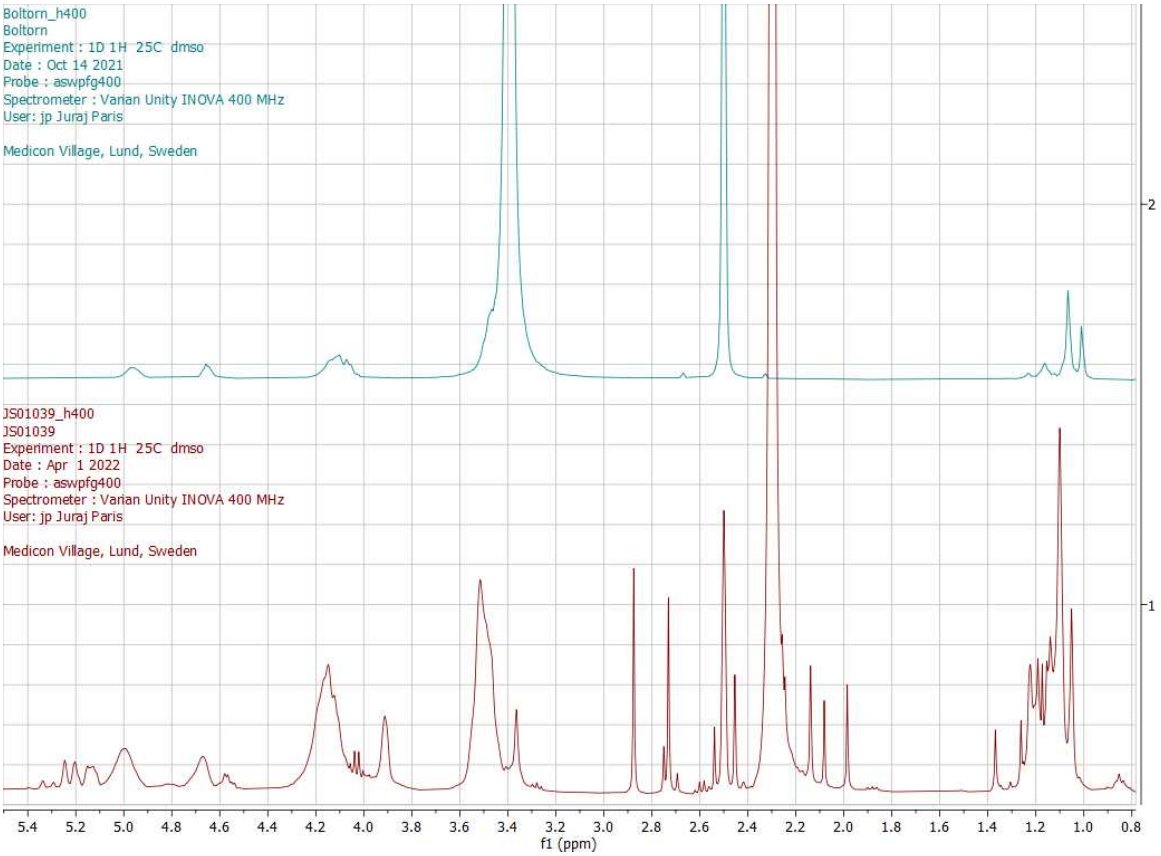


(a) Top, compound **1**. Bottom, compound **2** from reaction A1.

Figure 6: ^{1}H-NMR spectra of compound **1** (reference) and compound **2**. Subfigures: (a), (b) and (c).



(a) Top, compound **1**. Bottom, compound **2** from reaction B1.



(b) Top, compound **1**. Bottom, compound **2** from reaction C1

LC-MS of A1 and C1

LC-MS has the advantage of being able to determine the mass of each ion directly. By doing so it can confirm the qualitative analysis of the samples. However, if multiple components within the samples have the same mass, it can be difficult to fully analyze the spectra and this is likely to happen for samples containing multiple components. The compounds put within the sample and their fragmentation are known, therefore the method is not limited by this. Fragmentation of all sorts when using LC-MS is expected and well studied within the field (*11*, *12*). LC-MS data was recorded on products from the reactions A1 and C1 to further confirm that compound **1** reacted into compound **2**. Thus, excluding the possibility that the ^{1}H-NMR spectra shows a mixture of unreacted starting materials. When compound **2** is fragmented by methanolysis and analyzed by LC-MS the expected mass through charge (m/z) are found and are marked in Figures 8 and 9. Expected fragments of compound **1** with mono- and diallyl-groups attached are found. In reaction A1 the fragments are found at peaks 215.2, 251.1 (diallyl) and 225.5 (mono allyl). In reaction C1 the fragments are found at peaks 175.1, 197 (monoallyl) and 98.9, 99, 215.3, 251.3 (diallyl). The complete spectra from A1 and C1 also shows tBTMG, solvents and other contaminants (*13*). By comparing the spectra from the products to the expected fragmentation results of compound **1** and then combining them with the ^{1}H-NMR spectra, there is strong evidence that reactions A1 and C1 succeeded. The interpretation is that the reaction went as expected, the hydroxyl-functional groups of compound **1** were allylated by the reaction with allyl bromide.

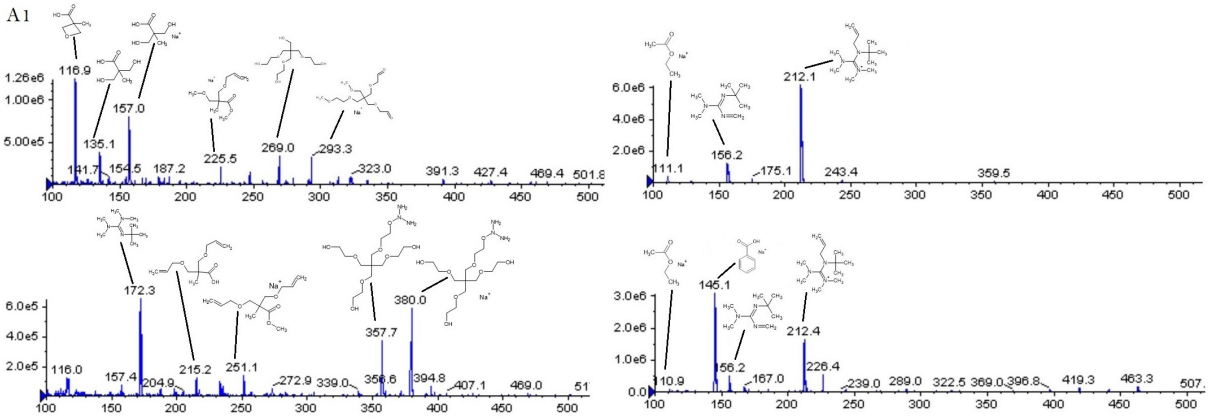


Figure 8: LC-MS of reaction A1 most prominent peaks: 1.41 5.16 6.12 6.63.

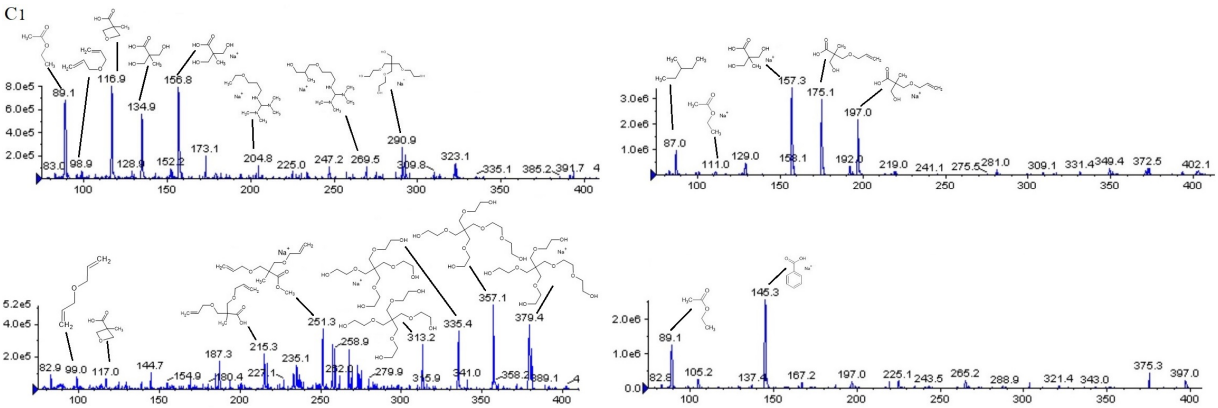


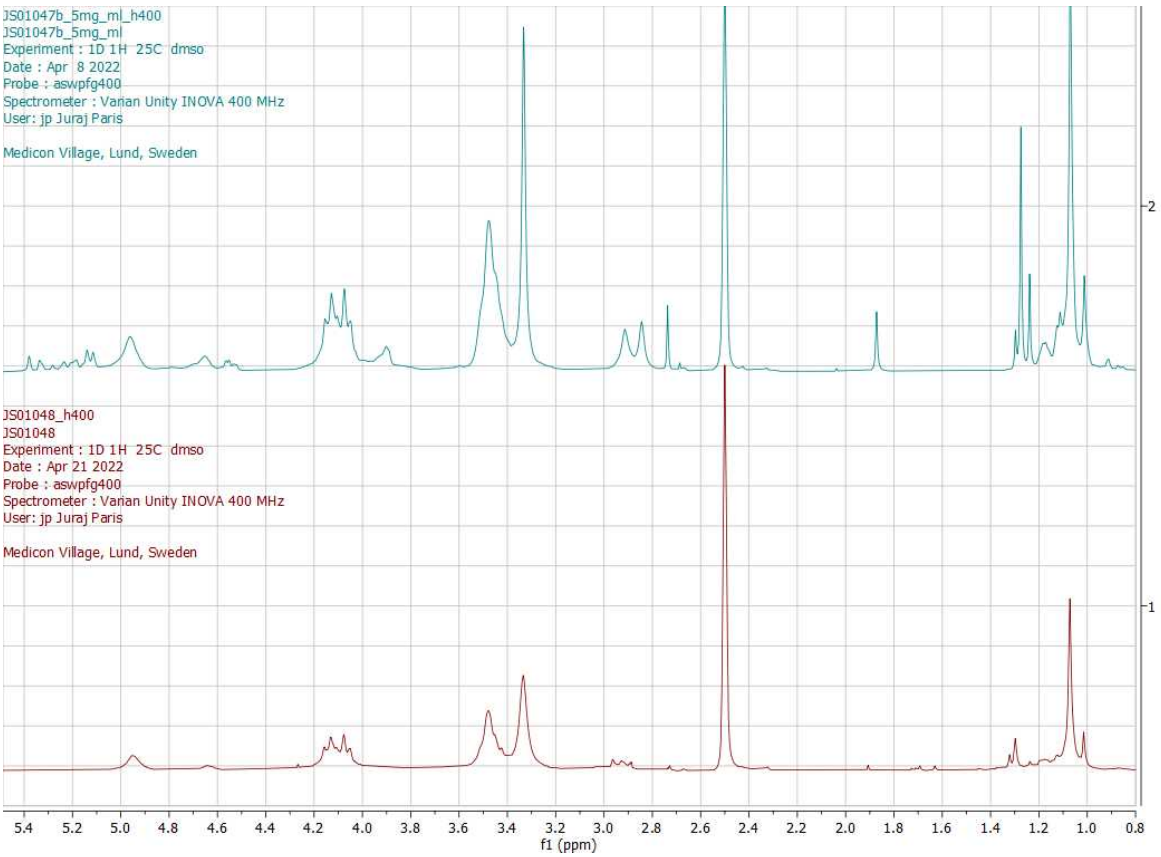
Figure 9: LC-MS of reaction C1 most prominent peaks: 1.41 5.16 6.12 6.63.

2.1.2 Epoxidation

By a reaction with mCPBA (Prilezhaev), the allyl-functional groups of compound **2** accepted an oxygen atom, thus epoxidized. The step is referred to as step 2 and the reaction is shown in Figure 28. The goal was to see if the epoxidized material was able to accept an amine bond. The decision to use ^{1}H-NMR and LC-MS for the analysis originated from the reasons stated in section 2.1.1.

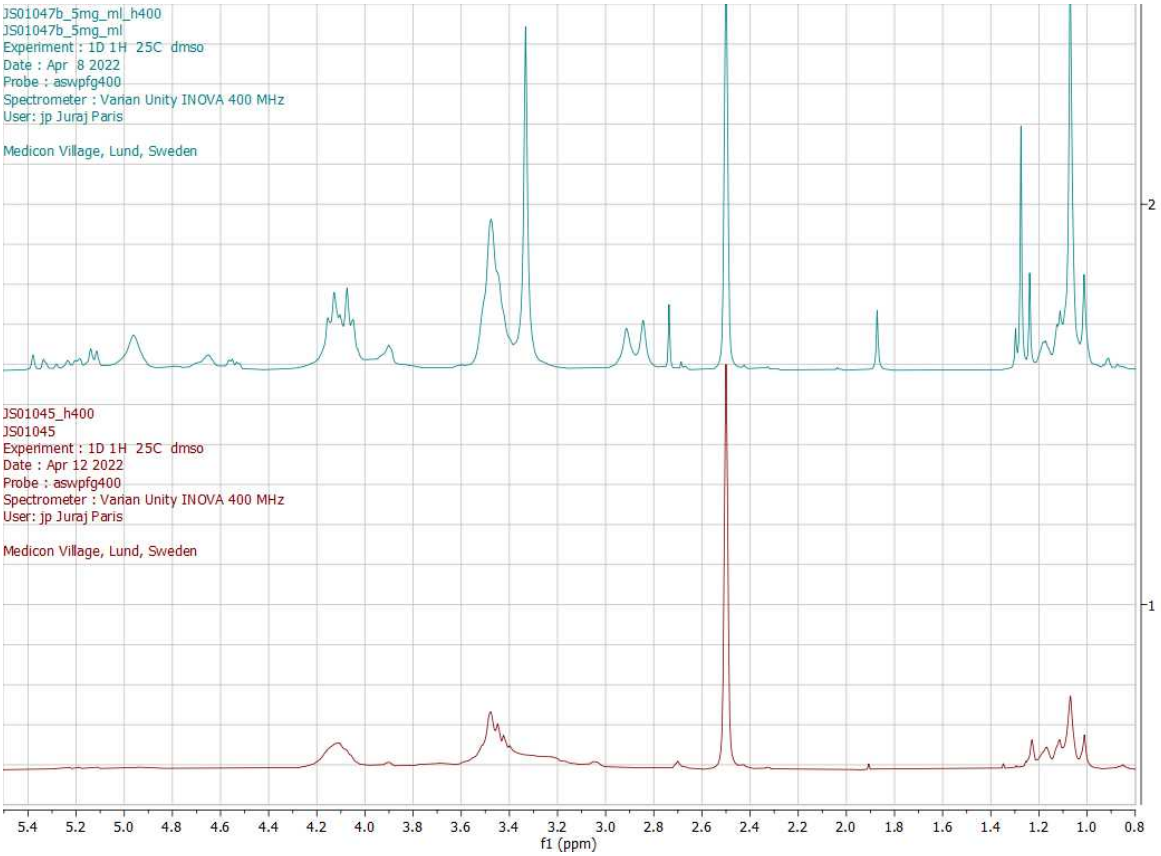
^{1}H-NMR

Both ^{1}H-NMR and LC-MS were not taken from reaction B2 in this step, reasoning behind this decision are found in A.1. The ^{1}H-NMR (400 MHz) spectra of compound **3** from the reactions A2, C2a and C2b compared to compound **2** from reaction A1 are seen in Figures 10a, 10b and 10c. When interpretations were made, each sample was compared to its previous reaction. Using compound **2** from reaction A1 as reference for all samples in this thesis was to make it clearer to the reader after the initial comparison. A decrease of the allylated areas in the spectra (mainly a decrease of overlapping multiplets between 5.1-5.4 ppm) and a change of peak position indicates that a reaction has occurred. References from epoxides in other solvents were used to interpret the spectra, implicating that the reaction went as expected. Due to shift of peaks, when using references in other solvents, the conclusions are to be further supported by LC-MS (seen in the following paragraph).

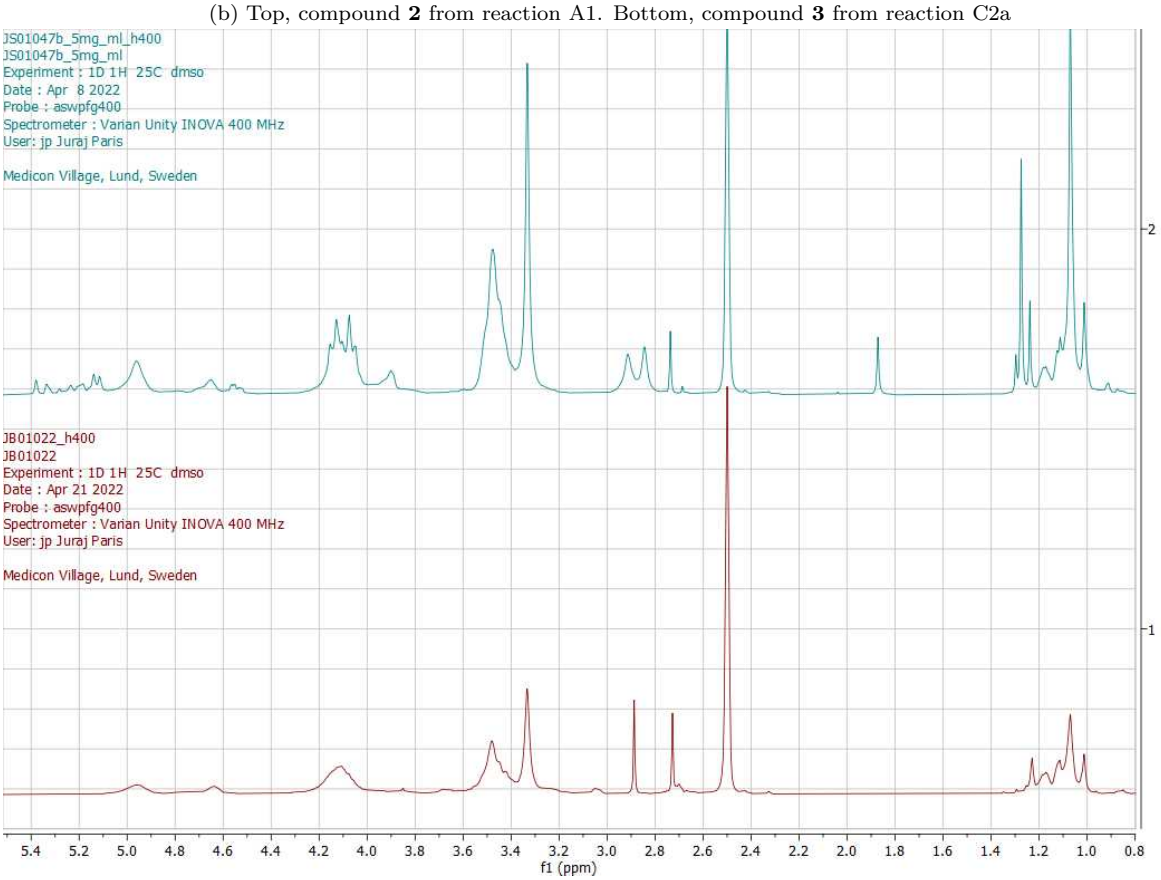


(a) Top, compound **2** from reaction A1. Bottom, compound **3** from reaction A2

Figure 10: Selected areas of ^{1}H-NMR spectra of compound **2** (reference from reaction A1) and compound **3**. Subfigures: (a), (b) and (c).



(b) Top, compound **2** from reaction A1. Bottom, compound **3** from reaction C2a



(c) Top, compound **2** from reaction A1. Bottom, compound **3** from reaction C2b

LC-MS of A2 and C2b

LC-MS data was recorded on products from the reactions A2 and C2b to confirm that compound **2** reacted in the expected way into compound **3**. Other than excluding the probability that the ^{1}H-NMR spectra show a mixture of unreacted starting materials, the analysis gives a conclusive result on the product from the reactions. When compound **3** is fragmented by methanolysis and analyzed by LC-MS the expected m/z are found and are marked in Figure 11. Fragments of compound **3** are found at 205 and 239 m/z in both spectra. In C2b (peak 1.41) seen in Figure 11 an expected fragment is found at 223 m/z and at 245 m/z its corresponding sodium adduct (Na^+ can be seen (*14*)). The complete spectra also shows tBTMG, solvents and other contaminants (*13*). By comparing the spectra from the products to the expected fragmentation results of compound **1** in combination with the ^{1}H-NMR spectra, there is strong evidence that the reaction went as expected. To further test the epoxidized functional groups, a diagnostic reaction with benzylamine was performed. When an epoxide forms a covalent bond to an amine, opportunities for more development and applications of NP:s within the biomedical field can be made.

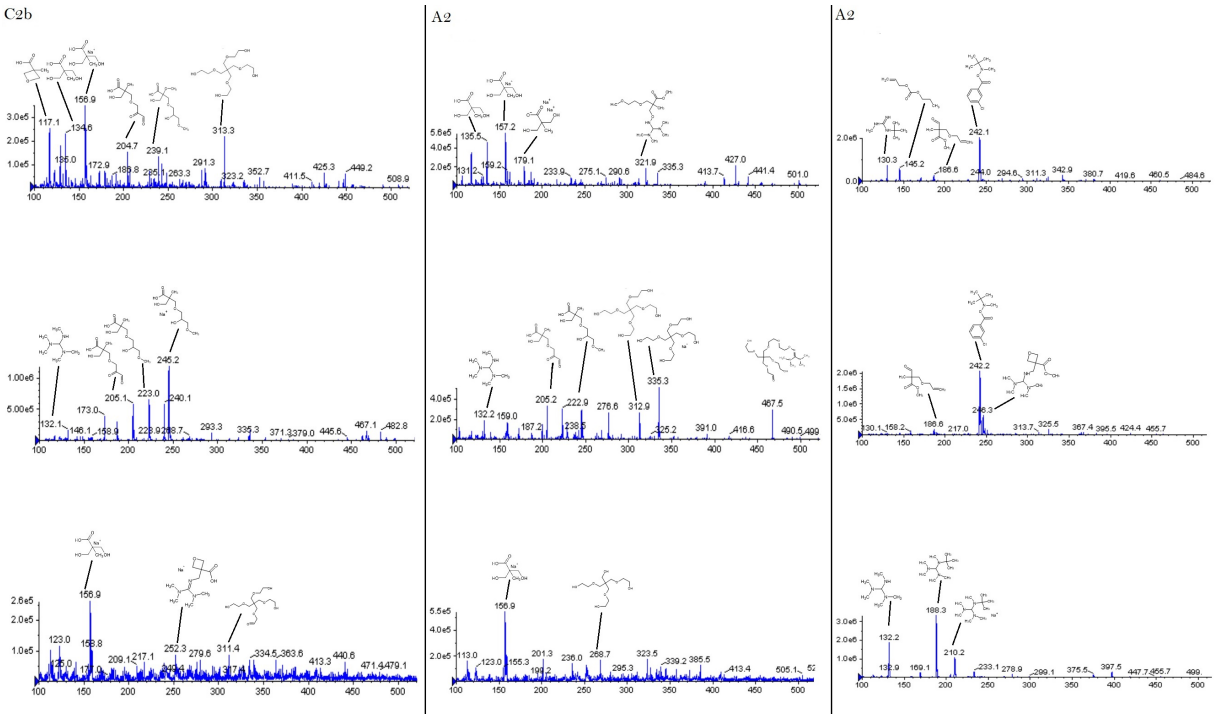


Figure 11: LC-MS of reactions C2b and A2. Left peaks: 1.41, 4.66, 8.48. Middle peaks: 1.41, 4.66, 8.46. Right peaks: 6.65, 6.85, 7.31.

2.1.3 Diagnostic reaction

By an S_N2-reaction with benzylamine, the epoxidized-functional groups of compound **3** broke one of the oxygen-carbon bonds in all end groups. The end groups went on to accept a nitrogen bond to the benzylamine. The step is referred to as a diagnostic reaction and the intent of these reactions is to see if the epoxidized material is able to accept an amine bond and by that synthesizing a polycation. This step is referred to as step 3 and the reaction is shown in Figure 29. The decision to use ^{1}H-NMR and LC-MS as two of the four analysis methods in this section originated from the reasons stated in section 2.1.1.

SEC

SEC was used to verify the molecular weight of the synthesized polycation. SEC is a quantitative method and the size distribution of the synthesized product was analyzed and compared to a slightly larger compound. The expected product, compound **4**, has a calculated Mw given in table 1 (calculations in Appendix D). The reference for the analysis, polyethylene oxide, is a polymer with a known Mw. The sample tested by SEC was product C3a. Other qualitative methods were necessary to analyze the structure of the product.

Table 1: Mw of Polymer 21k and compound **4**

Substance	**Molecular weight (g/mol)**
Polyethylene oxide	21 000
Compound **4**	17 800

In Figure 12 the result of product C3a is presented. The retention time of C3a is higher than 30 min, which is also higher than polyethylene oxide at 21 min. This confirms that the size of this product is smaller than polyethylene oxide. It also indicates that fragments or contaminations are within the sample, due to the multiple peaks at 29.76-33.01 min.

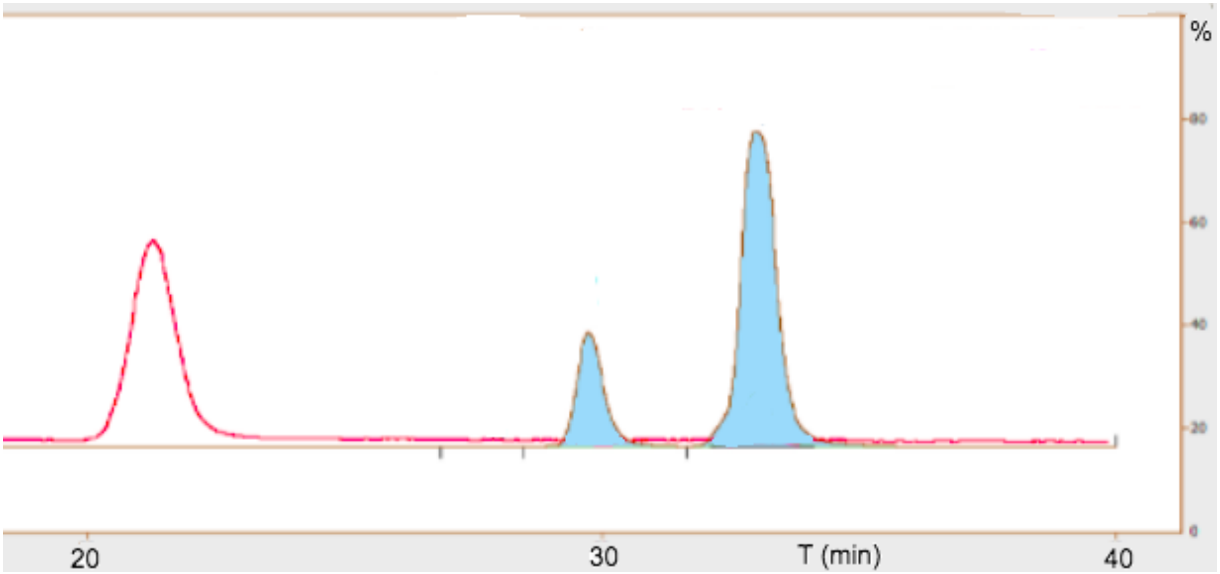
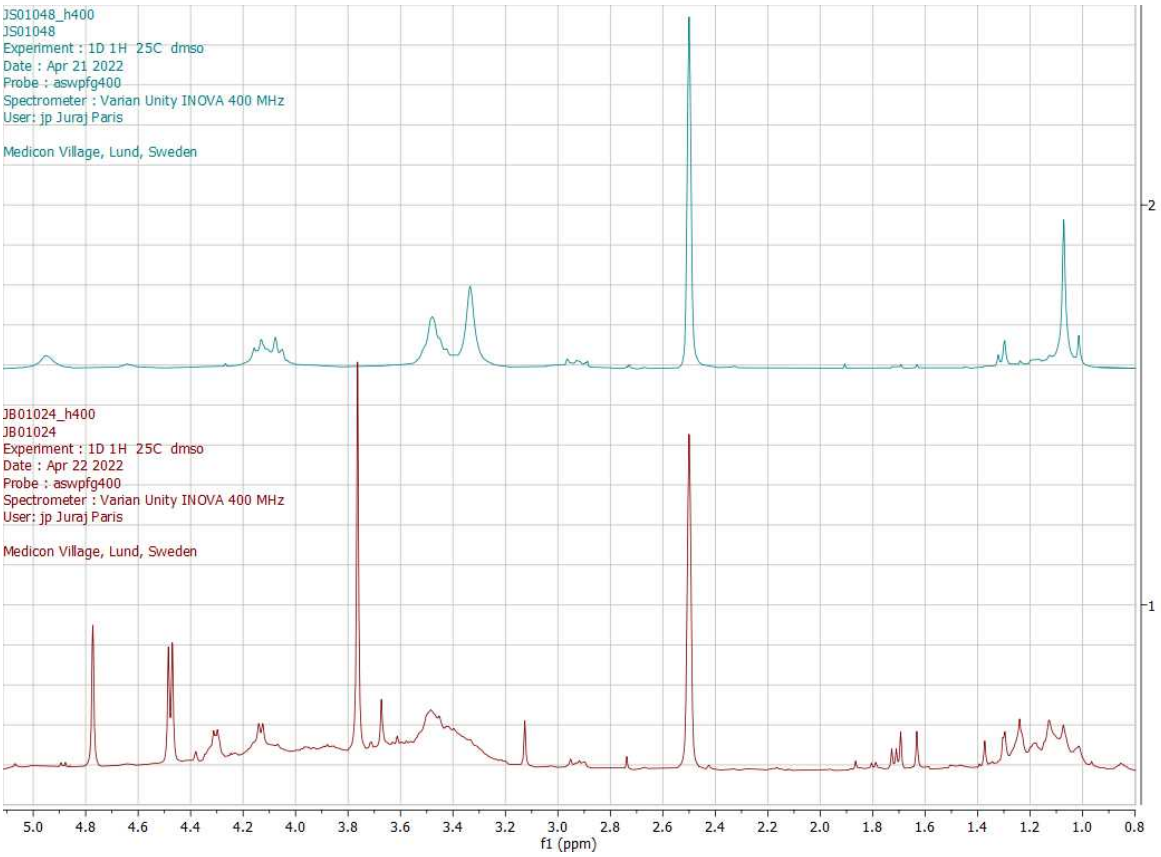


Figure 12: Red line polymer 21K, brown line compound **4** from reaction C3a.

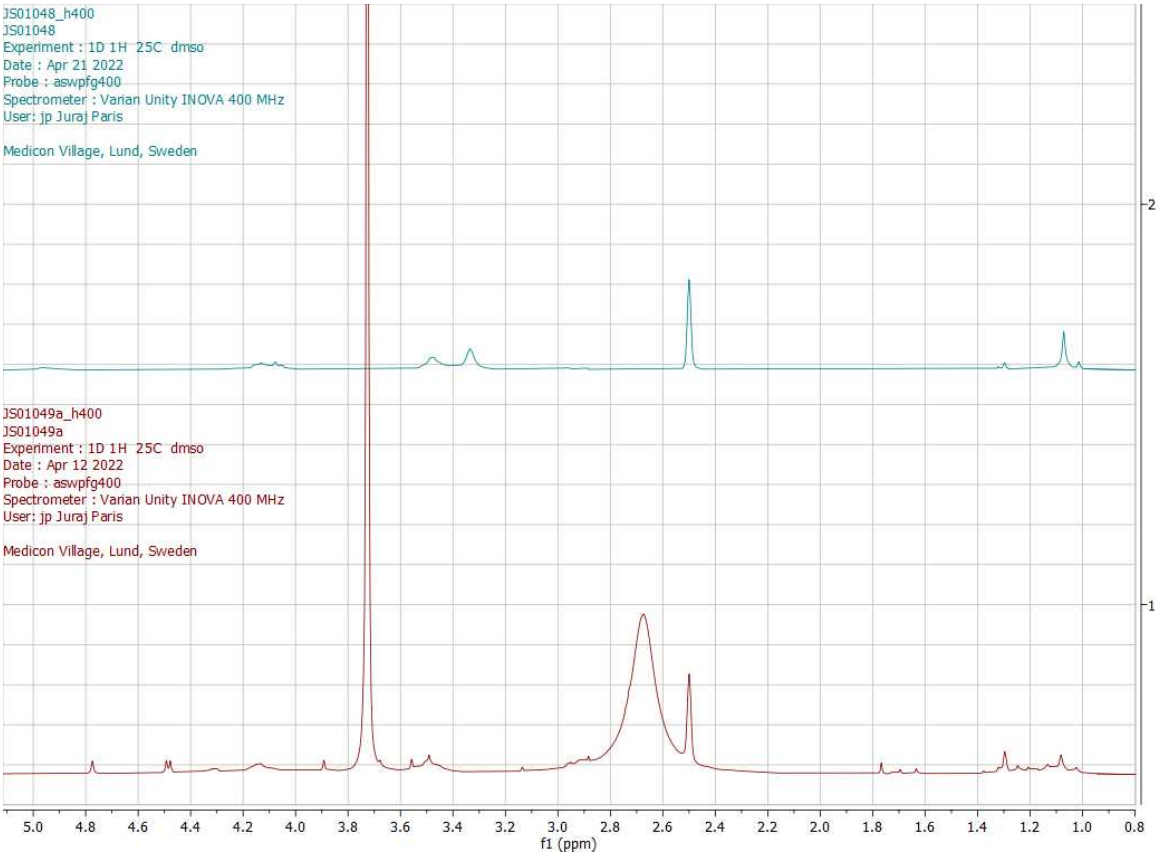
^{1}H-NMR

^{1}H-NMR (400 MHz) of compound **4** from the reactions A3 (both washed and unwashed with phosphate buffer), C3b and C3c were analyzed. Selected parts of the spectra are compared to compound **3** from reaction A2 in Figures 13 and 14. When interpretations were made, each sample was compared to its previous reaction. With peaks in Figure 13 being upfield with respect to peaks in Figure 14, wich are downfield. The use of compound **3** from reaction A2 as reference for all samples in this thesis were to make it clearer to the reader after the initial comparison. The first observation is that the intensity of the different spectra varies, but reaction C3c looks different from the rest and is missing a lot of the expected peaks. This is true in both the up- and downfield spectra (Figures 13d and 15c) and reveals that the molecular skeleton is missing. It could have been lost in a washing- or filtration step. This can be caused by either fragmentation or insufficient reaction time. The fragmentation can be caused by contaminants, too high temperature or that the sample was incorrectly stored before the analysis. The insufficient reaction time may cause the product to favor the not aimed for pH when washed and thus get extracted to the aqueous phase. The second observation is that the spectra from reactions A3 (before and after washing) and C3b still contain the skeleton in varying degrees. A change of peak position within the spectra leads to the third and last observation, that a reaction has taken place. By the same principle as the previous steps, confirmation of which reaction is needed, and LC-MS data was collected.

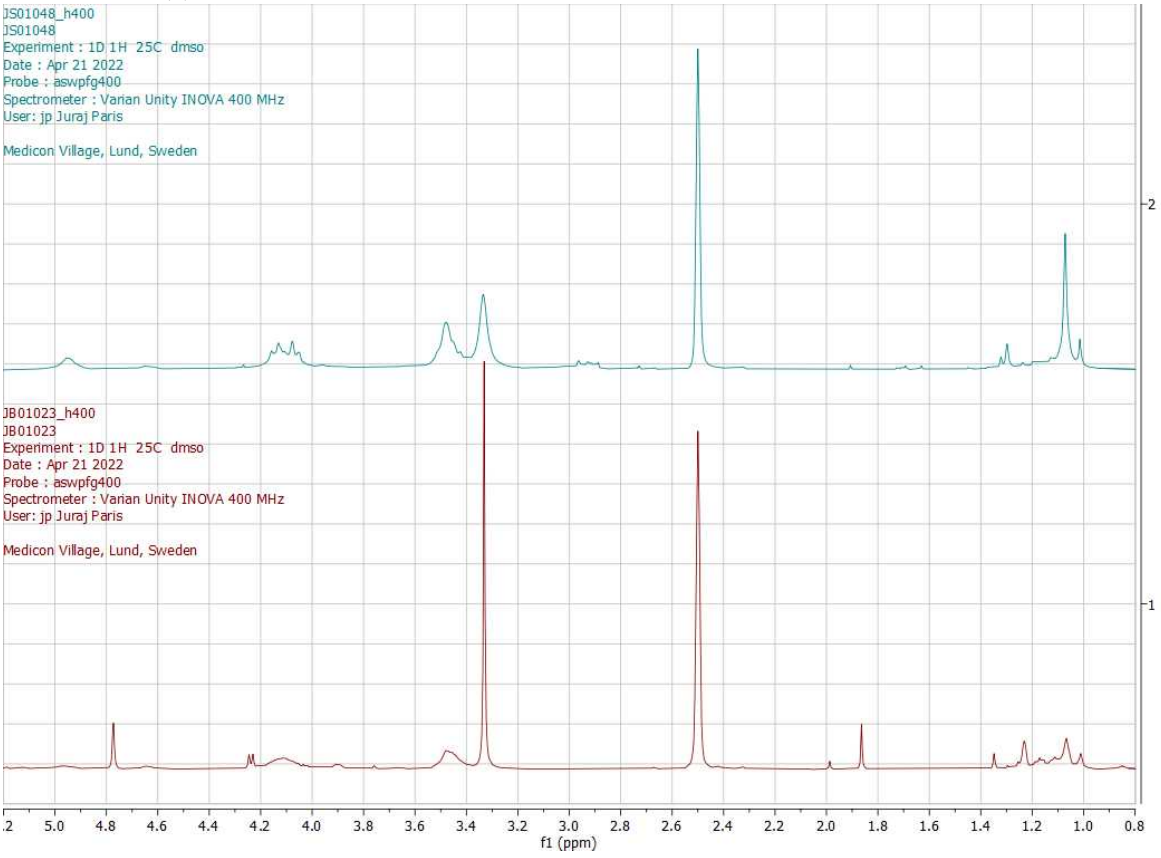


(a) Top, compound **3** from reaction A2. Bottom, compound **4** from reaction A3 washed

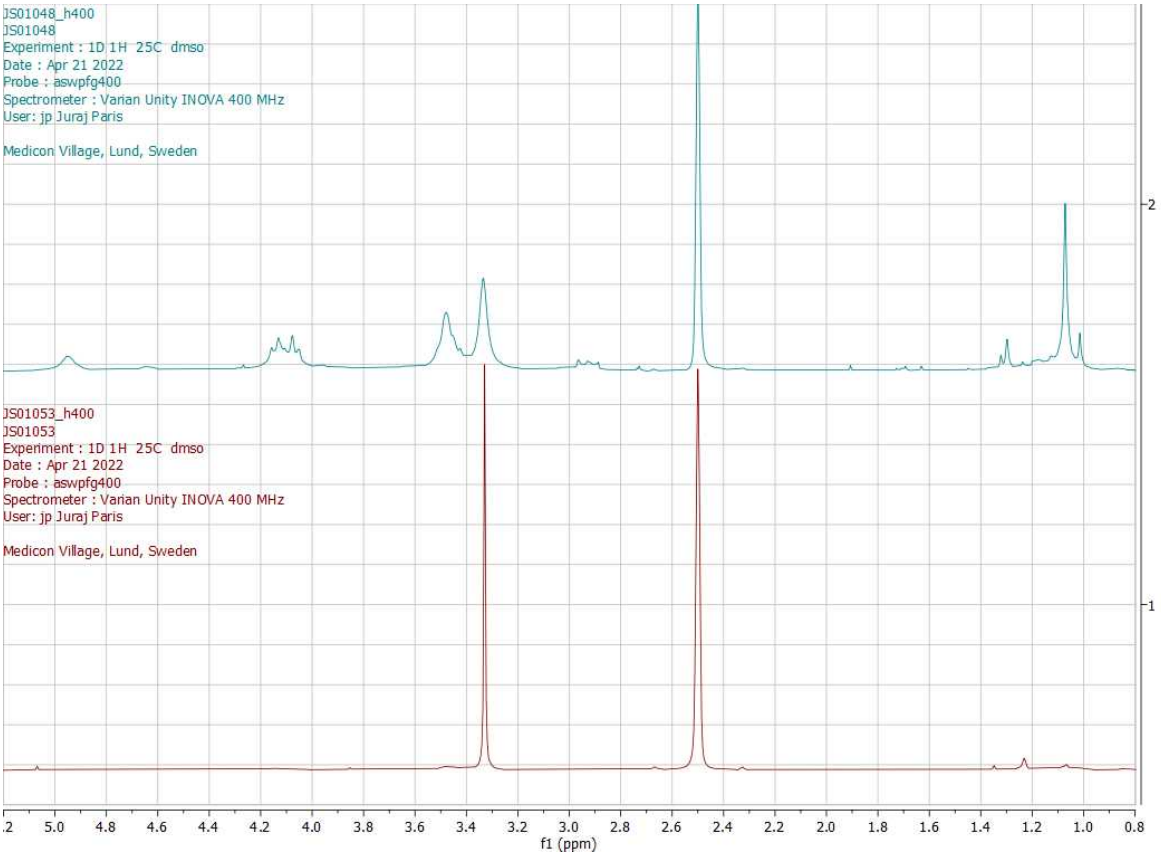
Figure 13: ^{1}H-NMR spectra from 0.8-5.2 ppm (upfield) of compound **3** (reference from reaction A2) and compound **4**. Subfigures: (a), (b), (c) and (d).



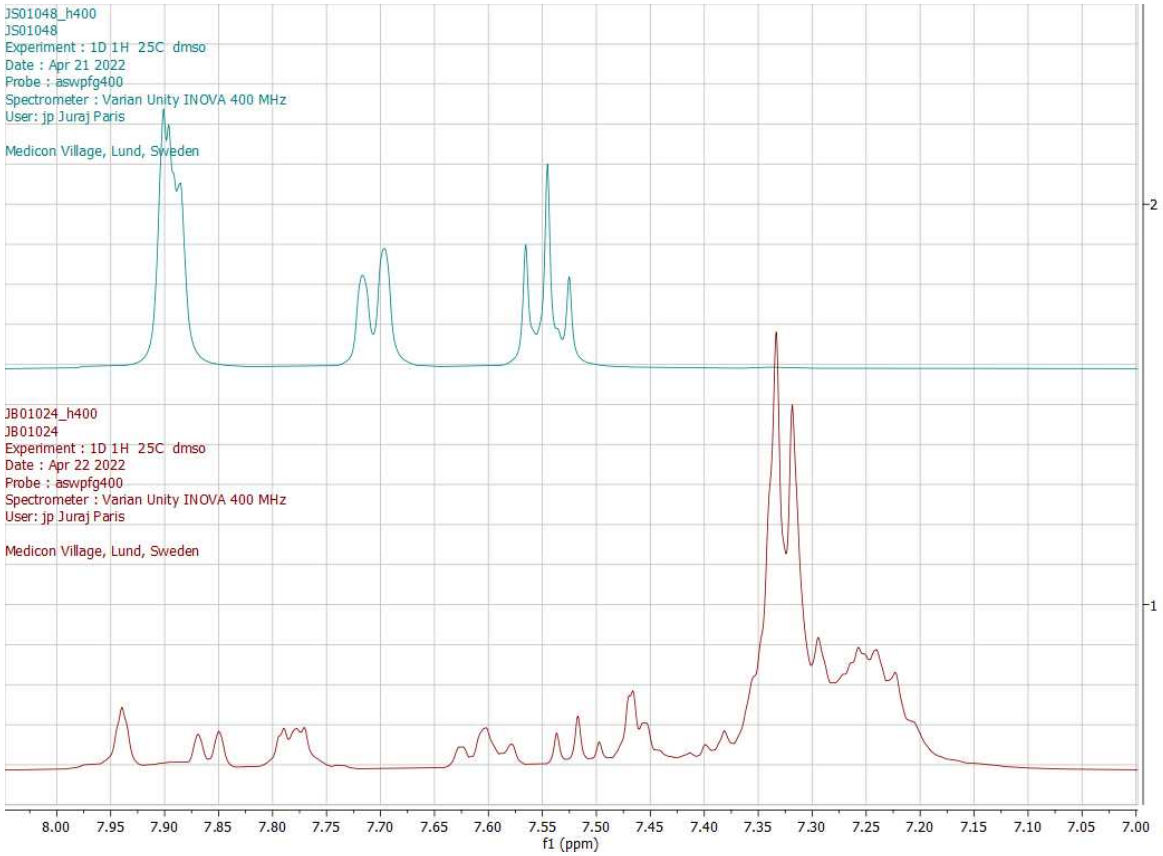
(b) Top, compound **3** from reaction A2. Bottom, compound **4** from reaction A3 unwashed



(c) Top, compound **3** from reaction A2. Bottom, compound **4** from reaction C3b

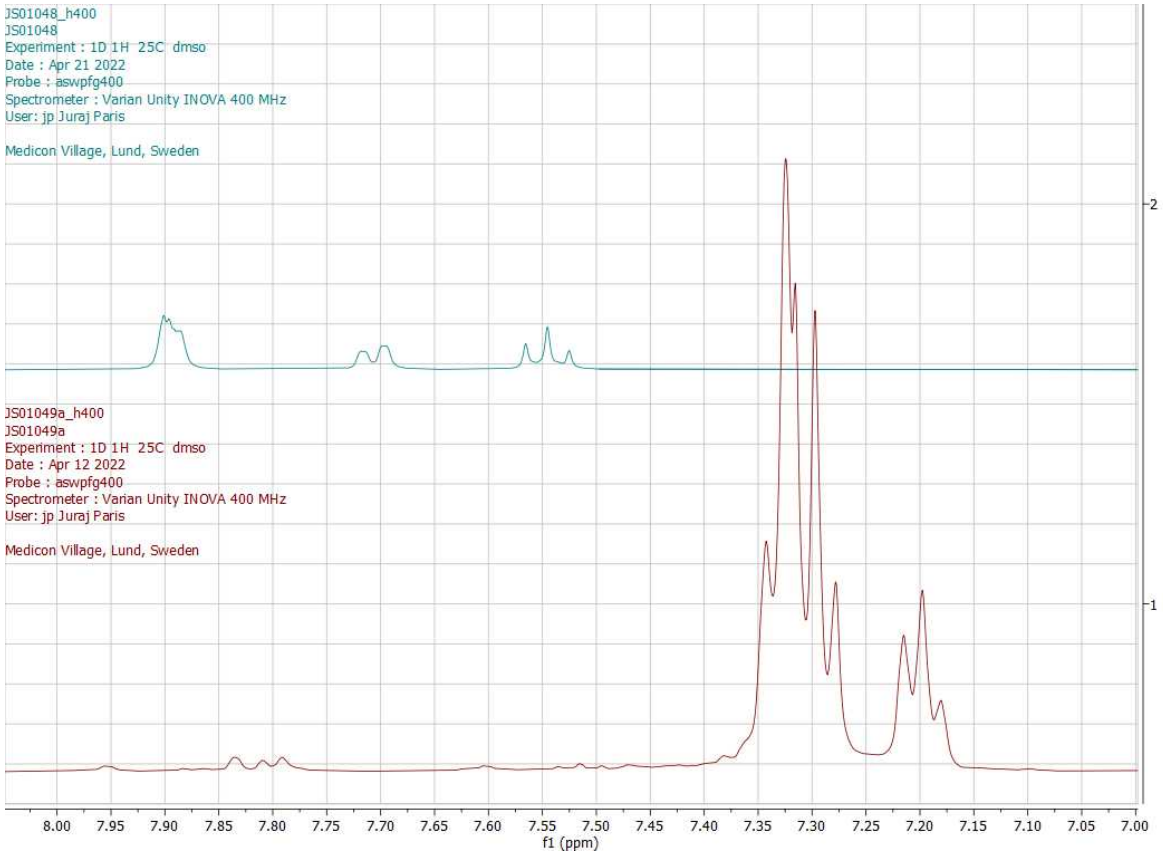


(d) Top, compound **3** from reaction A2. Bottom, compound **4** from reaction C3c

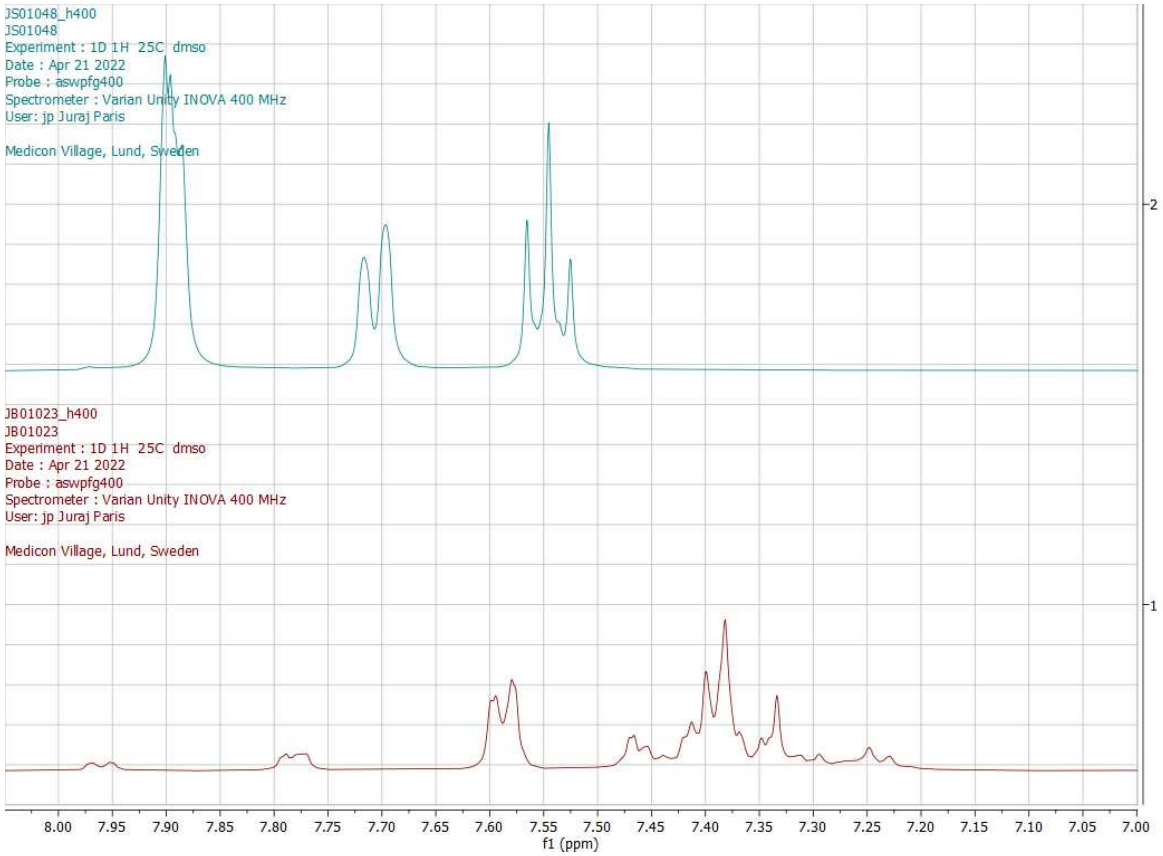


(a) Top, compound **3** from reaction A2. Bottom, compound **4** from reaction A3 washed

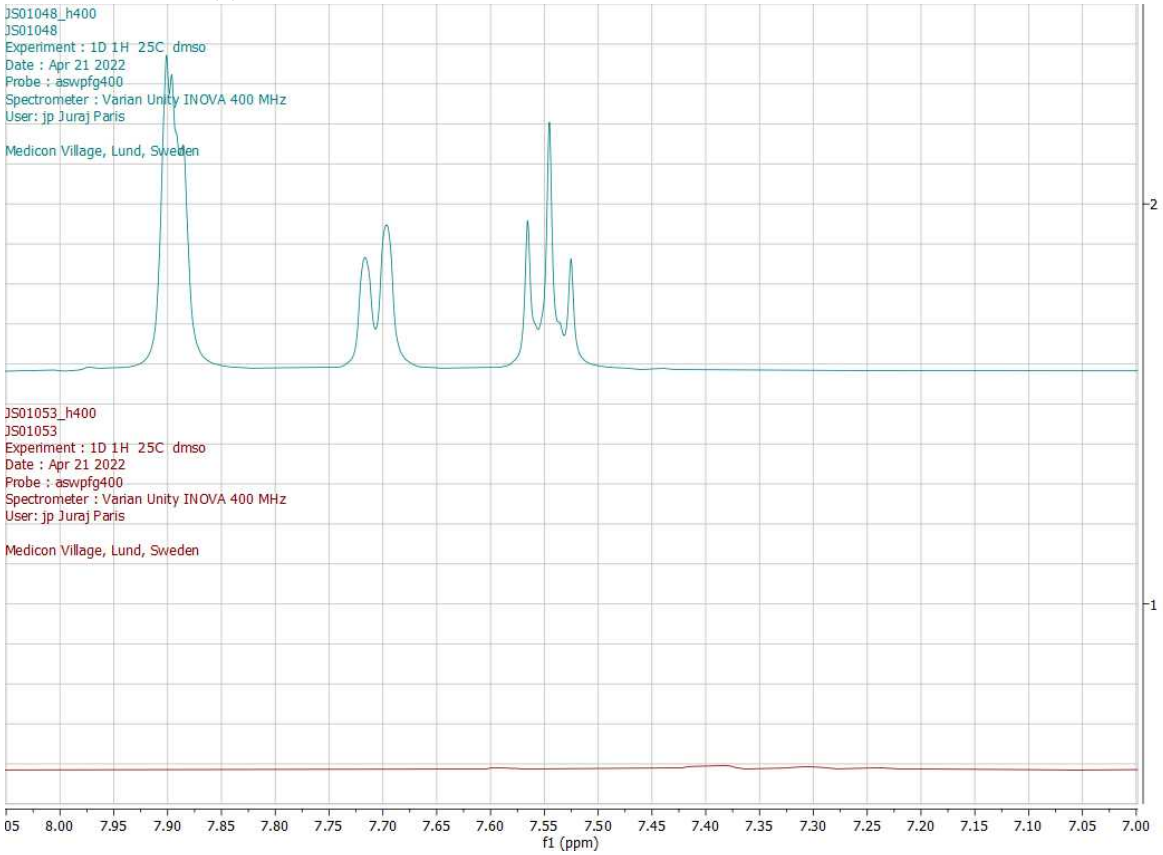
Figure 14: ^{1}H-NMR spectra from 7.0-8.0 ppm (downfield) of compound **3** (reference from reaction A2) and compound **4**. Subfigures: (a), (b), (c) and (d).



(a) Top, compound **3** from reaction A2. Bottom, compound **4** from reaction A3 unwashed



(b) Top, compound **3** from reaction A2. Bottom, compound **4** from reaction C3b



(c) Top, compound **3** from reaction A2. Bottom, compound **4** from reaction C3c

LC-MS of A3 and C3b

LC-MS data was recorded on products from the reactions A3 and C3b to confirm that compound **3** reacted in the expected way into compound **4**. The analysis was made to, as previously stated, give a conclusive result on whether the product from step 2 can bond to an amine. The complete spectra also show tBTMG, solvents and other contaminants (*13*). The expected m/z are found and are marked in Figures 16 17. Fragments of compound **4** are found at 300 m/z in both spectra and at 91 m/z a tropylium ion is clearly present (*11*). This in combination with the ^{1}H-NMR spectra concludes that the epoxidized functional groups can react with amines, forming a polycation. After this analysis, the synthesized products and a reference of compound **1** were tested to determine their cytotoxicity.

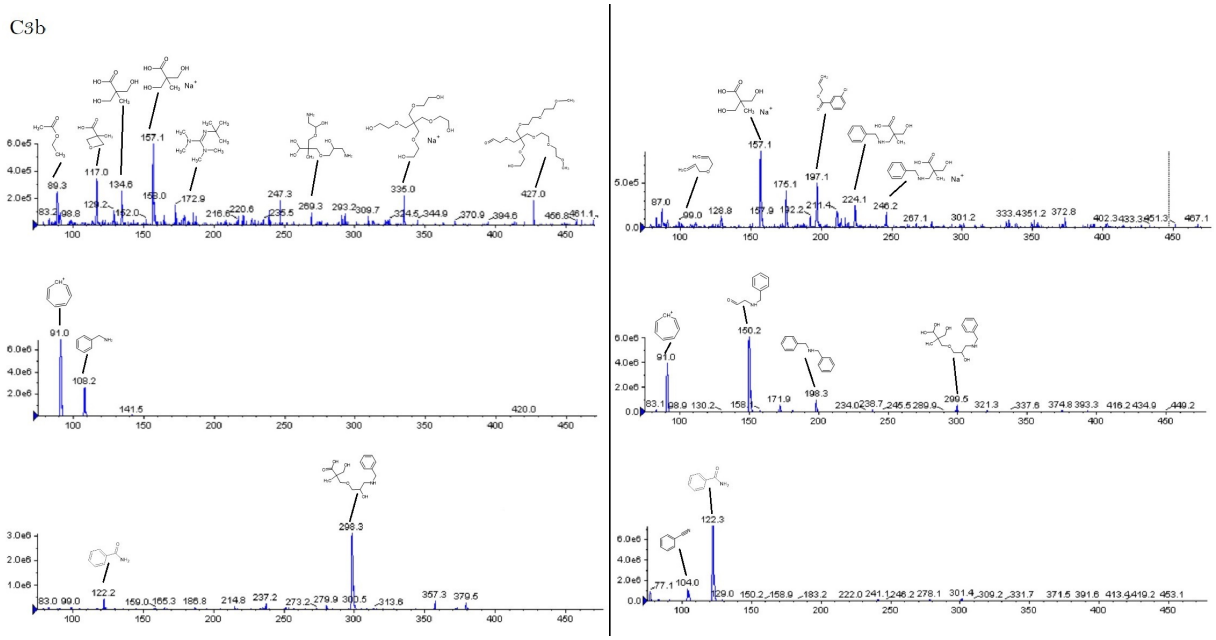


Figure 16: LC-MS of reaction C3b, Peak 1.41 2.43 5.13 6.12 6.43 7.57.

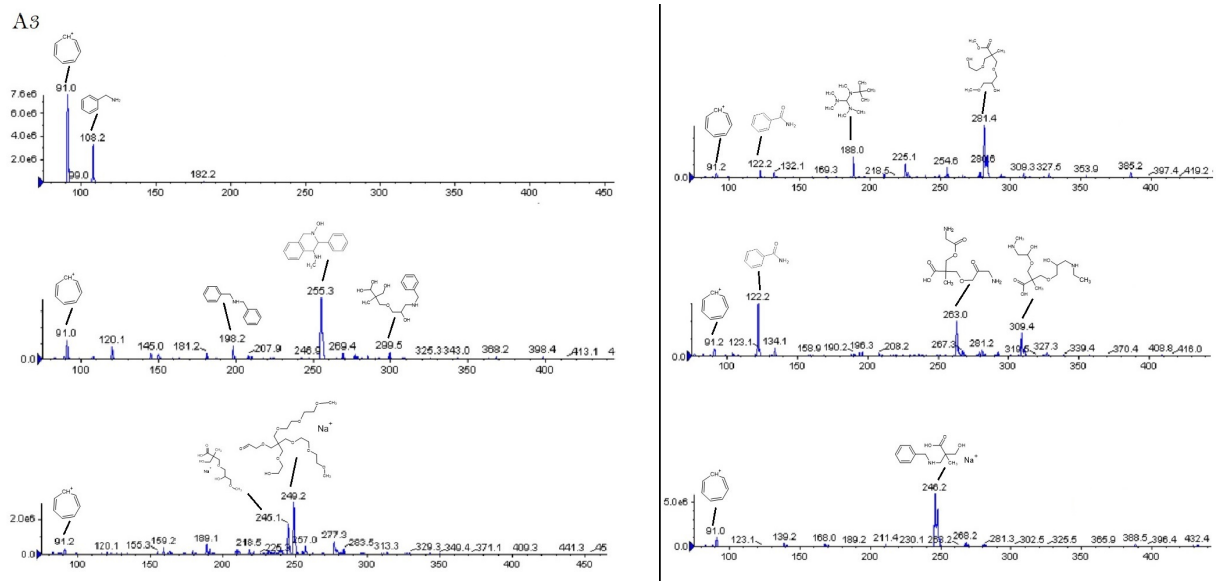
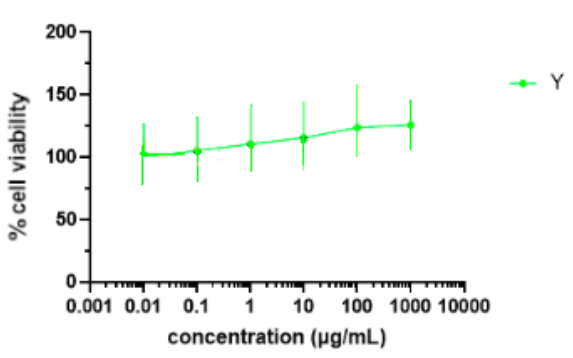
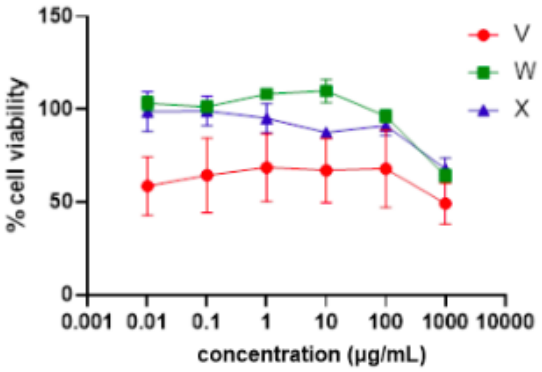


Figure 17: LC-MS of reaction A3, Peak 2.33 6.60 7.15 7.44 7.59 10.05.

Cytotoxicity
The products were tested for the cytotoxicity on a cell culture, according to Figure 18b, details of the cell culture are described in Appendix B. Reaction A3 started more cytotoxic than the other samples. All samples are clearly more cytotoxic at the last concentration than at the first, all are close to 50 % cell viability at the final concentration. Compound **1**, Figure 18a, has a high cell viability and after a 3 step synthesis, all products have lower cell viability than the starting material. The reference samples were prepared by using the starting material dissolved in milli-Q water. When the samples from step 3 of the synthesis were tested, an assumption were made that all samples, sent to the cell laboratory, contained a negligible amount of contaminations. The results confirms that a cytotoxic product is formed, which matches the aim of the synthesis of a polycation, which was been shown to be cytotoxic according to earlier statement (*9*).



(a) Y) Compound **1**



(b) V) Product A3, W) Product C3b, X) Product C3c

Figure 18: Cytotoxicity step 3.

2.2 PEGylation of a hyperbranched polyester

The PEGylations were analyzed by DLS, ^{1}H-NMR and LC-MS. The cytotoxicity was also tested in reference to compound **5**. The decision to use ^{1}H-NMR and LC-MS as two of the four analysis methods in this section originated from the reasons stated in section 2.1.1.

DLS
The size of the molecules within the product sample were measured by DLS. A successful PEGylated product should have a larger molecular size than the starting material. DLS was a suitable method to confirm that the reaction did occur. It was also suitable due to its efficiency for multiple samples. The results below represent the samples from reaction group E. The reference shows that the molecular size of a non PEGylated compound **5** is around 10 nm. In all of the samples from reaction group E, a size increase in comparison to the starting material indicates that PEGylation has occurred, according to Figure 19.

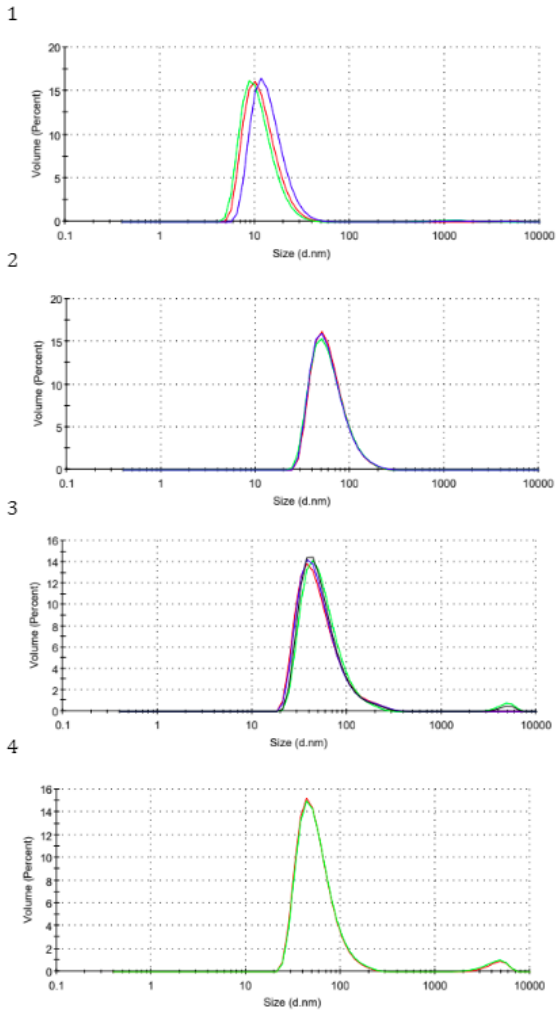
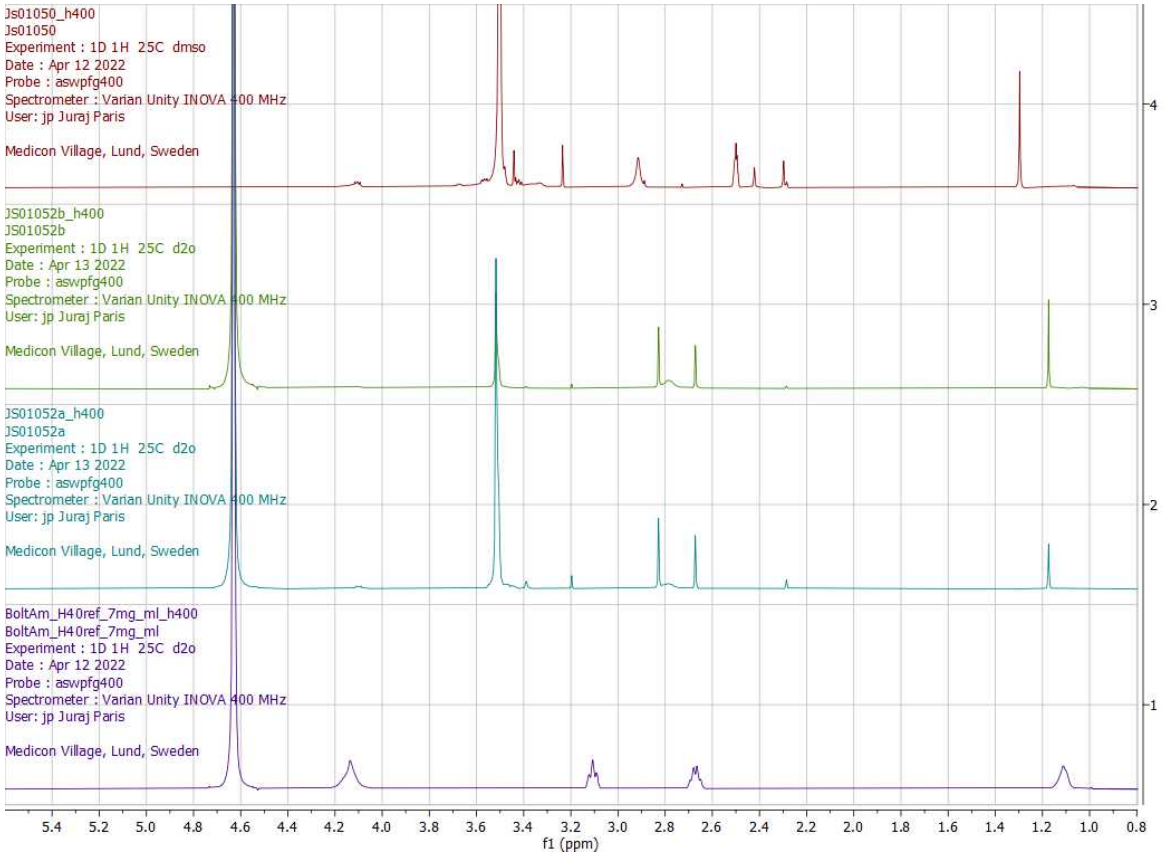


Figure 19: DLS results from reaction group E.
Subpictures: 1. Reference compound **5**; 2. E1; 3. E2; 4, E3.

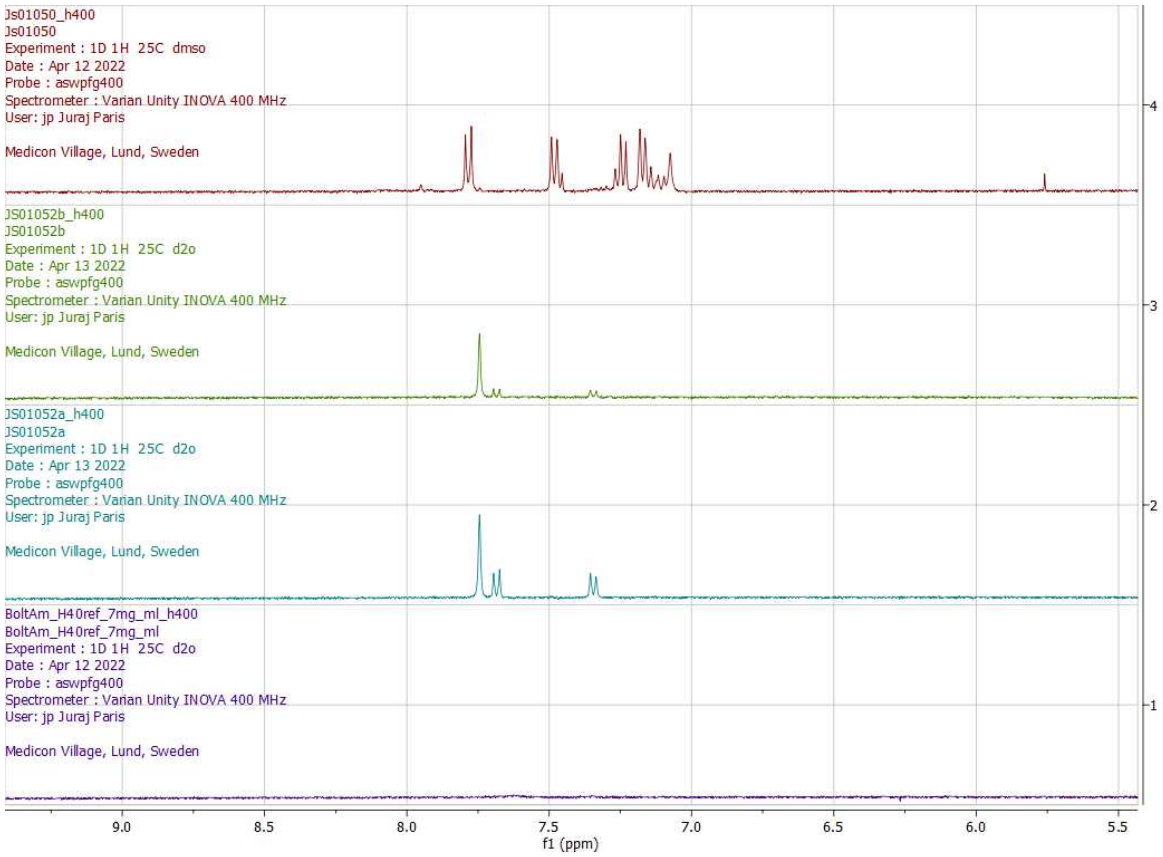
^{1}H-NMR

For this ^{1}H-NMR (400 MHz), D_2O was used as solvent for reaction groups, D, E and F with the exception of d6-DMSO used as solvent for reaction D3. The solvents used differed due to a shortage of d6-DMSO, which was the original plan to analyze all the products in. The amount of product from reaction D3 was too small and it would not be eco-friendly to repeat the experiment just to do an analysis already made. All spectra show an unambiguous change of the spectrum depending on the concentration of mPeg-tosylate added to the sample. Mainly a correlation between concentration and higher intensity from the peak at δ 3.50 seen in Figures 20a, 21a and 22a, and at the two multiplets at δ 7.2 and δ 7.5 seen in Figure 21b. In Figures 20b and 22b the multiplets are seen at δ 7.35 and δ 7.7. The peak at δ 4.62 is HDO and can be attributed to D_2O in all spectra analyzed in the solvent (*15*). The peak present at δ 3.50 can be attributed H_2O and the peak at δ 2.50 can be attributed d6-DMSO in the spectrum from reaction D3. At δ 7.75 reactions D1 and D2 have a prominent singlet which is not present in reaction D3. The two doublets close to earlier mentioned singlets in the spectra from reactions D1 and D2 shows that a reaction have taken place by not being present in the reference sample. Four doublets present in the spectrum from reaction D3 also indicate a reaction, by the same principle, but LC-MS is needed for further confirmation.

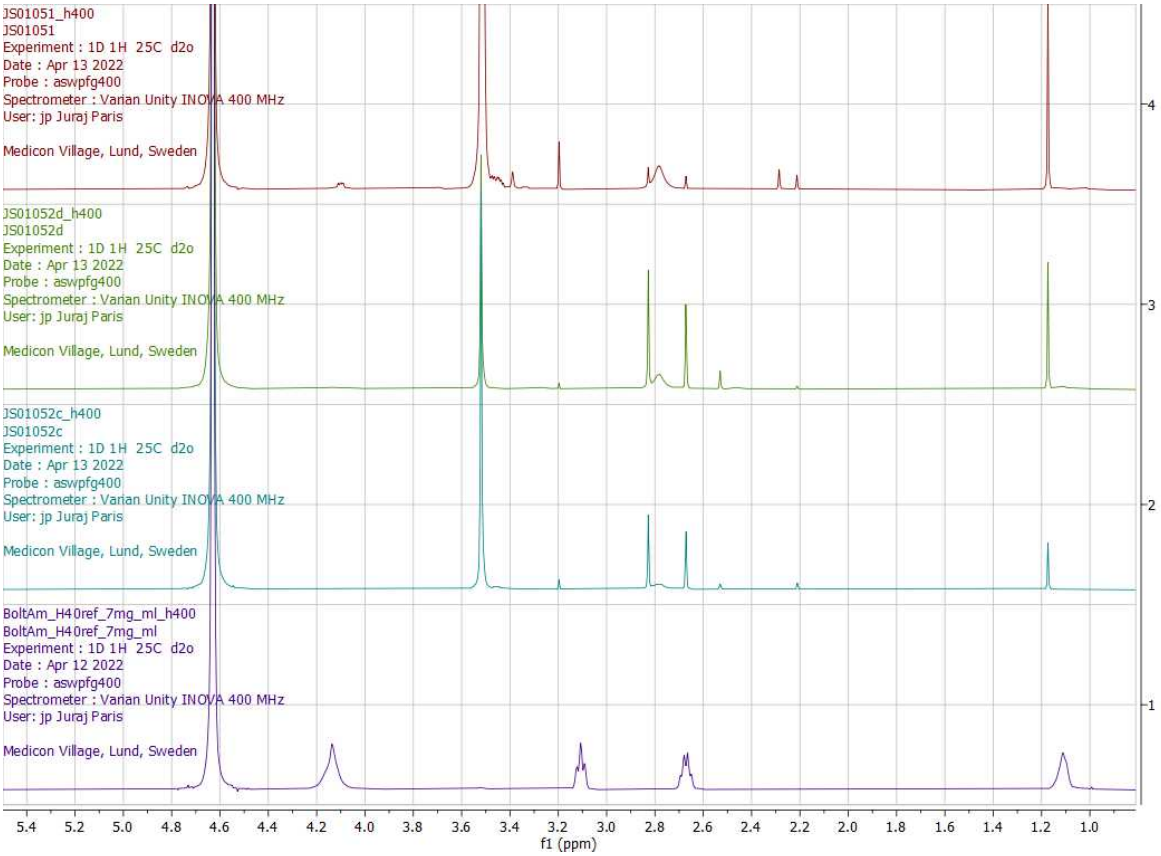


(a) Upfield. Top down: 1, D3; 2, D1; 3, D2; 4, ref compound **5**

Figure 20: Selected sections of ^{1}H-NMR spectra from reaction group D. Subfigures: (a) and (b).

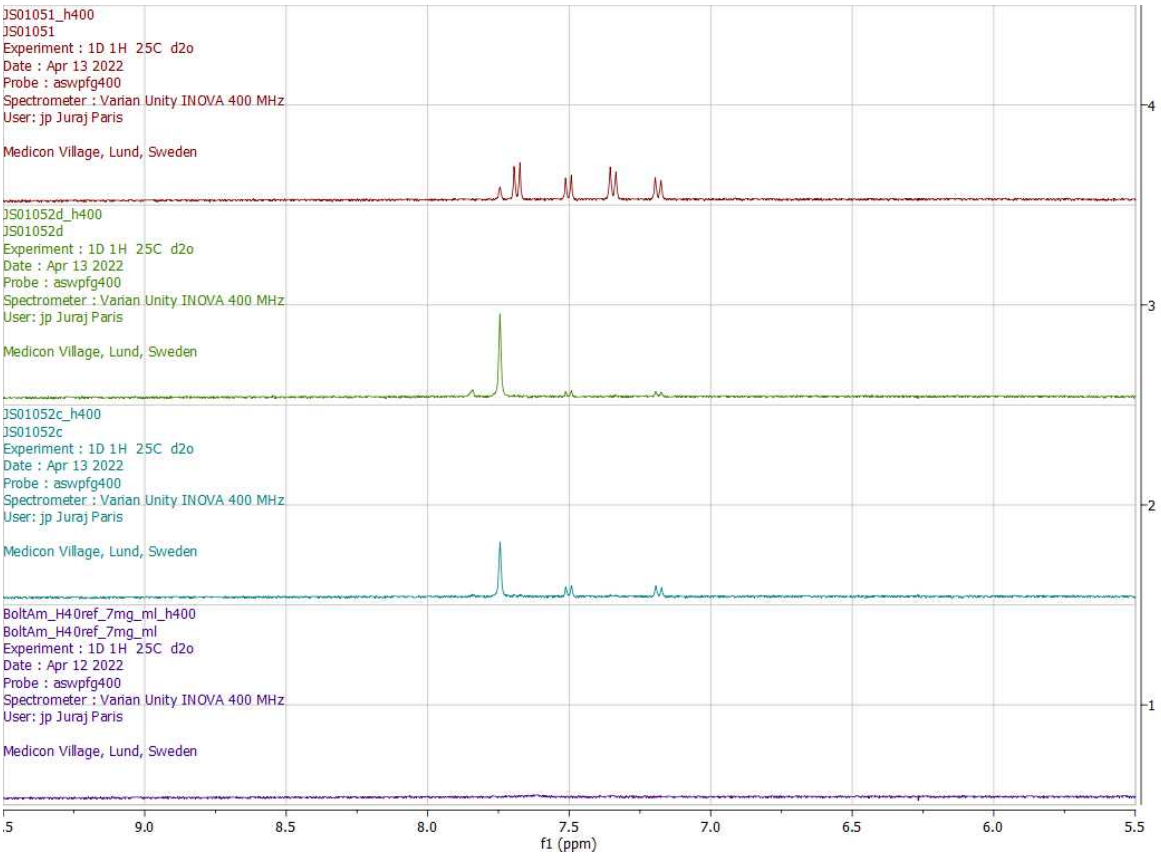


(b) Downfield. Top down: 1, D3; 2, D1; 3, D2; 4, ref compound **5**

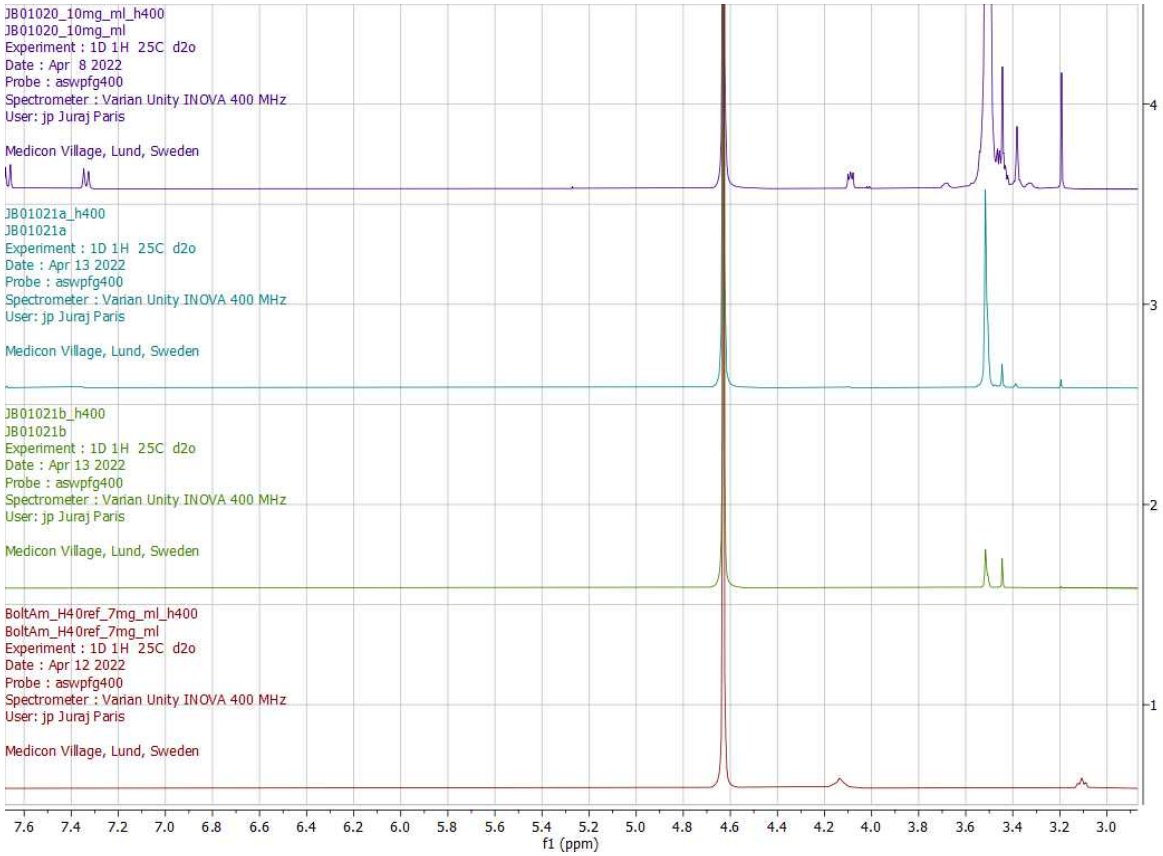


(a) Upfield. Top down: 1, E3; 2, E1; 3, E2; 4, ref compound **5**

Figure 21: Selected sections of ^{1}H-NMR spectra from reaction group E. Subfigures: (a) and (b).

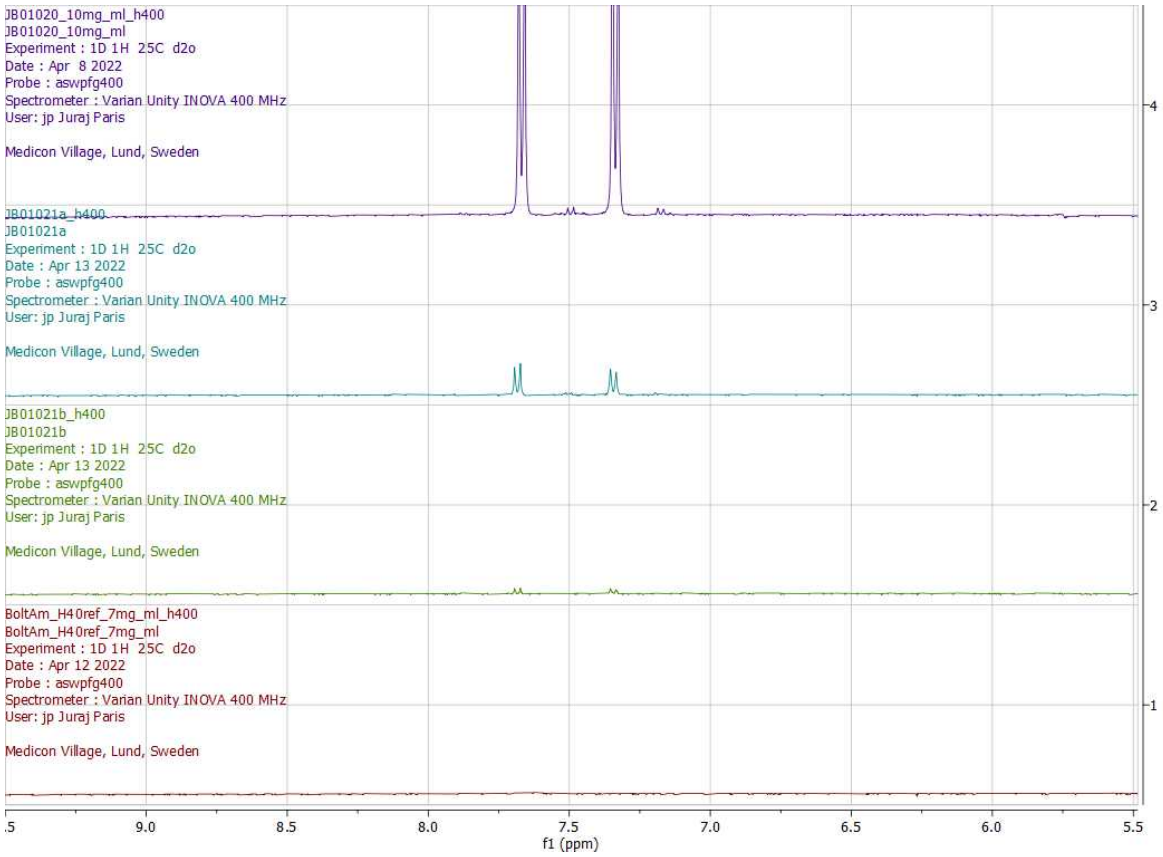


(b) Downfield. Top down: 1, E3; 2, E1; 3, E2; 4, ref compound **5**



(a) Upfield. Top down: 1, F3; 2, F2; 3, F1; 4, ref compound **5**

Figure 22: Selected sections of ^{1}H-NMR spectra from reaction group F. Subfigures: (a) and (b).



(b) Downfield. Top down: 1, F3; 2, F2; 3, F1; 4, ref compound **5**

LC-MS

To interpret the spectra, an estimation of the monoisotopic mass of compound **6** and its fragmentation are made. Fragmentation of all sorts when using LC-MS is, as previously stated, expected and well studied within the field (*11*, *12*). With the knowledge of this and having the expected fragments of the starting material confirmed by Spago Nanomedical the interpretation of the peaks are manageable. In peak 8.13 shown in Figures 24 and Figure 25 a methoxy adduct is seen, originating from the methyl ether from the tosylate that formed through an S_N2-reaction. Both spectra have a peak at 89 m/z which due to the low resolution can hide the three degradation products from the method used (*16*) seen in Figure 24. The first fragmentation yields beta alanine which then oxidizes to a hydroxylamine. Due to its low stability, water is quickly eliminated and an imine is formed. This is then hydrolyzed into an aldehyde. Beta alanine, the imine and the aldehyde are shown in Figure 23.

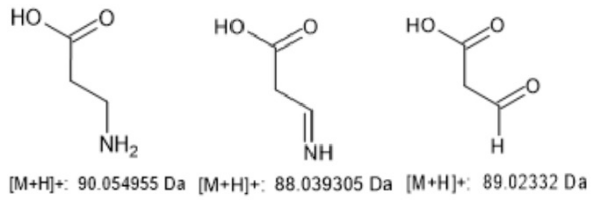


Figure 23: Illustrated degradation products in Peak 89 in LC-MS of reactions E3 and F3.
Left, beta alanine. Middle, imine. Right, aldehyde.

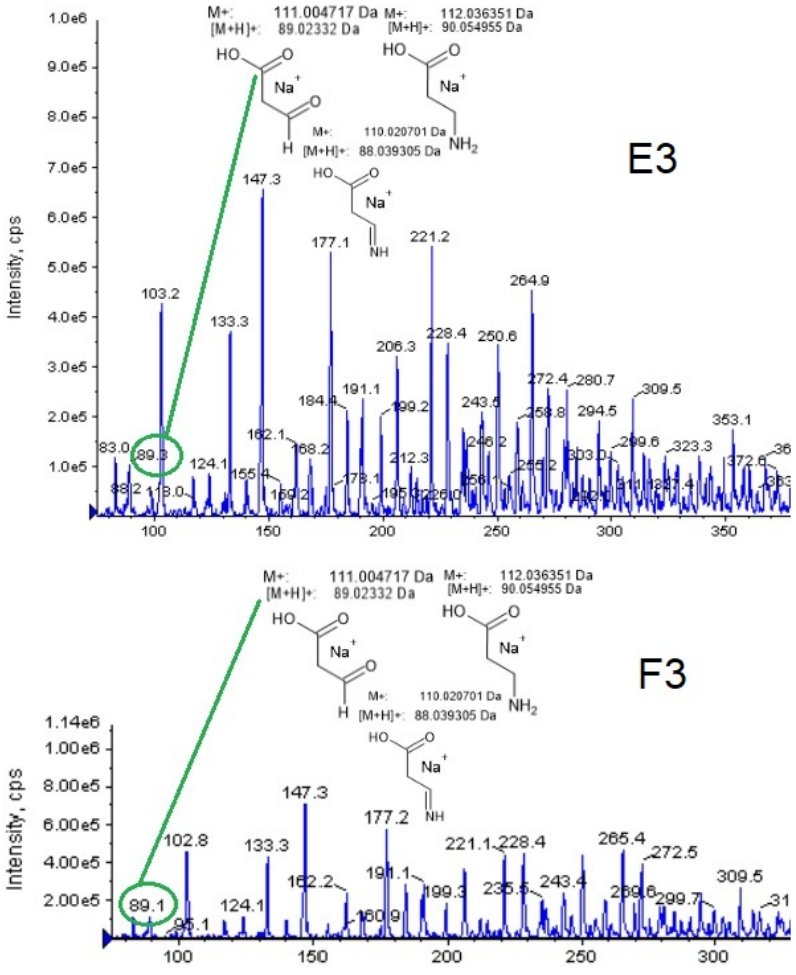


Figure 24: Selected sections of LC-MS spectra (peak 8.13) of compound **6** from reactions E3 (top) and F3 (bottom).

Circled in orange in Figure 25 are 3^+ monomer-fragments and three of those give a monoisotopic mass of 44. This is the mass of the monomer; CH_2-CH_2-O. The charge of these fragments can differ depending on where the chain is protonated. A charge of 3^+ gives a difference in between peaks of 14.67. With the same method a charge of 4^+ gives a difference in between peaks of 11. The many peaks are different chains from the original structure. By going through the spectra and using this method continuously it was found that the peak at 530 in the spectra is coming from a chain with a mono isotopic mass of 1587. Looking at the lower m/z (100-350) of the spectrum from reaction F3, there is a difference in between peaks of 14.67 m/z. This is showing bigger fragments of compound **6**.

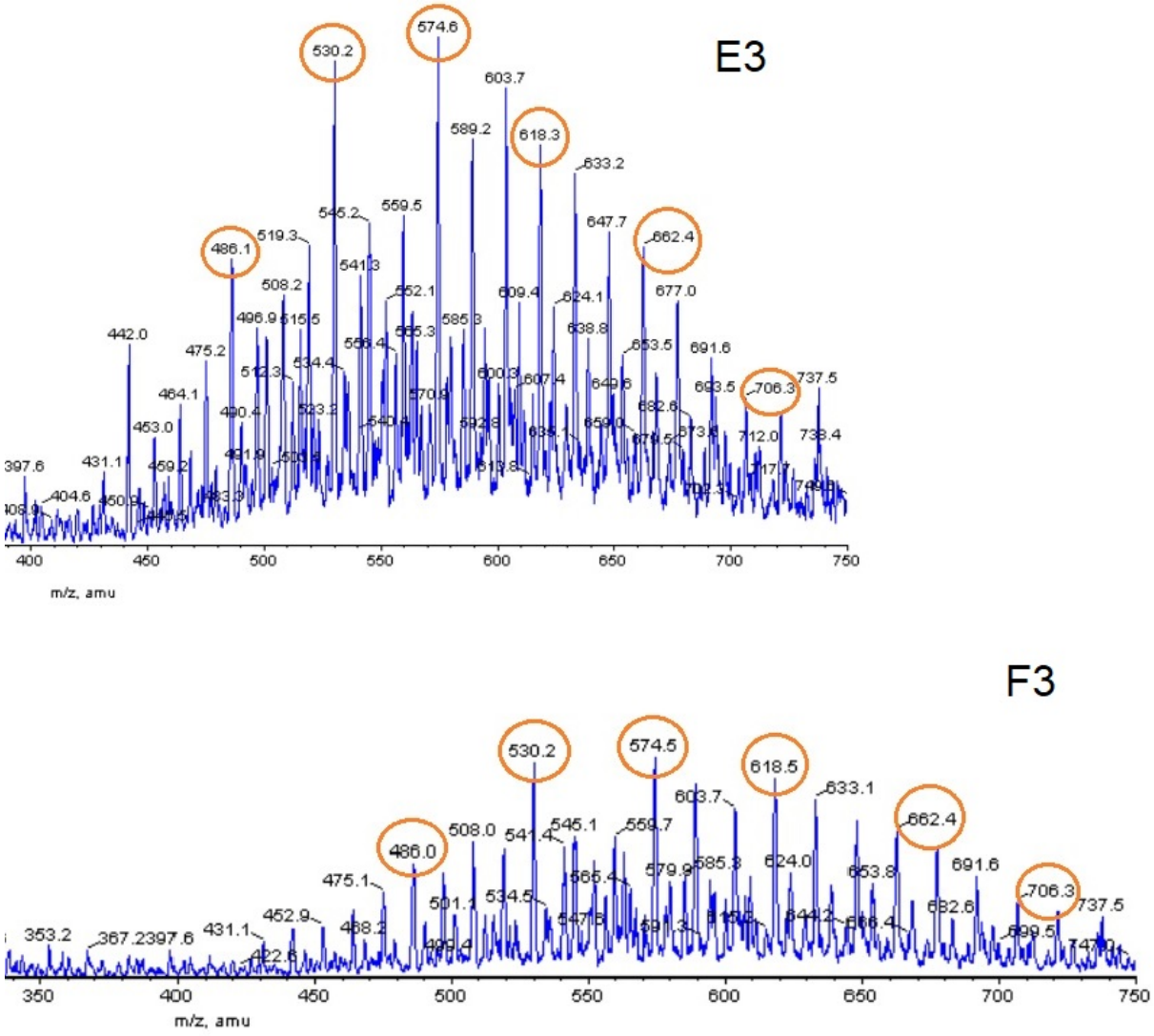
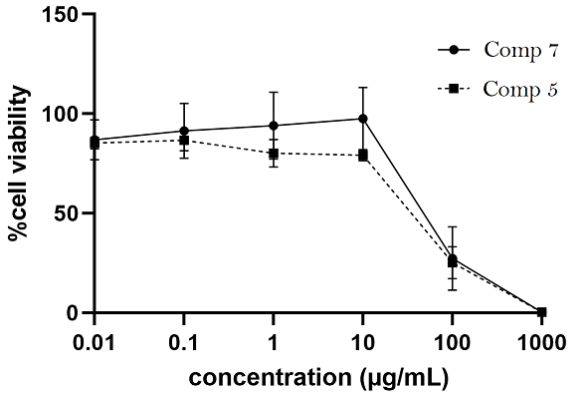


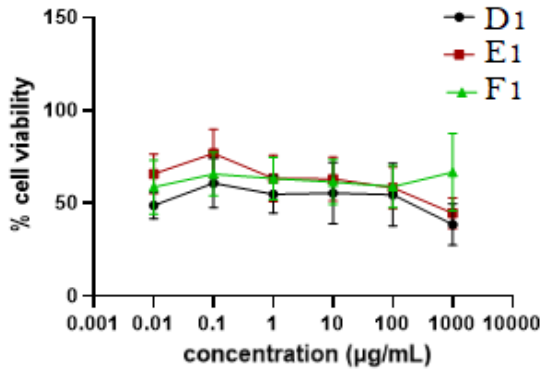
Figure 25: Selected sections of LC-MS spectra (peak 8.13) of compound **6** from reactions E3 (top) and F3 (bottom). The numbers above peaks refer to the intensity, cps (counts per second).

Cytotoxicity

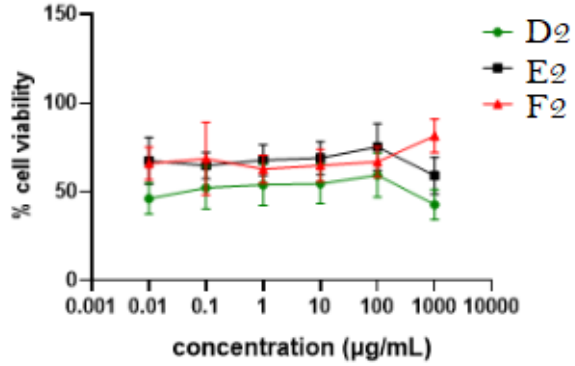
The starting materials of the PEGylation, compound **5**, was tested for its cytotoxic effects, compared to a smaller molecule, Amine Functional Boltorn™ H20, according to Figure 26a (see chemical structure of compound **7** in Appendix E). Until 10 µg/mL the cell viability varies between 65-80 %. At 100 µg/mL the cell viability decreases to below 25 %, which indicates high cytotoxicity at that concentration. Also indicated is that the larger the molecule the lower is the cell viability, as mentioned in the earlier statement (*5*). These reference samples were prepared using the starting material dissolved in milli-Q water. When the samples from the PEGylation were tested, an assumption were made that all samples, sent to the cell laboratory, contained a negligible amount of contaminations. The cytotoxic effects of the PEGylation was tested relative to the starting material and in Figure 26b the result of samples D1, E1 and F1 are presented. Samples D1 and E1 are following the same trend, the cell viability is decreasing with higher concentration, except for at 0.1 µg/mL. The cell viability of sample F1 is increasing at 100 µg/mL, which indicates that the sample is less toxic than the other two. Compared to the starting material of this PEGylation, compound **7**, the cell viability does not have a decrease lower than 25 %. Figure 26c shows the result of samples D2, E2 and F2. All samples have cell viability with the same trend up until 100 µg/mL, and vary between 50-65 %. Higher concentration indicates that sample F2 have an increase when sample D2 and E2 have a decrease. This demonstrates that sample F2 is less cytotoxic, which can be interpreted that this PEGylation is slightly more successful. Compared to the reference on non PEGylated starting material, compound **5**, the cell viability never drops under 25 %. This indicates that the PEGylated samples D2, E2, F2 are less cytotoxic at 100 µg/mL. Figure 26d is presenting the result of samples E3, D3 and F3. Sample E3 is showing the highest cell viability of all throughout the concentration interval, which indicates that the less cytotoxic sample was 100 % PEGylation in DMF at 80 degrees. Sample D3 has nearly as high cell viability, and is the second least toxic of the samples. Sample F3 does not show any difference from the other percentages in D_2O as a solvent. These results also indicate that the PEGylated products are less cytotoxic than the starting material, which was the aim of the reaction and agrees with earlier statement (*8*). They also indicate that the least toxic molecule is the polycation PEGylated 100 %.



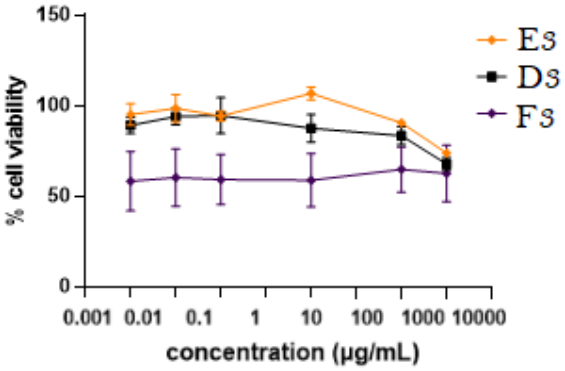
(a) Compound **7**, Compound **5**



(b) Reaction D1, Reaction E1, Reaction F1



(c) Reaction D2, Reaction E2, Reaction F2



(d) Reaction E3, Reaction D3, Reaction F3

Figure 26: Cytotoxicity graphs. Subfigures: (a), (b), (c) and (d).

3 Conclusion

In step 1 of the synthesis (see 4.2.1) the allylated product were confirmed by ^{1}H-NMR and LC-MS, as were the product from step 2 (see 2.1.1 and 2.1.2). The epoxides from step 2 of the synthesis proved to be able to form a bond to an amine in the subsequent step 3 (see 2.1.3) and thus forming a polycation. There is a rise in toxicity from the synthesized polycation in relation to Compound **1**. This was founded in that an assumption that all samples, sent to the cell laboratory, contain a negligible amount of contaminations was made. The result indicates that products from reaction path **A** proved more cytotoxic and had more polycationic properties than the reaction paths **B** and **C**. The correct formation of the polycation was supported by SEC, ^{1}H-NMR and LC-MS, and the aim of synthesizing a polycation was thereby achieved. Regarding the PEGylation, a decrease of the cytotoxicity in reference to Compound **5** confirms the facts stated in the introduction that PEGylation lowers the cytotoxicity of polycations. This was once more founded on that an assumption that all samples, sent to the cell laboratory, contain a negligible amount of contaminations were made. The success of the PEGylation was further supported by DLS, ^{1}H-NMR and LC-MS. The percentage of mPEG in relation to the molar equivalent of Compound **5** confirms that a higher percentage of mPEG attached to the polycation gives a less cytotoxic product, which also clears out the aim. A distinct trend of the reaction conditions of the PEGylation were not found, but the less cytotoxic product were 100% PEGylation in DMF. By using Compound **7** as a reference to Compound **5**, it was also confirmed that the cytotoxicity increased with an increase of molecular weight. The reproducibility of both the synthesis and PEGylation is estimated as good.

4 Experimental section

In this section the experimental procedure will be presented, for detailed time frame see Appendix A.1 and A.2. Calculations are presented in Appendix D.

4.1 General section

Index

- Synthesis
  - The capital letter indexes of the reactions indicate the conditions in the initial reaction and are found in table 3.
  - The number indexes refer to where a previous product has been used in the subsequent step and also makes it possible to differentiate between reaction steps and their corresponding products.
  - The lower case letter indexes show where a product has been divided from a main reaction when utilized in the following step.
- PEGylation
  - The capital letter indexes refers to a group of 3 reactions with different aims for degrees of PEGylation.
  - The number indexes is corresponding to the %age of the aimed for PEGylation within the group. With 1, 2, and 3 referring to 10, 55, and 100 %.

4.1.1 Chemicals

This section presents the chemicals used in reactions below, so are the suppliers of the chemicals. Provided by Spago represents chemicals that were prepared at Spago Nanomedical.

Table 2: Column 1, Chemical abbreviation. Column 2, Supplier.

Compound 1	Polymer Factory Sweden AB
Allyl Bromide	Sigma-Aldrich
tBTMG	Sigma-Aldrich
Acetate buffer	Provided by Spago
DCM	Sigma-Aldrich
EtOAc	Sigma-Aldrich
d6-DMSO	Sigma-Aldrich
mCPBA	Sigma-Aldrich
Carbonate-bicarbonate buffer	Provided by Spago
3-chlorobenzoic acid	Sigma-Aldrich
Benzylamine	Sigma-Aldrich
Phosphate buffer	Provided by Spago
Compound 5	Polymer Factory Sweden AB
Compound 7	Polymer Factory Sweden AB
mPEG-tosylate	Provided by Spago
Calcium hydroxide	Sigma-Aldrich
Polyethylene oxide	Agilent Technologies

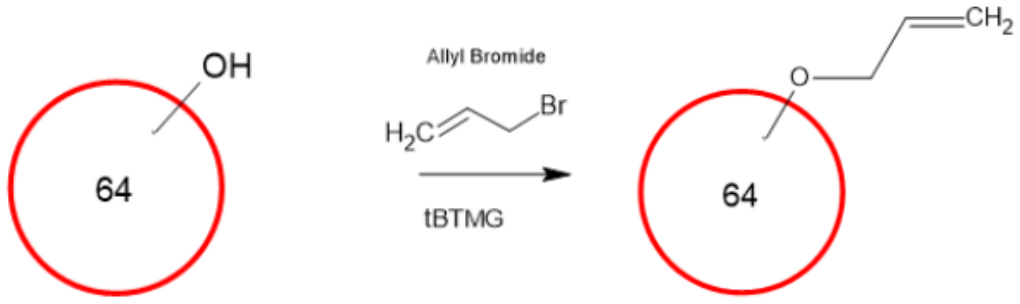
4.2 Synthesis of a polycation

4.2.1 Allylation method

Compound **1** was dissolved in different solvents (A1, DCM; B1, Acetone; C1, DMF) and dried over molecular sieves for at least 24 hours. The reasoning behind the solvents used to dissolve compound **1** are found in Appendix A.1. Reaction mixture B1 was rotary evaporated to remove the acetone which was replaced by DCM (50ml). Through a S_N1-reaction compound **1** reacted with allyl bromide (Figure 27). Allyl bromide and tBTMG were added at room temperature under vigorous stirring, during conditions according to table 3. The reaction time within said table differ due to the fact that it's not a time sensitive reaction even though it still follows the basic principle of that reactants decrease over time. Reaction A1 was washed with acetate buffer (3 x 100 ml, pH 4.51). Reaction B1 was washed with acetate buffer (3 x 50 ml, pH 4.51) and DCM (20 ml) was added. Reaction C1 was diluted with water (50 ml) and extracted with EtOAc (3 x 50 ml). The organic phase was dried with magnesium sulfate and the solids were filtered off on a Büchner funnel. The filtrate was collected and used for analysis and further synthesis.

Table 3: Reaction conditions for the allylation (step 1)

Reaction	**A1**	**B1**	**C1**
Synthesis	DCM	DCM	DMF
Compound **1**, mg (mmol)	1000 (0.137)	671 (0.0916)	500 (0.0683)
Allyl Bromide, mg (mmol)	1057 (8.74)	706 mg (5.86)	529 (4.37)
tBTMG, µl	1968 (9.614)	558 (6.45)	390 (1.91)
Reaction time, h	24	2	24
Compound **2**, mg	603 (0.0610)	-	281 (0.028)



(a) Mw, compound **1** = 7323 g/mol (b) Mw, compound **2** = 9887 g/mol

Figure 27: Illustration of the chemical reaction of compound **2** with Mw. Subfigures: (a) and (b).

4.2.2 Epoxidation method

Compound **2** reacted with mCPBA (Prilezhaev reaction), added under vigorous stirring (see Figure 28 for illustration of the chemical reaction). The amount of solvent used in reactions B2 and C2a was 50 ml each, DCM and EtOAc respectively. In reaction C2b 100 ml EtOAc was used and in reaction A2 100 ml of DCM was used. The amount mCPBA added was initially 64 times the molar equivalents of compound **2**, under reaction conditions stated in table 4. The reaction time within said table differ due to the reason stated in 4.2.1. In mixture A2 and C2b additional mCPBA was added, after indication

from TLC that all acid had reacted or decomposed to the corresponding carboxylic acid. Mixtures B2 and C2a were washed with a carbonate-bicarbonate buffer (3 x 100 ml, pH 8.41) to remove excess mCPBA. A small amount (20 ml) of reaction mixture A2 was washed (pH 8.41, 3 x 20 ml). A small amount (1.5 ml) of unwashed reaction mixture A2 taken out and used as a reference for TLC. TLC indicated no effect of the wash, resulting in that reaction mixtures A2 and C2b weren't washed.

Half of mixture B2, was used for the subsequent step of the synthesis. Half of mixture C2a, excluding samples for ^{1}H-NMR, was used for the subsequent step of the synthesis. The remaining half of mixture C2a was rotary evaporated to remove solvents and calculate the final mass of the product and 3-chlorobenzoic acid that still remained. Mixtures A2 and C2b were rotary evaporated to remove solvents and calculate the final mass of the product and 3-chlorobenzoic acid that still remained. Samples from mixtures A2 and C2b for ^{1}H-NMR and LC-MS were taken from the dried products.

Table 4: Reaction conditions for the epoxidation (step 2)

Reaction	**A2**	**B2**	**C2a**	**C2b**
Solvent	DCM	DCM	EtOAc	EtOAc
Compound 2, mg (mmol)	580 (0.059)	138 (0.014)	64 (0.00647)	106 (0.0107)
mCPBA, mg (mmol)	1248 (7.23)	173 (1.002)	105 (0.608)	236 (1.368)
Reaction time, h	24	4	4	4
Compound 3, mg (mmol)	1331 (0.121)	-	71 (0.0065)	271 (0.0248)
Compound 2, mg	603 (0.0610)	-	281 (0.028)	-

(a) Mw, compound **2** = 9887 g/mol (b) Mw, compound **3** = 10911 g/mol

Figure 28: Illustration of the chemical reaction of compound **3** with Mw. Subfigures: (a) and (b).

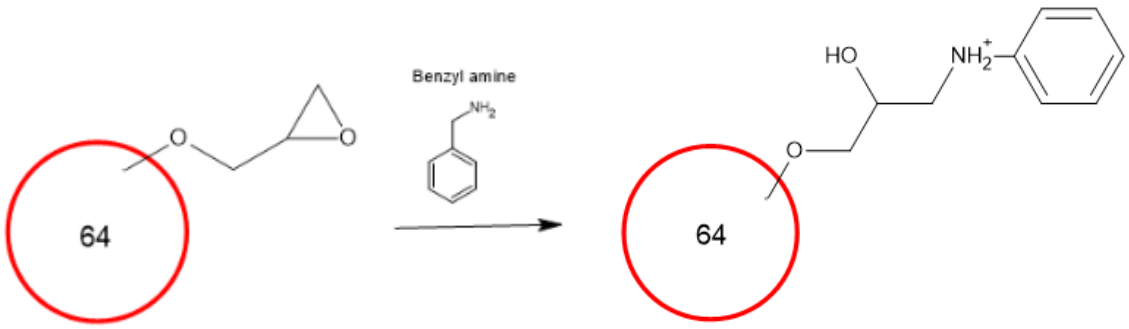
4.2.3 Diagnostic reaction method

Compound **3** reacted through a S_N2-reaction with benzylamine, added under vigorous stirring (see Figure 29 for illustration of the chemical reaction). The amount benzylamine added was initially 64 times the molar equivalents of compound **3**, under reaction conditions stated in table 5. The reaction in step 2 formed a byproduct in the form of the corresponding carboxylic acid (3-chlorobenzoic acid) to mCPBA. TLC was performed on reactions A3, C3a and C3b to ensure an excess of benzylamine in relation to 3-chlorobenzoic acid within the mixtures. Reasoning behind this are founded from analysis of the precipitate (benzyl ammonium dichlorobenzoic acid) formed in reaction B3. Additional benzylamine was added to ensure abundance within the reaction mixtures. In reactions A3, B3, C3b

and C3c a precipitate of benzyl ammonium dichlorobenzoic acid formed. No precipitate formed in reaction C3a after the addition of benzylamine. The precipitate in reaction B3 was collected by centrifugation, filtration and evaporation of the top solution. A ^{1}H-NMR of the precipitate showed that it was benzyl ammonium dichlorobenzoic acid and the expected product was lost from the reaction mixture. The excess of benzylamine added in reactions A3, C3a and C3b acted as a precipitant to ensure that all of the 3-chlorobenzoic acid from the previous step precipitated as benzyl ammonium dichlorobenzoic acid. A small amount of reaction A3 (0.5 ml) was taken out (once precipitated) and centrifuged to confirm that it was benzyl ammonium dichlorobenzoic acid. Both the top solution and precipitate was sent to ^{1}H-NMR for confirmation. By confirming the chemical composition a second time postulations regarding that the precipitates in C3b and C3c was the same compound. This knowledge led to that the precipitate in reactions A3, C3b and C3c was removed by centrifugation and filtration. To reaction C3a, in which no precipitate formed, heptane (20 ml) was added yielding two layers. The filtrate from reactions A3, C3b and C3c were washed with phosphate buffer (pH 7, 9 x 20 ml, 5 x 10 ml, 7 x 10 ml) until a consistent pH of 7 was reached in the aqueous phase, indicating that the excess benzylamine was extracted to the aqueous phase. Reaction mixtures A3, C3b and C3c were rotary evaporated to remove solvent and calculate the final mass of the product. When washing reaction C3a with phosphate buffer (pH 7, 1 x 20 ml), no layers formed and the reaction was deemed unsuccessful.

Table 5: Reaction conditions for the diagnostic reaction (step 3)

Reaction	**A3**	**B3**	**C3a**	**C3b**	**C3c**
Solvent	DCM	DCM	EtOAc	EtOAc	EtOAc
Compound **3**, mg (mmol)	1298 (0.119)	-	35.5 (0.0033)	243.9 (0.0224)	33 (0.00303)
Benzylamine, µl (mmol)	2495 (22.84)	32 (0.293)	23 (0.21)	314 (2.87)	70 (0.64)
Reaction time, h	24	19	2	5 days	1.5
Compound 4 mg (mmol)	78 (0.0044)	-	-	74 (0.00416)	7 (0.000394)
Compound **4**, mg	603 (0.0610)	-	-	281 (0.028)	-



(a) Mw, compound **3** = 10911 g/mol

(b) Mw, compound **4** = 17800 g/mol

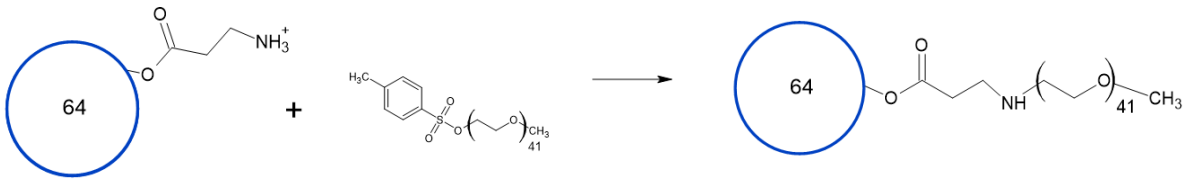
Figure 29: Illustration of the chemical reaction of compound **3** with Mw. Subfigures: (a) and (b).

4.3 PEGylation of a hyperbranched polyester

Compound **5** was dissolved in corresponding solvent with amounts and conditions according to table 6. To reaction groups D and E, mPEG-tosylate and tBTMG, were added under vigorous stirring. Calcium hydroxide (1 spoon) and mPEG-tosylate was added to reaction group F. The mixture was left stirring. Reaction group F was centrifuged and filtered to remove solids.

Table 6: Reaction conditions for PEGylation of compound **5**

Reaction Group Reaction	**D**			**E**			**F**		
	D1	D2	D3	E1	E2	E3	F1	F2	F3
Solvent	DMF	DMF	DMF	DMF	DMF	DMF	D2O	D2O	D2O
Volume solvent, ml	2	2	2	2	2	2	1	1	2
Temperature	RT	RT	RT	80°C	80°C	80°C	RT	RT	RT
Compound **5**,mg (mmol)	20 (0.01)	20 (0.01)	20 (0.01)	20 (0.01)	20 (0.01)	20 (0.01)	10 (0.005)	10 (0.005)	20 (0.01)
mPEG tosylate, mg (mmol)	13 (0.0067)	73 (0.037)	133 (0.067)	13 (0.0067	73 (0.037)	133 (0.067)	7 (0.0033)	37 (0.018)	133 (0.067)
Reaction time	1d	1d	3d	1d	1d	1d	1d	1d	3h



(a) Mw, compound **5** = 19200 g/mol

(b) Mw, compound **6** = 135693 g/mol

Figure 30: PEGylation compound **5**. Illustration of the chemical reaction of compound **6** with Mw. Subfigures: (a) and (b).

5 Recommendations for further studies

By altering parameters and performing the reactions under controlled conditions, a determination of vital parameters can be made. The repeatability of both the synthesis and PEGylation is satisfactory. Optimization of the conditions and identifying their respective impact on the synthesis and on the PEGylations is a good place to start.

6 Acknowledgements

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References

(*1*) Sharma, A., Kontodimas, K., and Bosmann, M. (2021). Nanomedicine: A Diagnostic and Therapeutic Approach to COVID-19. *Frontiers in Medicine 8*, 1–11.

(*2*) Salata, O. (2004). Applications of Nanoparticles in Biology and Medicine. *Journal of Nanobiotechnology 2*, 1–3.

(*3*) (2021). Let's Talk about Lipid Nanoparticles. *Nature Reviews Materials 6*, 1–99.

(*4*) Gascón, A. R., del Pozo-Rodríguez, A., and Ángeles Solinís, M. In Martin-Molina, F., Ed.; Gene Therapy - Tools and Potential Applications; InTech: 2013, p 756.

(*5*) Kim, K., Chen, W. C., Heo, Y., and Wang, Y. (2016). Polycations and Their Biomedical Applications. *Progress in Polymer Science 60*, 18–50.

(*6*) Pack, D. W., Hoffman, A. S., Pun, S., and Stayton, P. S. (2005). Design and Development of Polymers for Gene Delivery. *Nature Reviews Drug Discovery 4*, 581–593.

(*7*) Armstrong, B. Why mRNA Vaccines aren't Gene Therapies - Genomics Education Programme, Genomics Education Programme, 2021.

(*8*) Suk, J. S., Xu, Q., Kim, N., Hanes, J., and Ensign, L. M. (2016). PEGylation as a Strategy for Improving Nanoparticle-Based Drug and Gene Delivery. *Advanced Drug Delivery Reviews 99(Pt A)*, 28–51.

(*9*) Monnery, B. D., Wright, M., Cavill, R., Hoogenboom, R., Shaunak, S., Steinke, J. H., and Thanou, M. (2017). Cytotoxicity of polycations: Relationship of Molecular Weight and the Hydrolytic Theory of the Mechanism of Toxicity. *International Journal of Pharmaceutics 521*, 249–258.

(*10*) Mohapatra, A., Uthaman, S., and Park, I.-K. In Gajbhiye, V., M. Paknikar, K., and Kesharwani, P., Eds.; Polymeric Nanoparticles as a Promising Tool for Anti-cancer Therapeutics; Academic Press: 2019, pp 205–231.

(*11*) DGMS Fragmentierung - Informationen Der DGMS, Deutschen Gesellschaft Für Massenspektrometrie (The German Society for Mass Spectrometry), 2017.

(*12*) Crotty, S., Gerişlioğlu, S., Endres, K. J., Wesdemiotis, C., and Schubert, U. S. (2016). Polymer Architectures via Mass Spectrometry and Hyphenated techniques: a Review. *Analytica Chimica Acta 932*, 1–21.

(*13*) Keller, B. O., Sui, J., Young, A. B., and Whittal, R. M. (2008). Interferences and Contaminants Encountered in Modern Mass Spectrometry. *Analytica Chimica Acta 627*, 71–81.

(*14*) Kruve, A., Kaupmees, K., Liigand, J., Oss, M., and Leito, I. (2013). Sodium Adduct Formation Efficiency in ESI Source. *Journal of Mass Spectrometry 48*, 695–702.

(*15*) Gottlieb, H. E., Kotlyar, V., and Nudelman, A. (1997). NMR Chemical Shifts of Common Laboratory Solvents as Trace Impurities. *The Journal of Organic Chemistry 62*, 7512–7515.

(*16*) Dash, A. C., Dash, B., and Praharaj, S. (1981). Hydrolysis of imines: Kinetics and Mechanism of Spontaneous acid-, base-, and Metal ion-induced Hydrolysis of N-salicylidene-2-aminothiazole. *Journal of the Chemical Society, Dalton Transactions 19*, 2063–2069.

(*17*) NMR Sample Preparation | Chemical Instrumentation Facility | Iowa State University, Iastate, 2017.

(*18*) Lim, C.-K., and Lord, G. (2002). Current Developments in LC-MS for Pharmaceutical Analysis. *Biological and Pharmaceutical Bulletin 25*, 547–557.

(*19*) Cytiva. Fundamentals of Size Exclusion Chromatography, Cytiva, 2022.

(*20*) Jose, N., Deshmukh, G., and Ravindra, M. (2019). Dynamic Light Scattering: Advantages and Applications. *Acta Scientific Nutritional Health 3*, 1–3.

(*21*) Saha, D., Testard, F., Grillo, I., Zouhiri, F., Desmaele, D., Radulescu, A., Desert, S., Brulet, A., Couvreur, P., and Spalla, O. (2015). The Role of Solvent Swelling in the self-assembly of Squalene Based Nanomedicines. *Soft Matter 11*, 4173–4179.

(*22*) Nova. Why Is Ionic Strength Relevant to DLS Measurements?, Brookhaven Instruments, 2020.

Appendices

A Details of experiment

A.1 Synthesis

Step 1 of the synthesis started off with reactions B1 and C1. To improve and optimize the synthesis reaction A1 was made, this was after reactions B1 and C1 had reached step 3. The usage of different solvents for dissolving and, the allylating reaction of compound **1** are the following: DMF was used in reaction C1 both for dissolving and for the reaction to take place in. This is due to the fact that EtOAc would react through Claisen condensation with the deprotonated hydroxyl groups of compound **1**. By doing so EtOAc would form EtOAc anions that interfere with and hinder the base catalyzed reaction aimed for reaction. EtOAc was used for the following steps of reaction "C" due to the reproductive toxicity of DMF. Acetone was used in reaction B1, to dissolve and dry compound **1**, later evaporated and replaced by DCM in reaction "B". This proved to be an unnecessary step because DCM could be utilized in the drying step as well. That is why DCM was used all throughout reaction "A".

In the second step of the synthesis reaction A2 uses the remaining product from reaction A1 after samples have been taken for analysis. The product gained from reaction C1 are divided into C2a and C2b, correspondingly using 23 and 38 %. Reaction B2 was made in conjunction with C2a, which were the initial trial synthesizers. Reaction B2 were not analyzed. This was because it was deemed unnecessary to do so when C2a was already being analyzed. The different solvents were not taken into account. Reaction C2b was made after reaction A1 had reached step 3.

In the third and last step of the synthesis reaction A3 uses all remaining product from reaction A2 after samples have been taken for analysis. Reaction C3a using a tenth of the volume from reaction C2a and reaction B3 yields no sought after product. Reaction B3 does yield a precipitate of benzyl ammonium dichlorobenzoic acid which was mistaken for the sought after product. By centrifuging, filtering and evaporating the solvent the sought after product was lost. Reaction C3b uses the remaining product from reaction C2b after samples have been taken for analysis. The remaining product from reaction C2a not used in the initial trial and saved for analysis are used for reaction C3c.

A.2 PEGylation

The PEGylation was approached using different conditions. The solvent, ion exchange, reaction time and temperature was altered. The PEGylation used Compound **5** as starting material.

B Equipment

^{1}H-NMR
Instrument: Varian Unity INOVA 400 MHz
Method: 1D 1H 25C d6-DMSO/D_2O
Preparations: After evaporating the solvent, the samples were taken as a solid and then diluted in d6-DMSO (70 µl). ^{1}H-NMR was performed on the following reactions of the synthesis: A1, B1, C1, A2, C2a, C2b, A3 (both washed and unwashed), C3b and C3c. ^{1}H-NMR was also performed on following reaction groups of the PEGylation: D, E and F. Group F was diluted in D_2O and then sent to ^{1}H-NMR. All spectra have 1.0 exponential apodization to clean the processed spectra.

LC-MS
Instrument: API 3200, LC-20AD, SIL20A, SHIMADSU
Method: 8.5 min, 0.4 mL/min, 100-1000 nm, 40 degrees
Mobile phase: 0.1 % formic acid in H_2O + 0.1 % formic acid in acetonitrile
Column: Agilent Poroshell EC C18, 2.7µm, ID 3 mm, l 100mm
Preparations: To perform LC-MS a method of decomposition in methanol methoxide was used. LC-MS was performed on following synthesis reactions: A1, C1, A2, C2b, A3, C3b and C3c. LC-MS was also performed on following PEGylation series: D, E and F.

SEC
Instrument: YL9100 HPLC System, KOVALENT AB
Method: 40 min, 0.7 mL/min, 34 bar, 220-350 nm, RT
Mobile phase: 50mM NH_4OAc + 90:10 H2O:MeOH, pH 9
Column: Superose 6 Increase 10/300 GL (pH = 3-12), Tricorn Glass Column
Preparations: The samples were diluted in the mobile phase to 1 mg/ml. SEC was performed on mixtures B3 and C3a.

DLS
Instrument: Zeta Sizer, MALVERN INSTRUMENTS
Preparations: To analyze the samples in DLS, several preparations were performed. Reaction groups D and E were spin filtered with a 10k centrifugal filter. The top solution from reaction groups D and E was diluted with the solvent according to table 21 to a concentration of 2 mg/ml. To all series KNO_3 (10 mg, 0.0989 mmol) was added. Reaction groups D, E and F were filtered through a 0.2 µm syringe filter and analyzed in the solvent used in corresponding reaction according to tables 21.

Cytotoxicity
Reagents:
1. RAW264.7 cells
2. RPMI-1640 + 10 % FBS, Na-pyruvate
3. DMSO 25, 10, 5, 2.5, 1, 0.1 % (positive cell death control)
4. Cell Titer Blue reagent (Promega)

Preparations: The samples were diluted in water to a concentration of 10 mg/mL and sent to the cytotoxicity lab.

TLC

Sigma Aldrich - Silica gel on TLC Al foils
Preparations: The mobile phase (500 ml) for TLC was prepared in a large volume due to other tests being performed within the lab. The mobile phase contained DCM (450 ml), MeOH (50 ml) and acetic acid (5 drops). Reference samples of 3-chlorobenzoic acid and benzylamine were diluted in the solvent used in the corresponding reaction mixture from tables 3, 4 and 5.

Filters
Amicon Ultra - 0.5 ml, centrifugal filters, 10K
Amicon Ultra - 0.5 ml, centrifugal filters, 3K

Needles
100 Sterican - 1.20 x 40 mm
100 Sterican - 0.8 x 120 mm

Centrifuge
Thermo IEC - Medilite

C Limitations analytical methods

C.1 ^{1}H-NMR

Sensitivity and the concentration of samples administered is a limitation of this method (*17*). The ^{1}H-NMR spectra give both qualitative and quantitative information about the sample, including mixtures of starting material and the expected product. The exact structure of the sample could be difficult to interpret in some complex samples, even with a reference-spectra. Calculating the shift between different solvents that the sample is prepared in is also limiting. An advantage with ^{1}H-NMR is the simplicity to remove unrelated signals within the samples.

C.2 LC-MS

The method is both qualitative and quantitative, and provides exact data of the molecular weight of the compounds within the sample (*18*). The instrument has high sensitivity, which allows the samples to have a low concentration. A disadvantage of this method is that contaminants and impurities within the sample are analyzed and part of the mass-spectra, which can be difficult to separate from the wanted product (*13*).

C.3 SEC

This instrument has high sensitivity and separates the sample based on size, which is useful for samples with large polymers (*19*). It is efficient when the tested sample can be compared with standard references that have a known molecular weight. The limitation of this method is the lack of information about the exact data of structures and molecular weights within the sample.

C.4 DLS

The instrument is highly sensitive to temperature and viscosity variations of the measurement (*20*). Due to the fact that scattering intensity is proportional to the 6th power of the size of the macromolecules, presence of aggregates will affect the measurements negatively (*21*). Controlling the ionic strength of the sample, using salts (e.g. KNO_3), to collapse the electrical double layer and simultaneously suppress particle-particle collisions by dilution are proven to aid the analysis (*22*).

D Calculations

D.1 Molecular weights

Mw (Compound **2**) = Mw (Compound **1**) - (Mw (H^+) × 64) + (Mw (C_3H_6) *64) = 9887 g/mol
Mw (Compound **3**) = Mw (Compound **2**) + (Mw(O^-) × 64) = 10911 g/mol
Mw (Compound **4**) = Mw (Compound **3**) + (Mw($C_6H_{11}N$) × 64) = 17800 g/mol
Mw (Compound **6**) = Mw (Compound **5**) - (Mw (H^+) × 64) + (Mw (C_2H_4O) × 41 + Mw(CH_3)) × 64 = 33804 g/mol

D.2 Synthesis

n(allyl bromide) = n(Compound **1**) × 64
n(tBTMG) = n(allyl bromide) × 1.1
n(mCPBA) = n(Compound **2**) × 64 (+ additional for abundance)
n(benzylamine) = n(Compound **3**) × 64 (+ additional for abundance)

D.3 PEGylation

A and B trials:
n(PEG) = n(Compound **5**) × 16
m(tBTMG) = m(PEG) × 1.1

C and D trial:
n(PEG) = n(Compound **7**) × 64
n(tBTMG) = n(PEG) × 1.1

E trial:
n(PEG) = n(Compound **7**) × 64

E Structure Compound 7

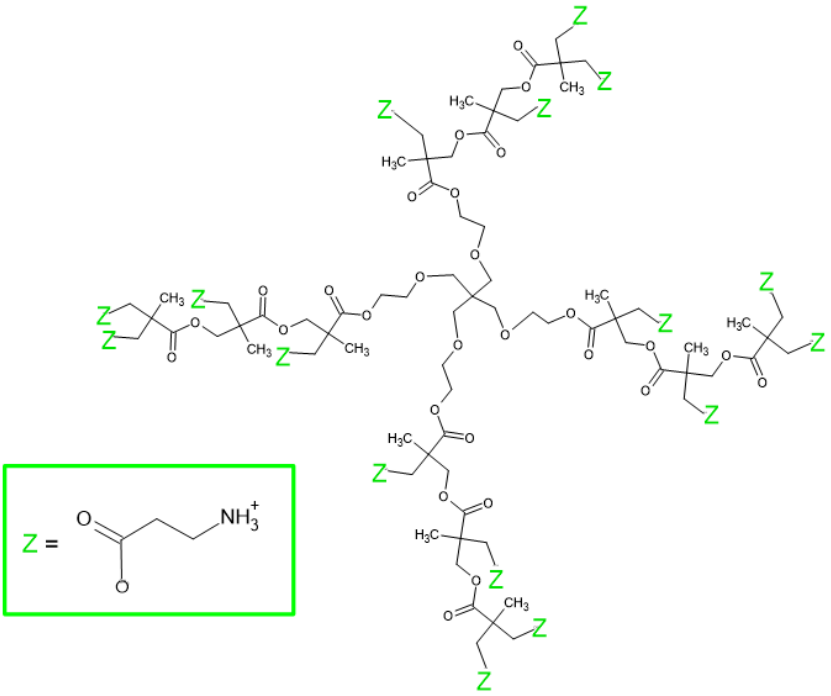

Figure 31: Idealized chemical structure of Compound **7** in 2D.