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Chemical Modifications of Hyaluronan using DMTMM-Activated Amidation

Sara Rydergren

UPPSALA
UNIVERSITET

Teknisk- naturvetenskaplig fakultet
UTH-enheten

Besöksadress:
Ångströmlaboratoriet
Lägerhyddsvägen 1
Hus 4, Plan 0

Postadress:
Box 536
751 21 Uppsala

Telefon:
018 – 471 30 03

Telefax:
018 – 471 30 00

Hemsida:
http://www.teknat.uu.se/student

Abstract

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An alternative approach to chemically modifying hyaluronan (HA) has been investigated. The triazine derivative 4- (4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium (DMTMM) has been used to activate carboxylic groups on HA, which react further to form stable amide bonds with primary and secondary amines. The reaction can either be used to couple monoamines to HA or to produce hydrogels by using diamines that form crosslinks between the HA chains. The reaction between HA and DMTMM has been investigated and optimized in regard to degree of substitution (DS). Analysis using SEC-LC-UV demonstrated that the reaction was successful in coupling benzylamine to HA with a DS of 40%. Gel formation was successful using hexamethylene diamine as a crosslinker. Results also show that the reaction can be controlled by either the DMTMM or the amine concentration so that a specific degree of substitution or crosslinking is achieved. The stability of DMTMM has also been examined, and degradation studies of DMTMM in H_2O at 50 °C with ^1H NMR analysis show that 11% of the starting material remains after 48 hours. The reaction has proven to be an effective alternative to other modification methods with cheap reagents, simple procedures and the ability to control the amount of modification. Further investigations are nonetheless required in order to determine the full potential the DMTMM-activated amidation of HA.

Handledare: Anders Karlsson
Ämnesgranskate: Gunnar Johansson
Examinator: Adolf Gogoll
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Sammanfattning

Hyaluronan (HA) är en polysackarid som förekommer naturligt i kroppen, där den ingår i den extracellulära matrisen. HA är bland annat viktigt för embryoutveckling och sårläkning, och ger vävnader deras mekaniska egenskaper. Genom att binda upp stora mängder vatten kan HA bilda hydrogeler och andra viskoelastiska lösningar som kan användas inom vävnadsteknik och för leverans av läkemedel. Den naturliga formen av polysackariden kan modifieras kemiskt för att ge det färdiga materialet specifika egenskaper, och modifikationerna går antingen ut på att koppla på nya grupper på HA eller skapa tvärbindningar mellan kedjorna. Eftersom HA bryts ned naturligt i kroppen av hyaluronidas behövs tvärbindningar för att öka livslängden hos injicerbara geler. Implantatens hållbarhet kan ökas från timmar till flera månader med hjälp av några enkla modifieringar.

I detta arbete har fokus legat på modifieringar av HA med hjälp av ett triazin-baserat reagens som kallas DMTMM (4-(4,6-dimetoxy-1,3,5-triazin-2-yl)-4-metylmorpholinium). DMTMM aktiverar karboxylsyragrupperna på HA och reagerar vidare med bland annat aminer för att bilda stabila amidbindningar. Reaktionen kan användas både för att koppla på aminer på HA och för att skapa tvärbindningar mellan HA-kedjorna med hjälp av diaminer. I nuläget används andra reagens för att tvärbinda HA, bland annat epoxider och divinylsulfon, medan DMTMM-reaktionen fortfarande är förhållandevis outforskad.

Reaktionen med HA och DMTMM har under arbetets gång undersökts och optimerats med avseende på hur mycket amin som substituerats på karboxylsyragrupperna på HA. Resultaten visar att både substitutions- och tvärbindningsreaktionerna varit framgångsrika och att substitutionsgraden beror på parametrar som pH, val av lösningsmedel och relativa koncentrationer av HA, DMTMM och amin.

Sammanfattningsvis är reaktionen ett möjligt alternativ till andra tvärbindningsmetoder som används på HA idag och är relativt billig, snabb och kontrollerbar. Det krävs dock vidare undersökningar för att avgöra precis hur användbar reaktionen är och för att utvärdera egenskaperna hos de geler som bildats.

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1 List of abbreviations

ACN	Acetonitrile
CDMT	2-chloro-4,6-dimethoxy-1,3,5-triazine
DMTMM	4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium
DMSO	Dimethyl sulfoxide
DS	Degree of substitution
EDCI	1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide
HA	Hyaluronan
HPLC	High performance liquid chromatography
NMM	N-methylmorpholinium
NMR	Nuclear magnetic resonance
SEC	Size exclusion chromatography
THF	Tetrahydrofuran
UV	Ultra-violet

2 Introduction

2.1 Background

Hyaluronic acid (HA), also referred to as hyaluronan, is a naturally occurring polysaccharide with remarkable viscoelastic properties. HA is a component of the extracellular matrix in the body and is a vital element for embryonic development, wound healing and for the biomechanical properties of tissues [1,2]. When chemically modified, HA can take many different physical forms, including viscoelastic solutions, hydrogels, flexible sheets, and nanoparticulate fluids. The modifications can alter the properties of the material leading to changes in hydrophobicity or biological activity, and the products are used in a range of clinical and preclinical applications. [3]

Chemical modifications can be made on the functional groups of HA by either performing a coupling of specific substances to the polysaccharide chain or by creating crosslinks between the chains. The crosslinking of HA is necessary in order to form injectable hydrogels since non-modified HA solutions are rapidly degraded in the body. The tissue half-life of HA-based implants is increased from hours or days to months or even years with the appropriate modifications [2,4]. Common modification reactions include; carbodiimide-mediated reactions, bis-epoxide crosslinking and crosslinking using divinyl sulfone. [5]

An alternative and relatively unexplored approach to modifying and crosslinking HA is the use of triazine-based reagents. The 1,3,5-triazine ring (Figure 1) can be modified with substituents on the carbon atoms, such as chlorine and methoxy groups to form carboxylic acid-activating reagents. In 1985, Kaminski et al. established that triazine-based activating species could be used for preparing reactive intermediates from carboxylic acids which under further treatment with amines and alcohols generated the corresponding amides and esters. [6]

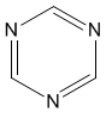


Figure 1 1,3,5-triazine.

The triazine derivative 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium, or DMTMM (Figure 2), effectively activates carboxylic acids. The reagent is commercially available, inexpensive and allows for a one-pot synthesis of amides and esters. Modifications of HA using this specific reagent will be the main focus of this thesis.

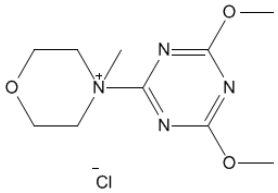


Figure 2 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium (DMTMM)

2.2 Aim of thesis

The aim of this thesis is to investigate the DMTMM-activated amidation of hyaluronic acid and to optimize the reaction in regard to degree of substitution of amines on the carboxylic groups on HA. The optimization will focus on parameters such as pH, temperature, time, reagent concentrations and solvent. The results from the investigation are to be applicable on the reaction of HA with diamines in order to create crosslinked hydrogels.

3 Theory

3.1 Hyaluronic acid

HA is a linear glucosaminoglycan (GAG) that consists of alternating units of glucuronic acid and N-acetyl-glucosamine (Figure 3). The molecular weight of the polysaccharide can vary from 10^3 to 10^7 Da and the ease with which the chain length can be regulated allows for a range of different properties and applications. [7]

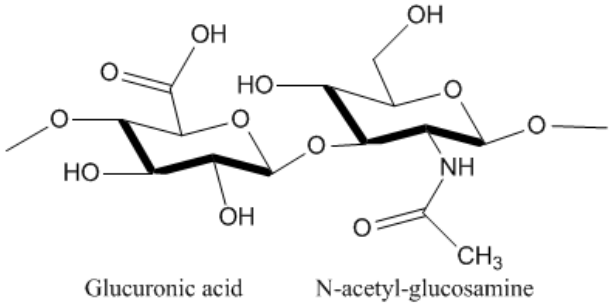


Figure 3 Disaccharide of hyaluronic acid

Due to its highly negative charge, HA attracts positive ions which creates an osmotic imbalance. This imbalance allows HA to retain water, making it ideal for producing hydrogels and other extracellular matrix surrogates. [2]

HA associates with cell-surface receptors and helps regulate cell movement and adhesion. It can also bear compressive loads in tissues and joints *in vivo*, and provides lubrication to articulating surfaces. These features can be utilized in a variety of applications, including tissue regeneration and tissue engineering [7]. HA can also be used for the design and delivery of drugs and DNA, producing slow-release hydrogel formulations. [1,7]

3.1.1 HA hydrogels

High concentrations of high molecular weight HA can form viscoelastic molecular networks. However, these networks lack long-lasting mechanical integrity, so in order to obtain HA-gels with specific and predictable properties, controlled chemical modification and crosslinking are necessary. [2]

HA is rapidly degraded by hyaluronidase in the body and non-crosslinked solutions have tissue half-lives ranging from hours to days [2]. The durability of HA hydrogels, however, can extend to months or even years in the body [4]

3.1.2 Chemical modifications

The many chemical modifications of HA have been reviewed comprehensively since its discovery nearly four decades ago, and mainly target three functional groups: the carboxylic acid group, the primary and secondary hydroxyls and the N-acetyl group [3]. The most common methods of modification of the carboxylate group are carbodiimide-mediated reactions, esterification and amidation. The hydroxyls have regularly been modified by etherification, esterification and crosslinking using divinyl sulfone or epoxides. Modifications of the N-acetyl groups are usually performed after deamidation. [3]

The focus in this thesis will be on modifications of the carboxylic group on the glucuronic acid part of HA, and specifically on the formation of amide bonds in this position using triazine-based activating reagents.

3.2 Triazine-based reagents

Triazines are benzene-like rings with three of the carbons replaced with nitrogens. Most common are the triazines with nitrogen atoms in the 1,3,5-positions, often with substituents on the 2,4,6-carbons. One of the main 2,4,6-substituted triazines is cyanuric chloride (Figure 4) where all three substituents are chlorine atoms.

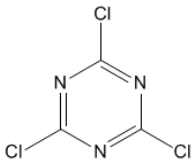


Figure 4 Cyanuric chloride

In 1985, Kaminski began investigating triazine-based reagents for their use in amide and ester synthesis and focused on cyanuric chloride and its derivatives. Cyanuric chloride had previously been used for the preparation of acyl chlorides and peptides. The studies indicated that a partial substitution of the chlorine atoms on cyanuric chloride by methoxy or phenoxy groups altered the original reaction with carboxylic acids. Instead of forming the expected acyl chlorides, the reaction between 2-chloro-4,6-substituted-1,3,5-triazines (Figure 5) and carboxyls afforded highly reactive ester intermediates. Further treatment of the intermediates with alcohols and amines gave the corresponding esters and amides. [6]

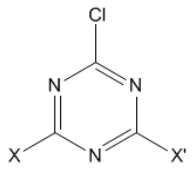


Figure 5 2-chloro-4,6-disubstituted-1,3,5-triazine. X, X'= OCH_3, OC_6H_5

3.2.1 CDMT

According to the early investigations by Kaminski [6] the triazine with two chlorine atoms replaced with methoxy groups, 2-chloro-4,6-dimethoxy-1,3,5-triazine, or CDMT (Figure 6), proved to be more reactive and more efficient in activating carboxylic acids than the other triazines. Kaminski suggested that the absence of the two chlorine atoms and the slower acylation rate of carboxylic acids and alcohol by CDMT compared to cyanuric chloride reduced the risk of potential side reactions, such as the formation of acyl chlorides. [6]

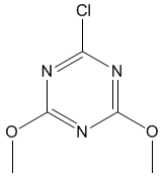


Figure 6 2-choro-4,6-dimethoxy-1,3,5-triazine (CDMT)

CDMT has wide applications as a condensing reagent in peptide chemistry. It is commercially available, but can also easily be synthesized from cyanuric chloride [8]. CDMT is commonly used with a tertiary amine such as N-Methylmorpholine (NMM, see Figure 7) in carboxylic acid-activating reactions. [9,10]

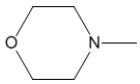


Figure 7 N-Methylmorpholine (NMM).

The reaction where CDMT activates a carboxylic acid depends on two important substitution steps: firstly, where the chlorine atom is replaced by a tertiary amine forming a quaternary ammonium salt (Figure 8), and secondly, the substitution of the amine leaving group by the carboxylate ion, affording a triazine "active ester" (see Figure 9). [9]

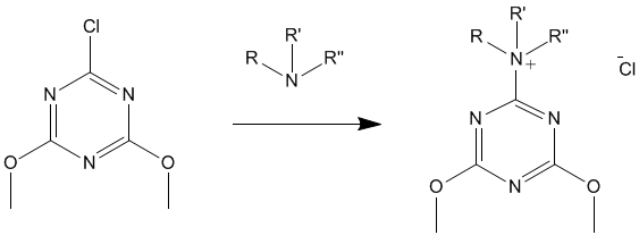


Figure 8 Reaction between CDMT and a tertiary amine.

Kaminski states that the formation of these triazinylammonium salts (such as the one formed between CDMT and NMM) strongly depends on the steric hindrance of the N-substituents on the tertiary amines. More hindered amines tend to be less reactive, and studies have shown that only a few tertiary amines react with CDMT to create effective carboxylic activating reagents. NMM was an efficient candidate, while triethylamine was incapable of activating benzoic acid in the reaction with CDMT. [9]

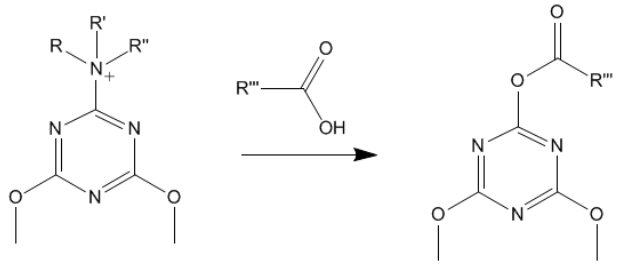


Figure 9 Reaction between the triazinylammonium salt and a carboxylic acid, forming an active ester.

Several publications investigate the use of CDMT in synthesis, though very few have focused on the modification of the carboxylic groups on polysaccharides. Bergman et al. [11], however, presented a method for preparing hyaluronic acid derivatives using triazine-activated amidation. The group successfully reacted a number of primary amines with HA carboxylic groups using CDMT as the activating species. They were also able to control the degree of modification by varying the amount of CDMT in the reaction. [11]

CDMT requires a two-step process to form the active ester, but the triazinylammonium salt formed with NMM in the first step is commercially available and is referred to as DMTMM.

3.2.2 DMTMM

4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium (DMTMM, Figure 10) is in fact the commercially available, inexpensive reaction product of CDMT and NMM. The reagent allows for a simple one-pot procedure in amide and ester synthesis and activates carboxyls as effectively as CDMT in the traditional, two-step process. [12, 13]

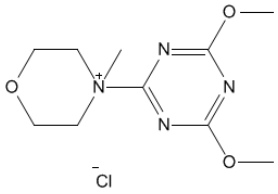


Figure 10 DMTMM

DMTMM is highly water soluble, which makes it a better alternative than CDMT in reactions where a water-based medium is used. DMTMM is also non-toxic and practically allows for complete removal of the products from the post-reaction mixture. [14]

In 2005, Farkas et al. [15] showed that DMTMM was a more efficient activator of carboxylic acids than carbodiimides (EDCI) by demonstrating a markedly higher degree of amidation. DMTMM was proved advantageous compared to carbodiimides due to the higher structural purity of the products as well as the higher reaction yields obtained. DMTMM was also established to be more stable in water than EDCI, which tended to decompose at lower pH. [15]

Very few stability studies have been performed on DMTMM, but Kunishima et al. [16] investigated its stability in a few different solvents over three hours. Little or no decomposition of DMTMM into DMTM (Figure 11) or DMT-OH (Figure 12) was detected in THF, hexane, Et_2O, methanol and water. According to the data, decomposition occurred in chloroform, DMSO and acetonitrile. [16]

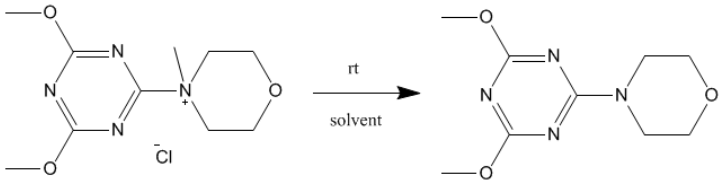


Figure 11 Demethylation of DMTMM to DMTM.

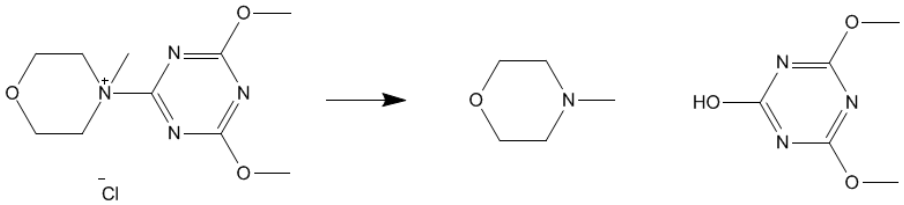


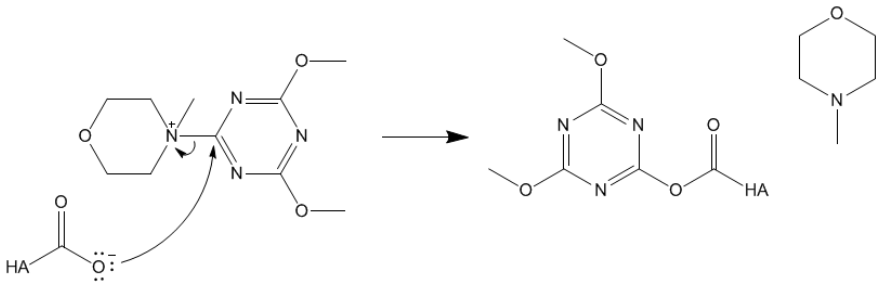
Figure 12 Hydrolysis of DMTMM to NMM and DMT-OH.

3.3 DMTMM reaction mechanism

The mechanism by which the triazine-activated amidation of HA occurs has been presented in various articles. Essentially, the first step is the formation of an active ester which occurs via an aromatic substitution on the triazine by a carboxylic acid. The second step is a nucleophilic attack on the carboxylic carbon by an amine. [13,15]

Step 1: Formation of the active ester

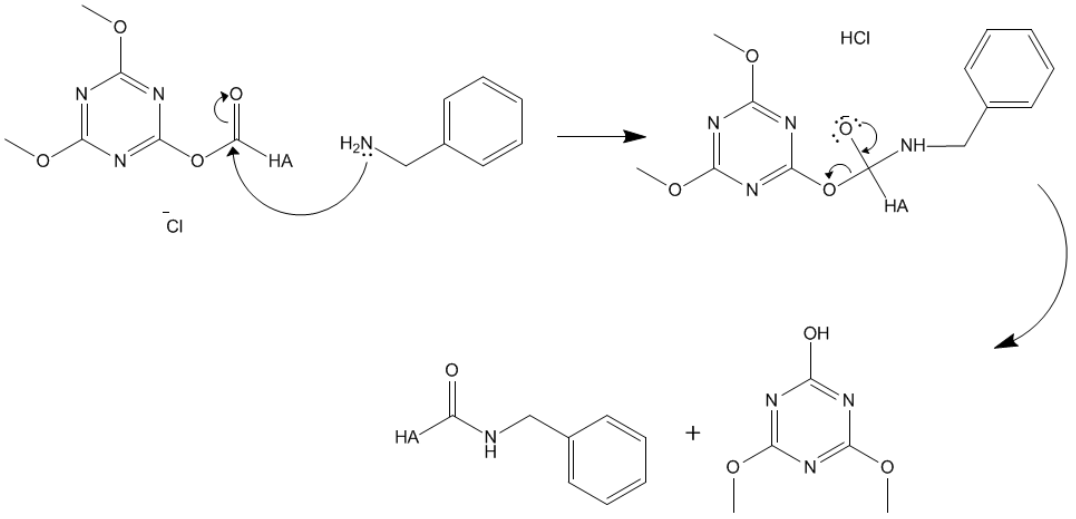
The carboxylic acid group on HA makes a nucleophilic attack on the triazine part of DMTMM. The morpholinium group, NMM, is a good leaving group and according to Montalbetti et al.,[13] a nucleophilic aromatic substitution takes place. An ester bond is formed between HA and DMTMM and NMM leaves. The resulting molecule is referred to as an "active ester" due to its ability to react further and form amides and esters [13]. The mechanism is shown in Scheme 1.



Scheme 1 Formation of the active ester.

Step 2: Nucleophilic attack on the active ester by a nucleophilic amine

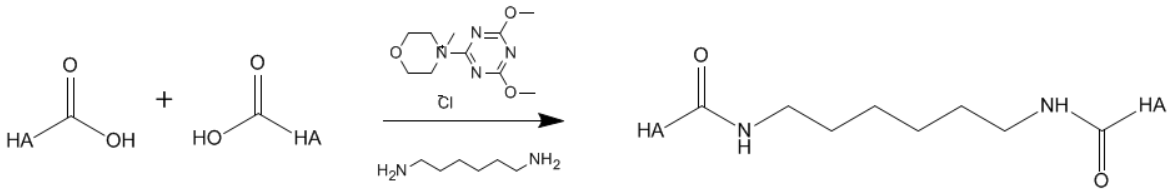
In the second step of the reaction, a nucleophilic amine (demonstrated here with benzylamine) attacks the carboxylic carbon on the active ester to form an amide bond as presented in Scheme 2 [13]. The amine is present in the solution even in the first step, which means that nothing is added after the reaction is initiated.



Scheme 2 Nucleophilic attack on the active ester by benzylamine.

3.4 Crosslinking reaction

By the same mechanism as the coupling reaction using a primary or secondary amine, a diamine can be used to form crosslinks between two carboxyl groups. For instance, if hexamethylene diamine is used along with DMTMM, the reaction in Scheme 3 would presumably occur.



Scheme 3 Crosslinking reaction using DMTMM and hexamethylene diamine on HA.

4 Method

The method was developed and optimized in regard to degree of substitution of the carboxylic groups on HA. The objective was to investigate the DMTMM-activated amidation reaction and to determine which conditions produced the highest degrees of substitution. Finding intervals where the reaction was predictable, reproducible and controllable was also of interest. The main focus was placed on optimization of parameters such as pH, temperature, time, choice of amine, DMTMM concentration, HA concentration, amine concentration and choice of solvent.

4.1 Reaction parameters

The challenges and points of interest for each of the parameters to be optimized in the coupling reactions are accounted for below.

4.1.1 HA solutions

The challenge here was to find the right HA concentration in the right medium. Too viscous solutions may be difficult to work with because of problems with obtaining homogenous mixtures. Very dilute solutions, on the other hand, may result in some modification but it will be hard to obtain any crosslinking with a diamine. Consequently, physical investigations had to be made in order to find HA solutions that were sufficiently, but not excessively, diluted.

The chain length of the polysaccharide in the solutions also had to be taken into consideration, since different masses (or lengths) give the resulting HA material different properties. Longer chains (10^6 Da for instance) will get entangled easier than shorter chains. In other words, lower mass of the original HA used in the coupling reactions should produce more reliable results for the crosslinking reactions.

Finally, HA had to be dissolved in an appropriate medium. Depending on the reaction conditions, the HA solutions had to either be water-based or contain organic solvents and be set to certain pH values. It was important that these conditions were mild enough that HA was not affected and that no crystallization or precipitation occurred in the solutions.

4.1.2 pH

Tests were required to establish the relationship between pH and the degree of substitution. Very acidic or alkaline solutions could cause increased degradation of the HA or the reagents, so pH values between four and eight were investigated.

Another aspect to consider was the solubility of the reactants. Certain coupling reagents were soluble in specific pH regions, but not in others. This meant that the results of the optimization may not reflect the reaction potential correctly by for example presenting a lower DS at a higher pH simply due to precipitation in the reagent mixture.

Adjusting the pH in the reagent solutions proved to be slightly difficult since the pH electrode was too large to be able to measure the value of the 1.5-2 ml mixture. This was resolved by preparing larger volumes of reagent mixture, which is reflected in the experimental procedure where a two-step procedure is used to adjust the pH.

4.1.3 Temperature

Performing the reaction at room temperature is convenient in many aspects, but if the reaction rate could be increased by an increase in temperature that would be preferred. Placing the syringes with reaction mixture in tempered water baths would allow for easy experiments at different temperatures.

4.1.4 Choice of amine

Choosing the right amine for the optimization proved to be a challenge since many of the amines tested either interacted with DMTMM or with HA directly. Other factors to be considered about the amines were; their solubility in phosphate buffer solution (both with and without DMTMM), their detectability in UV, their toxicity, their functional groups (they were not to contain groups that could cause side reactions with HA, DMTMM or with other amines) as well as their commercial availability.

4.1.5 Amine concentration

The challenge in finding an appropriate amine concentration was on the one hand to ensure that the amount of amine did not become a rate determining factor in the reaction, and on the other hand to avoid using an unnecessary excess of reagent. Also, determining whether the reaction could be controlled with precision using the amine concentration was of interest (specifically finding out if the degree of substitution was increased with an increase in amount of amine).

4.1.6 DMTMM concentration

Based on previous attempts, by for instance Bergman et al., [11] the amount of triazine activator in relation to the amount of carboxylic groups to be modified is an effective way to control the degree of substitution. In other words, it would be interesting to see if there was an evident, positive relationship between the DMTMM concentration and the degree of substitution.

4.1.7 Time

Depending on the temperature that the reactions were performed in, a suitable time was chosen so that the reaction had gone to completion.

4.1.8 Solvent

Water-based buffers as reaction media would be ideal, but if organic solvents give a higher degree of substitution, they could be taken into consideration.

4.1.9 Gels

Firstly, it is important to see if hydrogels are actually formed using the reaction with DMTMM and diamine. If this is successful, the next step would be to investigate if the hydrogels produced using the optimized reaction conditions give the materials coveted properties.

5 Experimental

5.1 Materials and equipment

The hyaluronan used in the experiments was provided by Q-Med, a Galderma Division and was from the Streptococcus strain with the molecular weight 170,000 Da. Amines, DMTMM, solvents and other substances were all ordered from Sigma-Aldrich. The analytical balance used was an XS205 Dual range and the pH meter was an SG2-ELK, both from Mettler Toledo. The water bath used was a Grant TX-150. The HPLC apparatus was from Shimadzu and consisted of a system controller (SCL-10A), a liquid chromatograph (LC-10 AD), an auto-injector (SIL-10AD) and a UV-DAD detector (SPD-M10A). The column used was a Tosoh Bioscience TSK-GEL $GMPW_{XL}$ (7.9 x 300 mm). Prior to injection, the samples were filtered through Acrodisc® 0.45 µm membranes. ^{1}H NMR spectra were recorded by Elin Säwen and Lars Nord at Q-Med. The chemical shifts of the signals were adjusted to that of D_2O (δ 4.79).

5.2 Experimental procedure

DMTMM and amine were weighed into a 50 ml Falcon tube and phosphate buffer (1 mM) with a set pH was added. The tube was vortexed for 30 seconds or until the reagents had completely dissolved. The pH of the reagent solution was adjusted with NaOH or HCl. A plastic 10 ml-syringe was filled with 2 ml of reagent solution and another 10 ml-syringe was filled with 6 ml of HA-solution (HA in phosphate buffer (1 mM), adjusted pH). The two syringes were joined by a plastic connector and the reaction was initiated by transferring the solutions back and forth between the syringes until a homogenous mixture was obtained. The syringe containing the reaction mixture was sealed with a plastic stopper and placed in a water bath with a set temperature. After completion of the reaction, the syringe was removed from the water bath and a small amount of reaction mixture was squeezed from the syringe into a 50 ml Falcon tube. The sample was diluted with H_2O to terminate the reaction and to reach an appropriate concentration for HPLC analysis. The diluted sample was finally transferred to a 1,5 ml-HPLC-vial and cooled to 4ºC before injection.

5.2.1 Crosslinking reactions

The same experimental procedure was applied for the gel formation reactions, except that instead of an amine, a diamine was added to the reagent solution. Also, the sample was analyzed by particle size reduction and swelling in water and HPLC was not used as an analysis method.

5.3 Evaluation and analysis

5.3.1 LC-SEC-UV

Size exclusion chromatography (SEC) is a common method for evaluating polysaccharides since larger molecules are eluted first and smaller substances are delayed in the pores of the packing material in the column. The elution time depends on the pore size of the packing material; smaller molecules fit into the pores (delaying their passage) while larger molecules pass by unaffected. SEC has proven to be especially efficient for the analysis of coupling of groups on HA since the focus of the evaluation is on the HA peak (which is conveniently separated from other substances in the sample).

The mobile phase used for all the HPLC runs was a 10 mM phosphate buffer, pH 7 with 80 mM Na_2SO_4. The flow rate was set to 0.50 ml/min and the injection volume was typically 100 µl. All samples were diluted to HA concentration 2.5 mM prior to injection.

UV is frequently used as a detection method in conjunction with HPLC. Analysis is both convenient and flexible due to the possibility of selecting specific wavelengths in the range 200-400 nm, depending on the target of the analysis. The UV spectra of the compounds used can be found in Appendix II. HA has no specific absorption maxima, but absorbs well in the 205nm region and very little above 230nm. DMTMM has maxima at 230 and 235nm, but absorbs relatively well above that region as well. Benzylamine, which was the most commonly used amine in the experiments, has a slight maximum at 255nm.

5.3.2 Degree of substitution

The degree of substitution (DS) refers to the amount of amine that is coupled to HA compared to the total amount of carboxylic groups on HA. In order to evaluate how much of the amine that had been coupled to HA, the area of the HA peak in the HPLC chromatogram was determined at a specific wavelength where the amine had an absorption maxima and where HA had little or no absorption (see Figures 13 and 14 where benzylamine is given as an example). The area was then compared to a standard curve of that specific amine and converted into a corresponding amine concentration. That concentration was then related to the concentration of HA in the sample to obtain the degree of substitution by Equation 1.

$$\mathrm{DS} = \frac{\text{Calculated amine concentration (mM)}}{\text{Total concentration of COOH (mM)}} \qquad \text{(Equation 1)}$$

Reference samples with only DMTMM and HA were also included in the experiments in order to disregard any contributions arising from DMTMM coupling to HA. The area of the HA peak for these reference samples was integrated at 254 nm and subtracted from the area for the amine-HA-DMTMM samples. All chromatograms can be found in Appendix III.

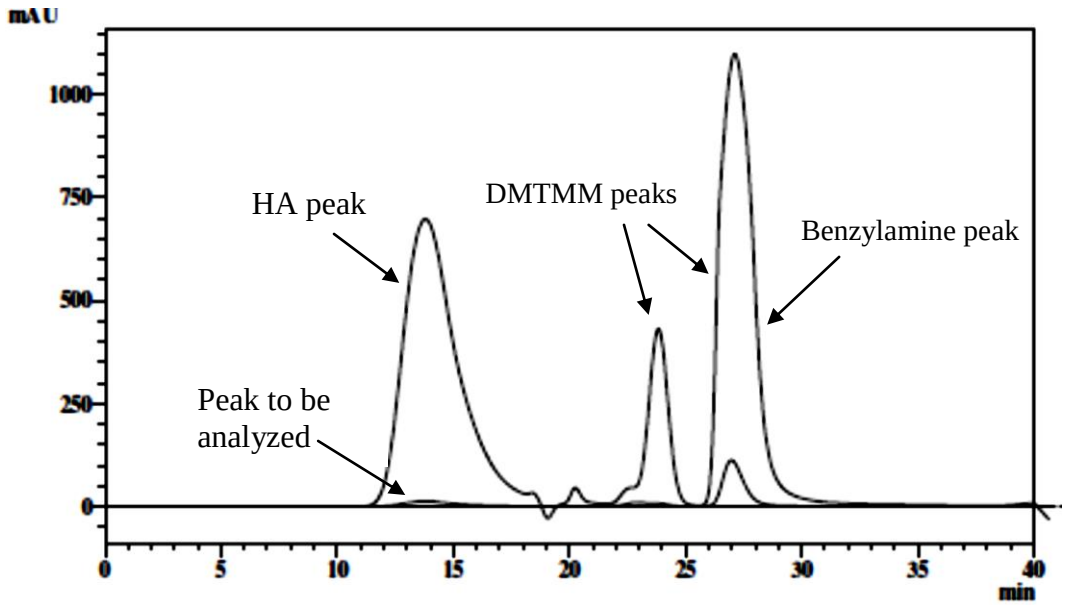


Figure 13 Chromatogram of HA-DMTMM-benzylamine reaction at 205nm. Indicated under the HA peak is the peak to be analyzed at 254nm corresponding to the carboxyls substituted by benzylamine.

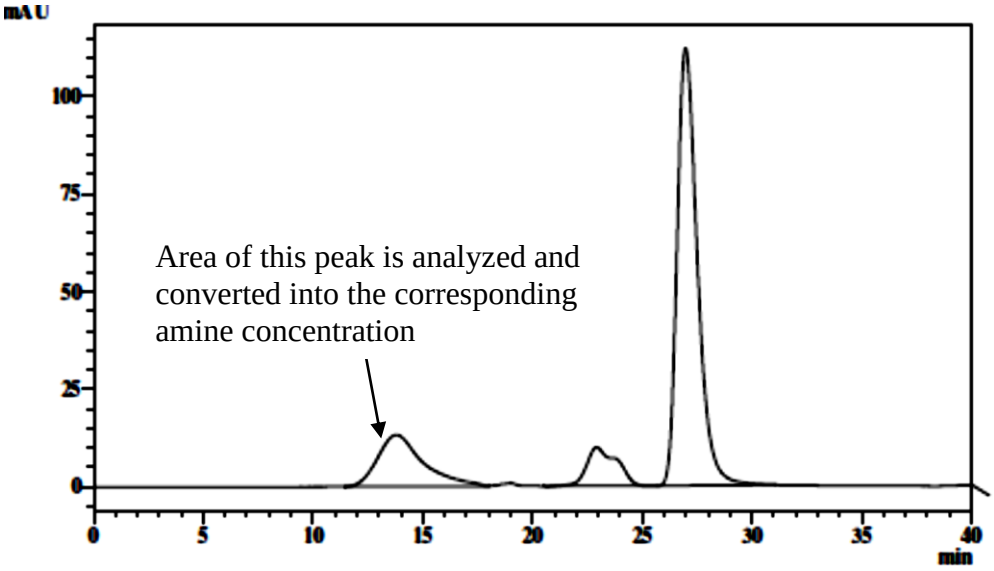


Figure 14 Chromatogram of HA-DMTMM-benzylamine reaction at 254nm.

5.3.3 Evaluating the amines

To investigate the solubility of each amine, the substances were dissolved to approximately 25 mg/ml in phosphate buffer (1 mM) and any visible precipitation was recorded. If the substance proved to be soluble in this first step, it was dissolved in buffer solution along with DMTMM to see if any changes in solubility or precipitation would occur. In order to determine whether the amine used reacted with DMTMM, the two substances were dissolved in phosphate buffer pH 6 (1 mM) and injected into the HPLC, both separately and in the same vial. If any other peaks than those corresponding DMTMM or the amine were observed in the combined sample, it was concluded that some form of side reaction had taken place.

To confirm that no reaction had taken place between DMTMM and an amine, exact amounts of each substance was injected, first separately and then together. The areas of the two separate substances should correspond to the area of the combined peaks.

5.3.4 Evaluating the gels

To establish if a gel had been formed in the reactions with diamines, visual and tactile observations were made. Essentially, the solution was considered a gel if it was viscous enough to no longer behave like a liquid and maintained its mechanical integrity even when the syringe was tilted or shaken. Also, when the gel was swelled in water, it absorbed the water and dispersed into particles when a small amount was placed in a Petri-dish filled with water and gently shaken.

6 Results

6.1 Amines tested

An abundance of different amines were tested before benzylamine was chosen as the best candidate. A list of requirements had to be fulfilled in order for the amine to be suitable for synthesis and analysis. As mentioned previously, the amine had to be soluble in water-based phosphate buffer in pH 6-8 and it had to be distinguishable in UV-detection (with a specific absorption maxima or absorption in a region where neither HA or DMTMM absorbed). The amine also had to couple to HA by a stable amide bond and should not react with DMTMM directly. Only benzylamine (Figure 15) fulfilled these requirements, and all of the candidates are accounted for in Appendix I.

Benzylamine was, as mentioned, the best candidate for use in the coupling reactions. It passed the tests described in section 4.3.3, confirming that no reaction had taken place between the amine and DMTMM and that no solubility problems were observed.

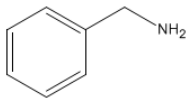


Figure 15 Benzylamine

6.2 Optimized parameters

In this section, the results of the optimization attempts are demonstrated in their separate categories and are illustrated in graphs showing the degree of substitution (DS) as the response. The methods used to calculate the DS values are accounted for in section 4.3. The concentrations of DMTMM and amine are referred to as equivalents to HA, where HA is one molar equivalent (since each disaccharide of HA contains one carboxylic acid group to be modified). HA concentrations are given in % where 1% (w/v) is 10 mg of dry HA/ml phosphate buffer.

6.2.1 pH

Results show that the highest degree of substitution was afforded when the reaction was performed at pH 6 (Figure 16). It should however be noted that the pH of the reaction dropped to approximately pH 5 at the end of the reaction, despite the adjustments that were made prior to starting the reaction. Also, the lower DS observed for pH 8 may be caused by a decrease in solubility of the reagents at pH above 7.

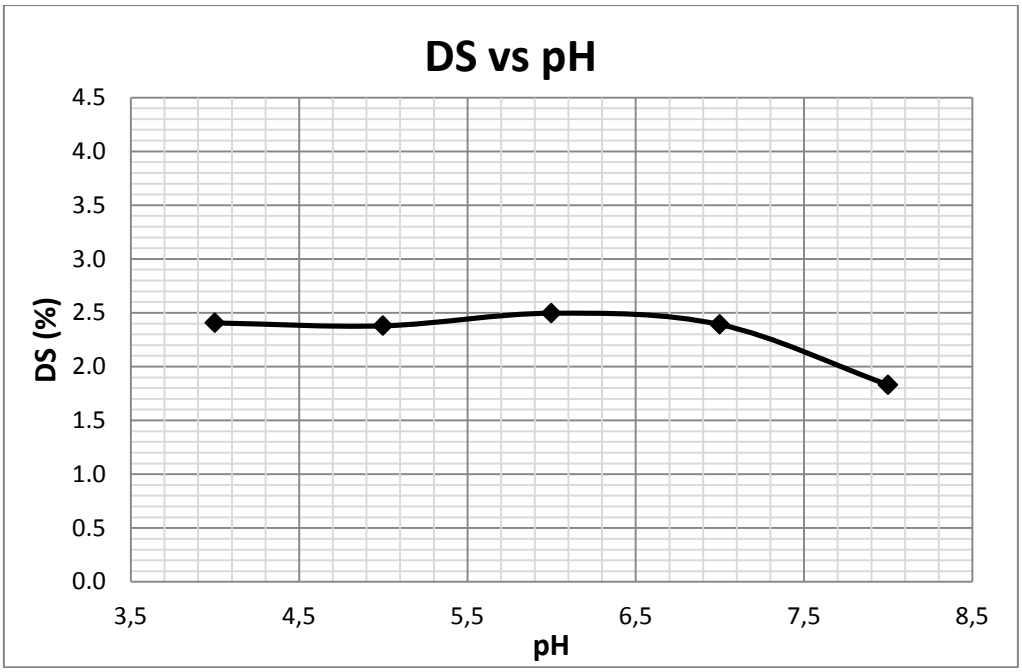


Figure 16 Degree of carboxyls on HA that have been substituted by benzylamine as a function of pH 4-8 in phosphate buffer (1 mM). Reaction conditions: 3.5% w/v HA-solution, 2 equivalents of benzylamine to HA, 0.5 equivalents of DMTMM, 70 ºC, 2 h.

6.2.2 Temperature

As Figure 17 shows, the reaction rate is drastically increased with an increase in temperature. A degree of substitution of approximately 40% is reached earlier for reactions performed at 50 and 70 ºC than at room temperature (23 ºC). Although it is evident that the reaction eventually proceeds in room temperature, it is completed after one hour in 70 ºC which saves both time and resources.

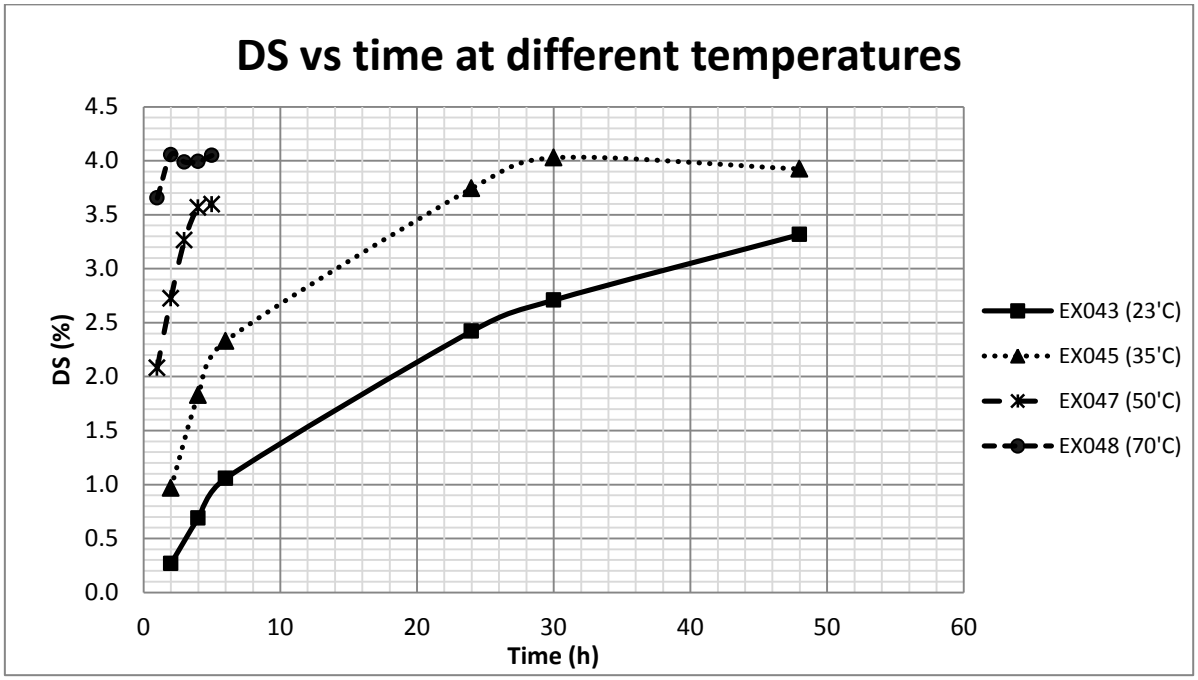


Figure 17 Degree of carboxyls on HA that have been substituted by benzylamine as a function of time for temperatures 23, 35, 50 and 70 ºC. Reaction conditions: 3.5% w/v HA-solution, 2 equivalents of benzylamine to HA, 0.5 equivalents of DMTMM to HA, pH 6 in phosphate buffer (1 mM).

6.2.3 Time

When the reaction was carried out in room temperature, it would take at least two days before the products could be analyzed. Working at higher temperatures was therefore a necessity to save time. To ensure that the reactions were completed, the syringes were removed from the 70 ºC water after two hours, which was a convenient time span in a practical regard. Reactions could be started and analyzed in the same day.

6.2.4 DMTMM concentration

A near-linear relationship between the degree of substitution and the DMTMM concentration was observed when less than one equivalent of DMTMM to HA was used. Essentially, higher concentrations of DMTMM afforded higher degrees of substitution. Figure 18 shows the relationship by comparing the molar equivalents of DMTMM to the degree of substitution calculated from the corresponding peak areas in the chromatograms. Concentrations above one equivalent of DMTMM led to precipitation of the reagents (and possibly of the HA) in the syringe. 1.5, 2 and 3 equivalents of DMTMM were tested, but all resulted in heavy precipitation in the syringes as the reaction progressed. Consequently, the results showed very low DS and are not included in the graph.

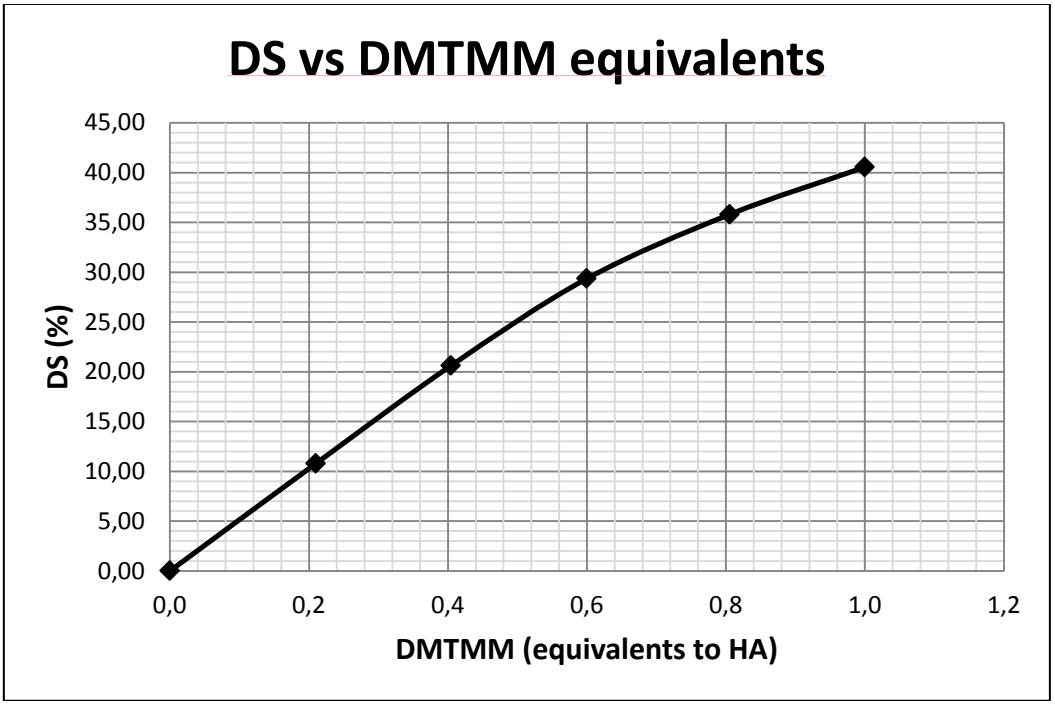


Figure 18 Degree of carboxyls on HA that have been substituted by benzylamine as a function of equivalents of DMTMM to HA. Reaction conditions: 3.5% w/v HA-solution, 2 equivalents of benzylamine to HA, pH 6 in phosphate buffer (1 mM), 70 ºC, 2 h.

6.2.5 Amine concentration

As is shown in Figure 19, higher concentrations of benzylamine gave higher DS, up to 35% where the curve levels out. The amine concentration is given in equivalents compared to HA. It is evident that it is redundant to use an excess of amine in order to achieve a higher DS.

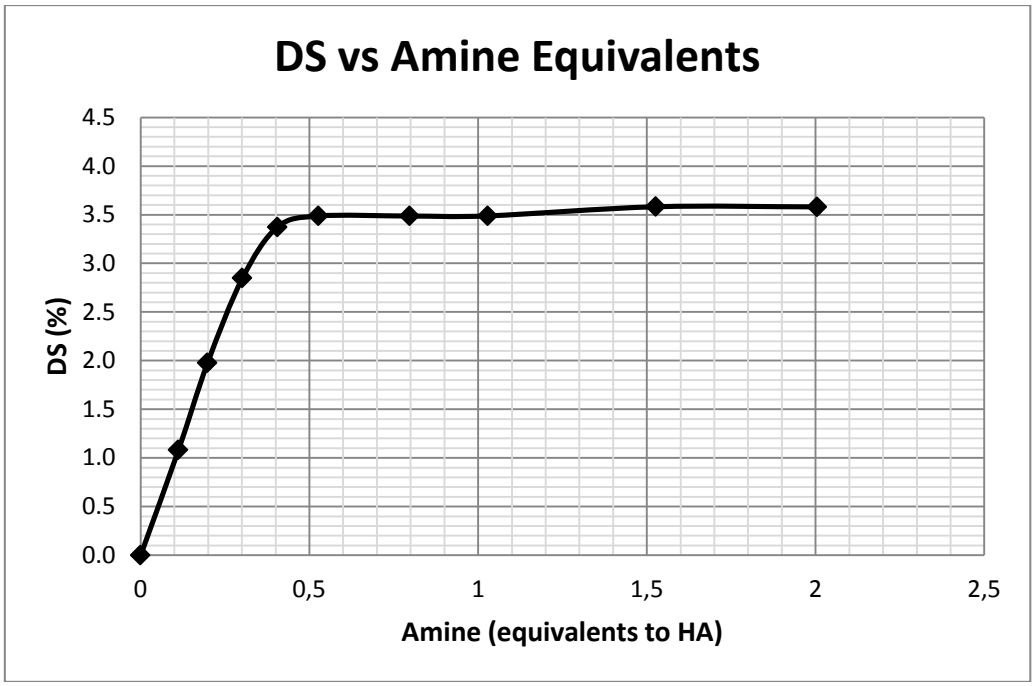


Figure 19 Degree of carboxyls on HA that have been substituted by benzylamine as a function of equivalents of benzylamine to HA. Reaction conditions: 3.5% w/v HA-solution, 0.8 equivalents of DMTMM to HA, pH 6 in phosphate buffer (1 mM), 70 ºC, 2 h.

6.2.6 HA-concentration

The equivalents of DMTMM and amine to HA were adjusted in regard to the HA concentration so that the same ratios were used and the only variable was the actual HA concentration. The results (Figure 20) show that HA concentrations below 2% (20 mg/ml) give a lower DS. Higher concentrations than 3.5% (35 mg/ml) were not investigated due to physical limitations; more viscous HA-solutions were difficult to squeeze through the two syringes in order to give homogenous reaction mixtures.

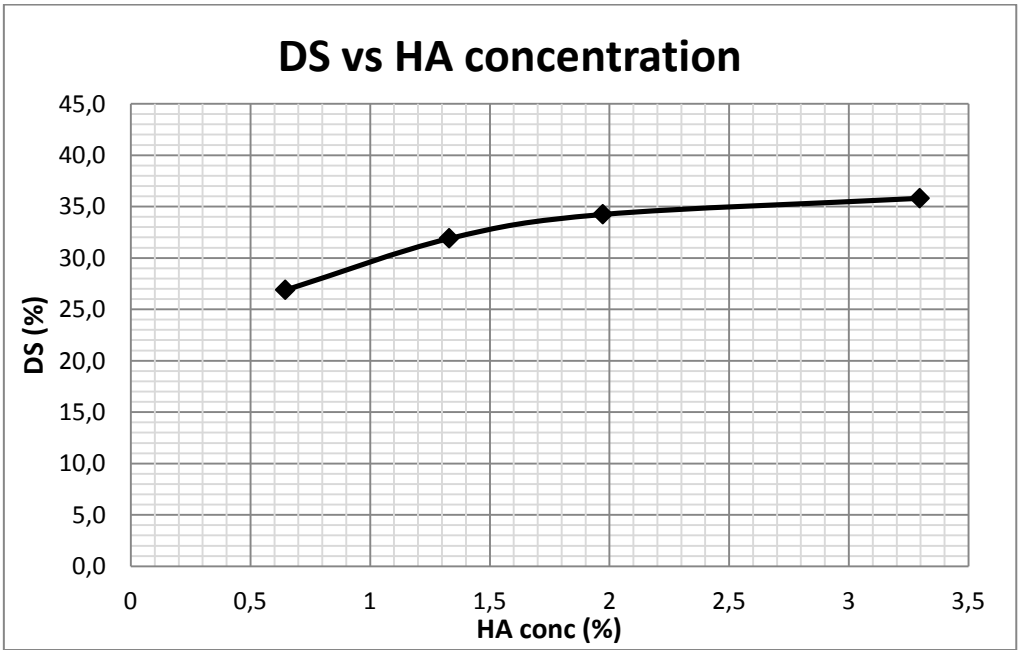


Figure 20 Degree of carboxyls on HA that have been substituted by benzylamine as a function of HA concentration. Reaction conditions: 2 equivalents of benzylamine to HA, 0.8 equivalents of DMTMM to HA, pH 6 in phosphate buffer (1 mM), 70 ºC, 2 h.

6.2.7 Solvent

Water-based buffer solutions with altered pH values dissolved HA, DMTMM and benzylamine well. The phosphate buffer concentration was lowered from 100 mM to 1 mM in order to solve the reagents without precipitation. Experiments were performed using phosphate buffer/acetonitrile mixtures in order to investigate if organic solvents would increase the DS of the reactions. Samples using 10, 20 and 30% acetonitrile (ACN) were investigated, but as Figure 21 demonstrates, an increase in ACN lowered the DS drastically. The temperature set to 40°C as a safety precaution since the boiling point of ACN is lower than that of water, and the reaction time was extended to compensate for the lower temperature.

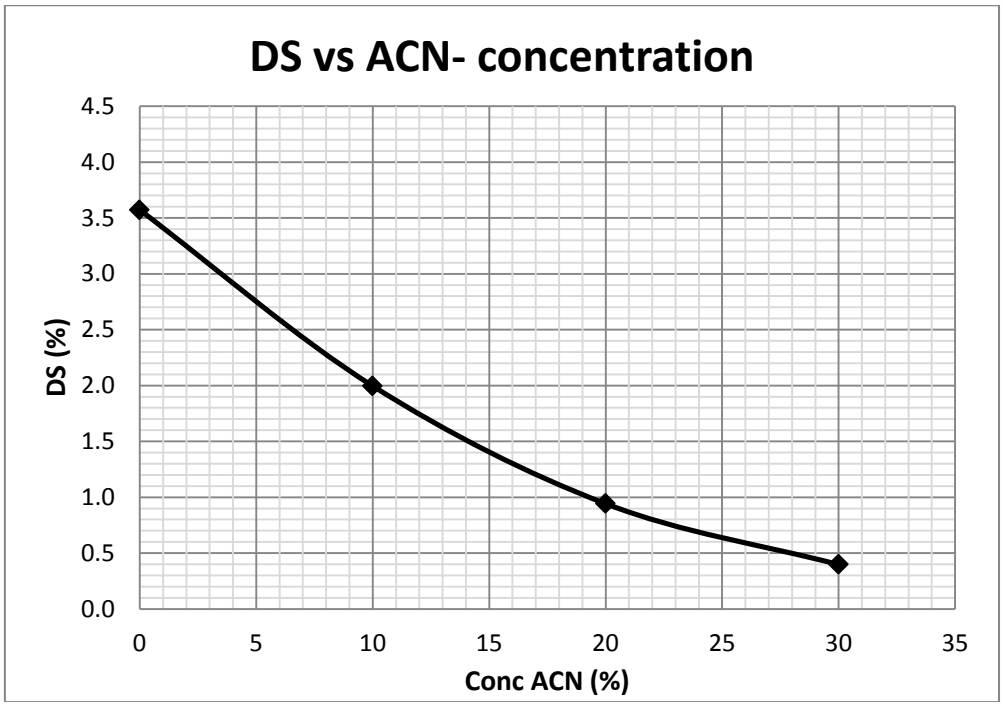


Figure 21 Degree of carboxyls on HA that have been substituted by benzylamine as a function of percentage acetonitrile in solvent. Reaction conditions: 3.5% HA-solution, 1 equivalent of benzylamine to HA, 0.8 equivalents of DMTMM to HA, pH 6 in ACN/phosphate buffer (1 mM), 40 ºC, 20 h.

6.3 DMTMM stability

The stability of DMTMM was investigated using both HPLC and NMR spectroscopy. Both methods demonstrated changes in the structure of the molecule. The chromatograms from the HPLC study are presented in Appendix IV where DMTMM in phosphate buffer was placed in a 70 ºC water bath for five hours with samples collected hourly. Results show that the ratio between the DMTMM peaks (see Figure 22) changes, where the first one decreases, the second one grows substantially and the third peak grows slightly over time.

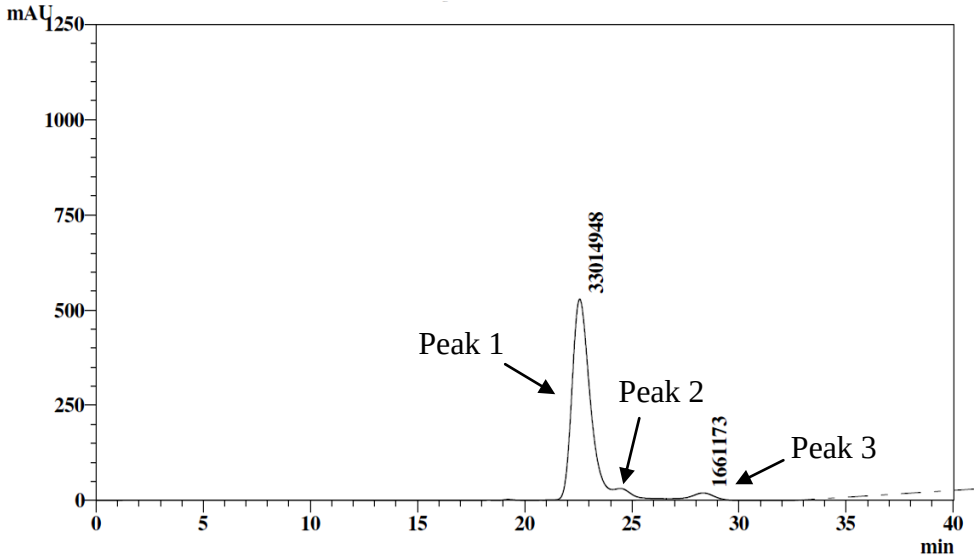


Figure 22 Chromatogram of DMTMM in phosphate buffer pH 6 (1 mM) before degradation at 235 nm.

NMR analysis also shows changes in the structure of DMTMM over time. The NMR spectra can be found in Appendix V. Although not all peaks have been assigned, it is apparent that more than one degradation product is formed and that the peaks from the starting material decrease over time. Information from ^{1}H, ^{13}C, HSQC and HMBC experiments indicated that two of the methyl peaks corresponded to the methyl on the morpholinium group (one from the starting material at 3.6 ppm and one from the degradation product at 3.0 ppm). The peaks are indicated in Figure 23 as **a** (starting material) and **b** (degradation product).

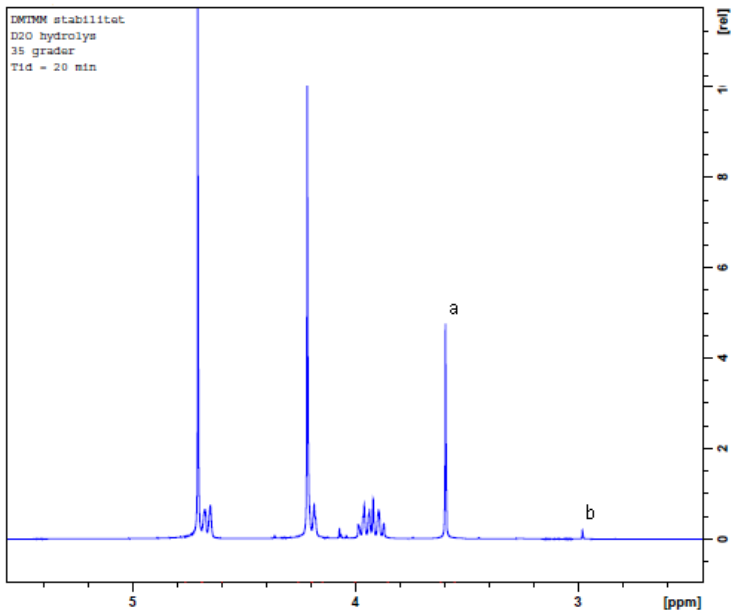


Figure 23 ^{1}H NMR spectrum of DMTMM before degradation (after approximately 20 min). The methyl peak at 3.6 ppm (a) is the methyl group on NMM in the starting material and the peak at 3.0 ppm (b) is the methyl on the degradation product.

The two methyl peaks were integrated and their sum was normalized and set to one. The percental decrease of **a** over time gave an approximation of the degradation rate and is illustrated in Figure 24. The stability study was performed over 90 hours in 35°C.

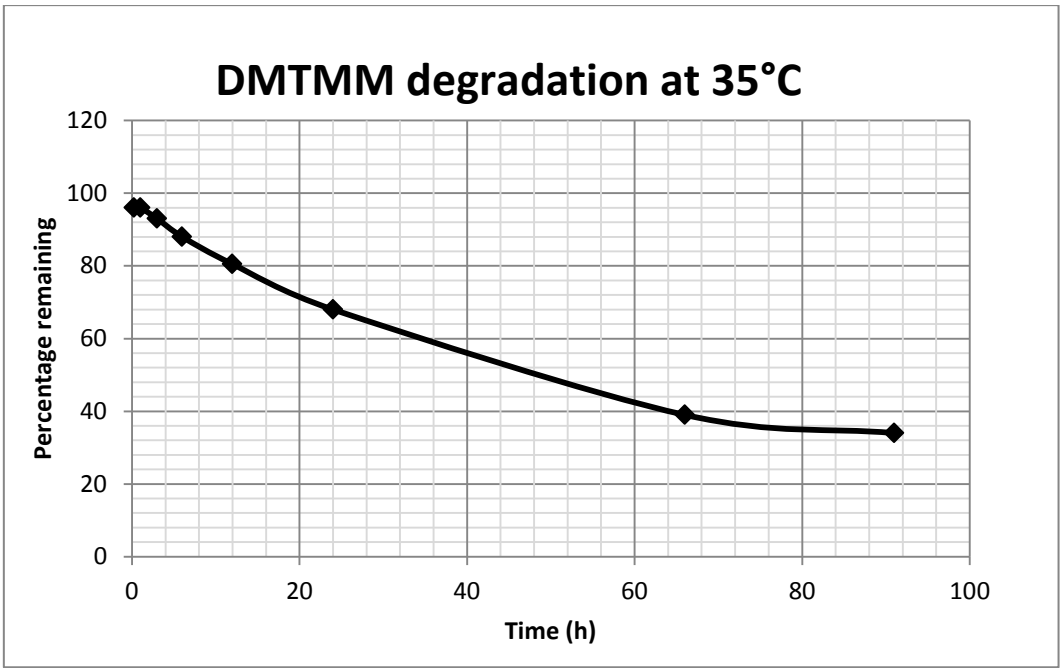


Figure 24 DMTMM stability study showing the percentage of starting material that remains over time (methyl at 3.6 ppm is integrated and compared to methyl from degradation product at 3.0 ppm). The study was performed in D_2O at 35°C.

A study was also performed where a freshly made sample was compared to a sample that had been placed in 50°C for 48 hours. The results showed that only 11% of the starting material remained after 48 hours (see spectra in Appendix V).

6.4 Gel formation

Reactions with HA, DMTMM and hexamethylene diamine resulted in gel formation, which means that the method was successful in crosslinking HA with diamines. Parameters from the optimized coupling reaction were applied on the crosslinking of HA with hexamethylene diamine. Gels were formed using phosphate buffer pH 6 (1 mM), 10% HA-solution, 0.5 equivalents of diamine, varying DMTMM concentration and performing the reaction in syringes as described in 3.2.1 in 70 °C for 2 h. The viscosity of the gels increased with larger equivalents of DMTMM to HA. For instance, 0.05 equivalents of DMTMM was practically a slow-flowing liquid while 0.5 equivalents produced a firm gel that, after homogenization, dispersed into particles in water. In other words, the results from the coupling reactions where more DMTMM gave more substitution also applied to gel formation.

7 Discussion

The DMTMM-activated amidation of HA has proven to be successful in both coupling amines to HA and in hydrogel formation. There are nevertheless aspects that need to be investigated further and sources of error that need to be evaluated in order to fully comprehend the reaction and its areas of utilization.

7.1 CDMT versus DMTMM

Choosing to work with DMTMM instead of CDMT has proven to be advantageous since higher concentrations could be used without causing solubility problems. Also, the reaction rate was not limited by the initial formation of DMTMM by CDMT and NMM. Practically, the experimental procedure was simplified by not having to work with NMM, which is a solution that demands the use of syringes and preparing solutions for exact administration. Both CDMT and DMTMM are solid, white powders that allow for easy weighing and convenient procedures. Considering that DMTMM is commercially available, there are no apparent reasons why CDMT should be used for the activation of carboxylic acids instead of DMTMM.

7.2 Finding the right amine

The reaction might be limited since the list of demands on the amine used is extensive. Benzylamine worked well in most conditions, but it would have been helpful if other amines could have been included for reference purposes. In general, the results are highly dependent on the properties of the amine used in the analysis. If the solubility of the amine was affected by a change in pH, HA concentration or solvent, incorrect conclusion might have been drawn regarding the effectiveness of the reaction. This would need to be cleared up in future investigations, for instance by choosing amines that did not need to be detectable in UV and analyzing the products with NMR.

7.3 Predicting the amine concentration coupled to HA

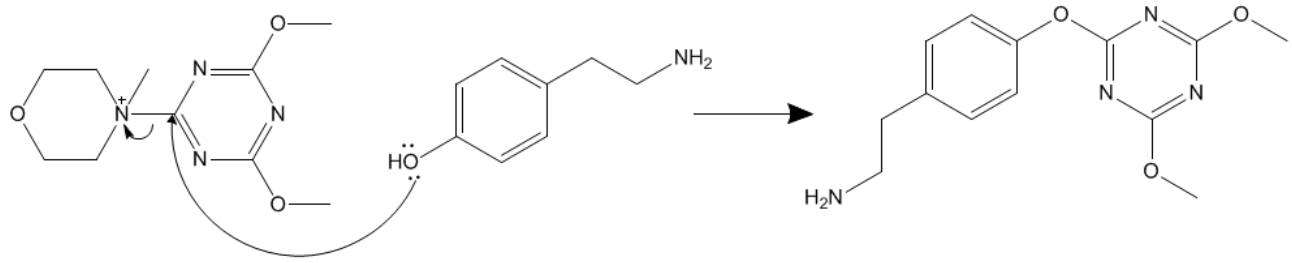
The evaluation method described in section 4.3 is a quick and accessible way to analyze the products in the reaction. There are however aspects to consider when it comes to the stability of the method. Even though reference samples with HA and DMTMM are used to estimate the amount of DMTMM that is coupled to HA, it is impossible to know exactly how much of the integrated area that corresponds to coupled benzylamine. The results may be both over- and underestimated since DMTMM absorbs in the 255 nm region as well. In other words, the area under the HPLC curve at 255 nm might represent 100% coupled benzylamine or 20% of the area could be DMTMM coupled to HA in the form of the “active ester”. Using reference samples to reduce the risk of overestimating the results is a simplified solution that postulates that the same amount of DMTMM will couple to HA whether benzylamine is present or not. A better quantification method is required to determine the degrees of substitution more accurately, such as NMR.

7.4 pH in reactions

Even though the pH was adjusted in both the HA solutions and in the reagent mixtures, lower pH values were recorded in the reaction syringes after completion. In other words, assuming that pH 6 is the optimal value may be incorrect because the reaction actually takes place at pH 4 or 5. A solution to this would have been to use a higher buffer concentration than 1 mM, but the higher ionic strength in the solution caused solubility problems for the reagents.

7.5 Possible side reactions

When the extra peak(s) appeared in the HPLC chromatogram for several of the amines, it was presumed that these belonged to side reaction products. The first interaction that was discovered was between DMTMM and tyramine. One suggestion of the reaction that may have taken place is presented in Scheme 4 where a nucleophilic attack by the hydroxyl group of tyramine on DMTMM caused the new peak.



Scheme 4 Possible reaction between DMTMM and tyramine. Nucleophilic attack by the hydroxyl group, NMM leaves.

The second interaction that was observed seemed to occur between pyridine-based molecules and DMTMM. The question is if the side reaction took place because of the tertiary nitrogen in the ring. According to the literature, CDMT will react readily with a list of tertiary amines. Kaminski [9] showed that CDMT would not react with triethylamine or dimethylaniline (Figures 28 and 29), and presumed that it was due to steric hindrance from the N-substituents.

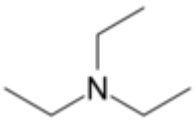


Figure 25 Triethylamine

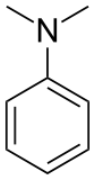


Figure 26 Dimethylaniline

Tetramethylenediamine (Figure 30), on the other hand, reacted with CDMT in the tests but the most efficient tertiary amine proved to be the familiar NMM (Figure 31).

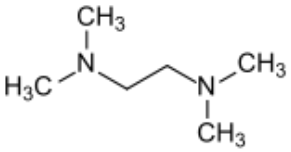


Figure 27 Tetramethylenediamine

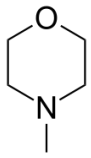
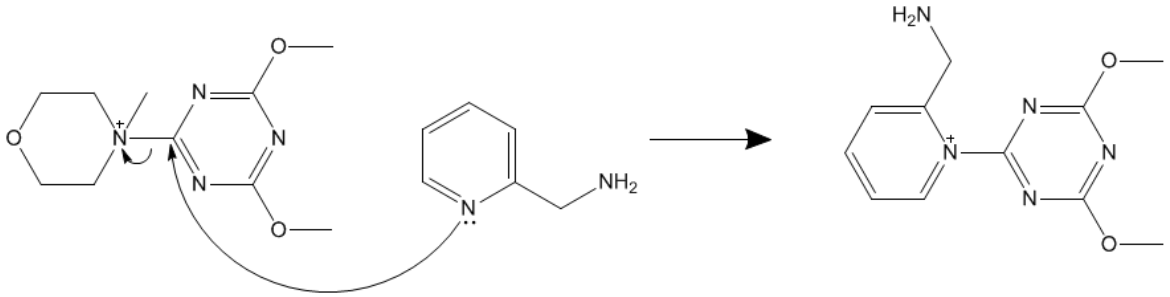


Figure 28 NMM

Based on the observations made by Kaminski regarding tertiary amines, the hypothesis regarding the interactions between DMTMM and pyridine rings is that the NMM-part of DMTMM was replaced by a pyridine ring to form a new triazinylammonium salt, as in the reaction shown in Scheme 5.



Scheme 5 Possible reaction between DMTMM and a pyridine nitrogen.

To determine if DMTMM reacted with tertiary amines and not with primary or secondary amines, three very similar molecules were compared. Benzylamine, N-methylbenzylamine and N,N-dimethylbenzylamine (Figures 25, 26 and 27 respectively) were chosen since they had aromatic structures that were detectable in UV and the only difference between them was the amine part. Results showed that the primary and secondary amines did not form extra peaks in the chromatogram when combined with DMTMM. The tertiary amine, on the other hand, showed a significant, wide peak that could not be identified as DMTMM or the amine itself. This was an indication that interactions actually occur between DMTMM and tertiary amines, but additional analysis is necessary.

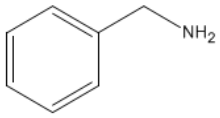


Figure 29 Benzylamine

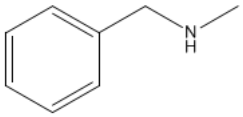


Figure 30 N-methylbenzylamine

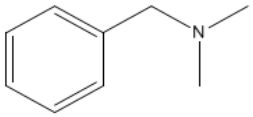


Figure 31 N,N-dimethylbenzylamine

In general, the side reactions clearly need to be investigated further and require better analysis techniques in order to be confirmed, e.g. NMR. Understanding which interactions that take place is crucial when using DMTMM for synthesis.

7.6 Highest possible DS

Despite attempts using higher DMTMM concentrations, the highest possible DS reached in the experiments was 40%. There may be several reasons why 100% substitution was not obtained. For example, it has already been shown that precipitation occurs at high DMTMM and amine concentrations. Furthermore, as HA becomes more substituted, it also becomes more hydrophobic and this decrease in solubility may contribute to the precipitation at high reagent concentrations. Also, there is a chance that HA complicates the procedure by forming clusters and tangled chains where the reagents cannot attack freely or that the degradation of DMTMM inactivates the reagent before all of the carboxylic groups have been activated.

7.7 DMTMM stability

As was shown in the stability studies in section 5.3, DMTMM is degraded in water. In other words, solutions of DMTMM should not be prepared in advance to avoid initiating the degradation process before the reacting is started. The stability studies have only been analyzed briefly, and the degradation rates are only estimations. Also, the degradation products have not been determined apart from the structures presented in the literature (see section 2.2.2). This means that the new products do not necessarily have to be inactive, but could react in unexpected ways. Essentially,

further analysis of the hydrolysis and degradation of DMTMM needs to be performed, preferably by combining techniques like NMR and mass spectrometry.

7.8 Future investigations

The focus of this thesis has been on the optimization of the coupling reactions with DMTMM, yet the crosslinking reactions have merely been looked at very briefly. There are many questions to be answered regarding the crosslinking of HA using DMTMM. For instance, what happens when the diamine is modified, i.e. a different chain length, a more branched molecule or molecules with more than two amines. Additional investigations are also needed in order to determine if and how the molecular weight of HA is affected throughout the reaction.

Another area that requires further attention is the analysis of the reaction products. It is essential that the products and by-products are determined so that predictable and reproducible results can be obtained. It would also be interesting to establish whether the by-products can be removed by relatively simple procedures.

8 Conclusions

The aim of this thesis was to investigate and optimize the DMTMM-activated amidation of HA. Considering the versatility and usefulness of HA, any new methods for developing stable, crosslinked hydrogels is of great interest and these investigations have shown that DMTMM-activation may be good alternative. Both the coupling and crosslinking reactions were successful, reaching DS values of up to 40%. The results from the optimization generally showed consistent correlations between the investigated parameters and the degree of substitution.

The optimal reaction conditions for the amidation reaction using DMTMM can be summed up as the following:

- pH 6
- 70 °C
- 2 hour reaction time
- 35 mg/ml HA concentration
- Phosphate buffer (1 mM) as solvent
- Benzylamine for coupling reactions

It can also be concluded that the reaction can be controlled by either the DMTMM or the amine concentration so that a specific degree of substitution or crosslinking is achieved.

In summary, there are still several aspects of the DMTMM-activated amidation of HA that need to be investigated, but the reaction is quick, effective and works under relatively mild conditions. The crosslinking attempts of HA were successful and the many applications of the reaction are still to be discerned.

9 Acknowledgements

First and foremost, I would like to thank my supervisors Hotan Mojarradi and Anders Karlsson at Q-Med, a Galderma division, for making this master thesis possible. Their generous support and guidance has been invaluable and I am so grateful for the time, expertise and encouragement that they have supplied. I would also like to thank Elin Säwen for her help with the NMR analyses and Gunnar Johansson for reviewing my work. The substances and equipment provided by Q-Med are also gratefully acknowledged.

Finally I would like to thank everyone at Q-Med for making me feel at home and for the many laughs along the way!

10 References

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11 Appendices

11.1 Appendix I: Results of amines tested

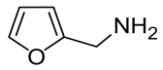
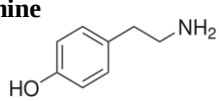
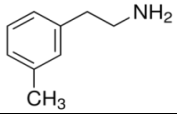
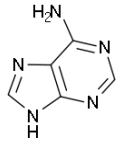
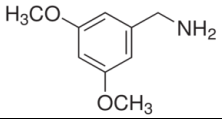
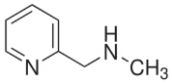
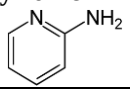
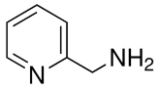
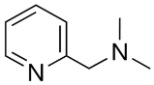
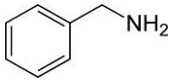
Amine	Solubility problems	Possible reaction with DMTMM	Distinguishable UV-spectra	Ability to couple w\ HA
Furfurylamine 	None	-	No	-
Tyramine 	Precipitates at pH 8 (1 mM phosphate buffer)	Yes	Yes	Yes
3-methylphenethylamine 	Precipitates in phosphate buffer (1 mM)	-	-	-
Adenine 	Precipitates in phosphate buffer (1 mM)	-	-	-
3,5-dimethoxybenzylamine 	Precipitates in phosphate buffer when DMTMM is added (1 mM)	-	-	-
2-picolylamine 	None	Yes	-	-
2-aminopyridine 	None	Yes	-	-
2-[(methylamino)methyl]-pyridine 	None	Yes	-	-
2-(dimethylaminomethyl)-pyridine 	None.	Yes	-	-
Benzylamine 	None	No	Yes, absorption maxima at 255 nm	Yes

Table 1 Amines tested and results for each requirement. Boxes marked with (-) have not been tested for that requirement due to solubility issues or unwanted interactions.

11.2 Appendix II: UV spectra

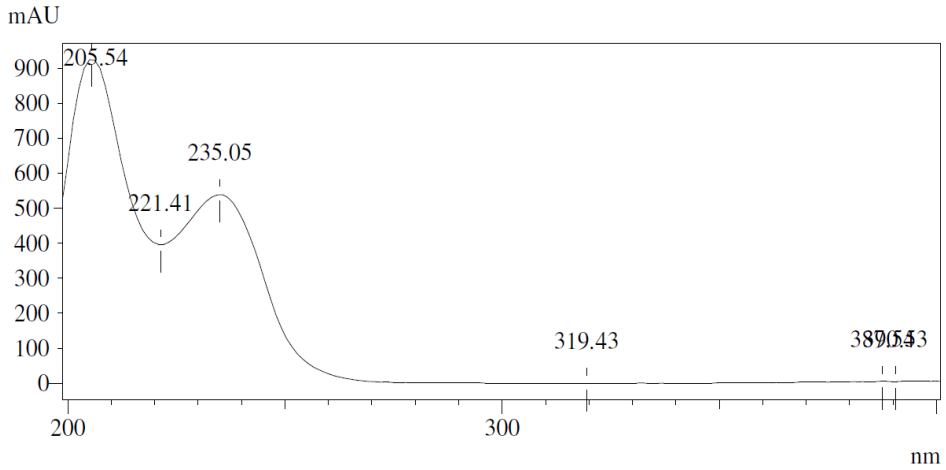


Figure 32 UV-spectrum of DMTMM.

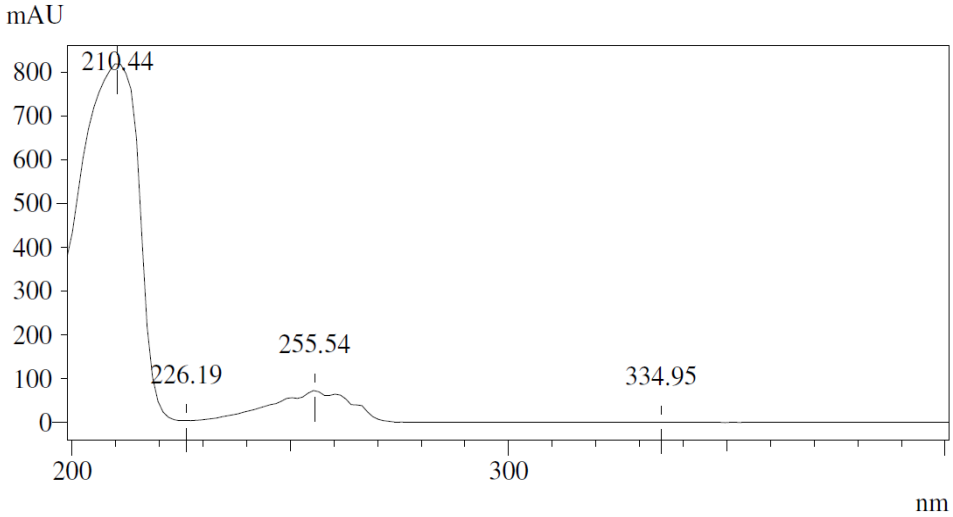


Figure 33 UV-spectrum of benzylamine.

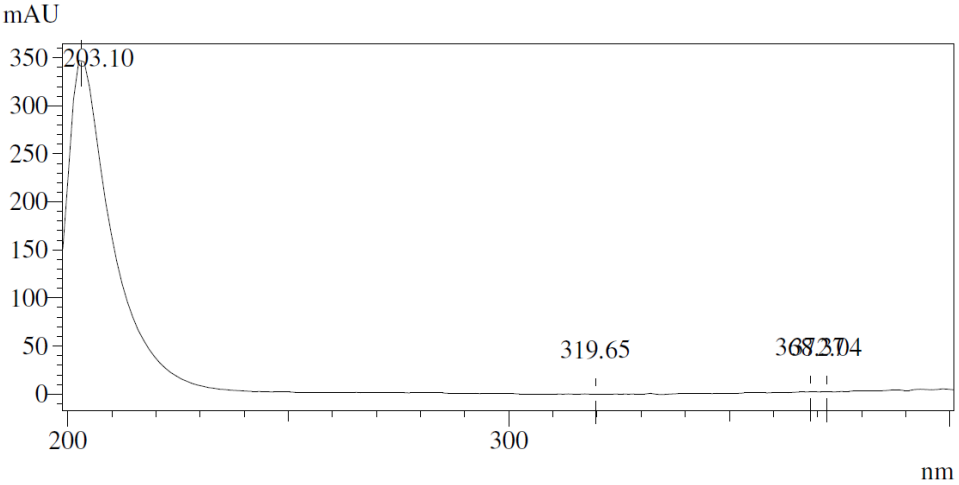


Figure 34 UV spectrum of 170 kDa HA.

11.3 Appendix III: HPLC chromatograms

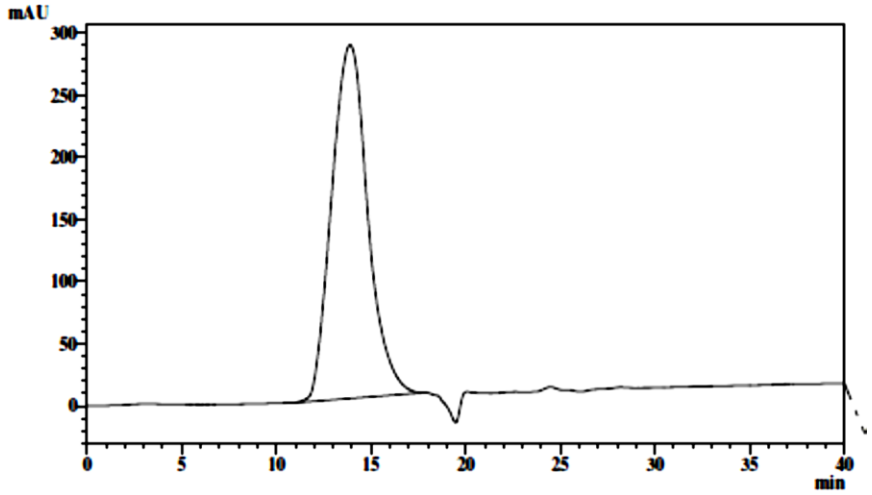


Figure 35 Chromatogram of 170 kDa HA in H_2O at 205 nm. Retention time = 13.5 min.

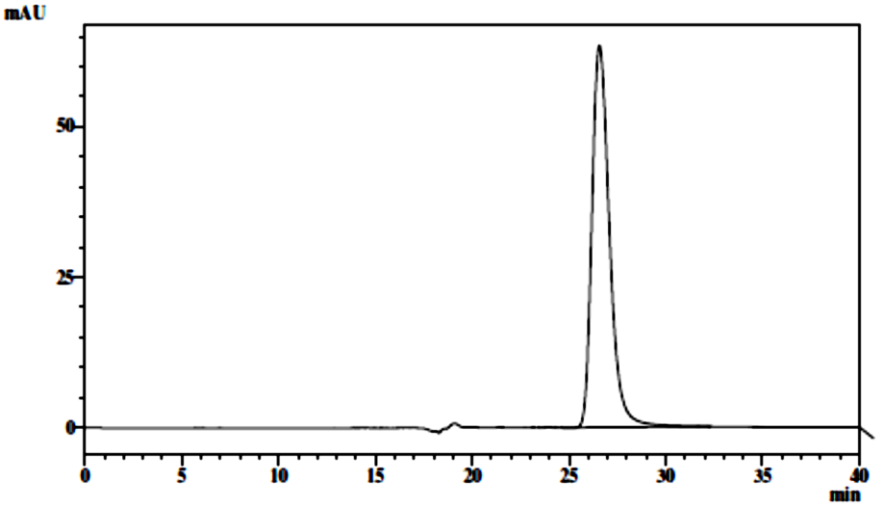


Figure 36 Chromatogram of benzylamine in H_2O at 254 nm. Retention time = 27 min.

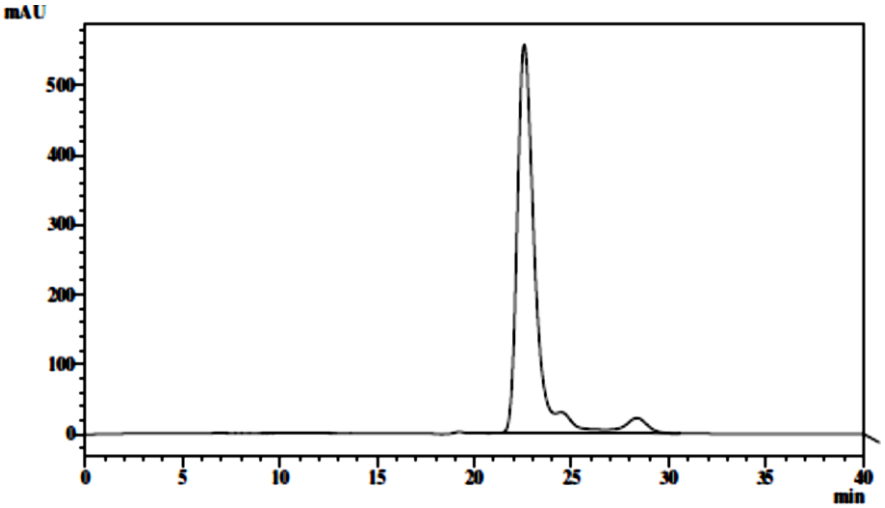


Figure 37 Chromatogram of DMTMM in H_2O at 235 nm. Retention time = 22.5 and 28.5 min.

11.4 Appendix IV: HPLC stability study DMTMM

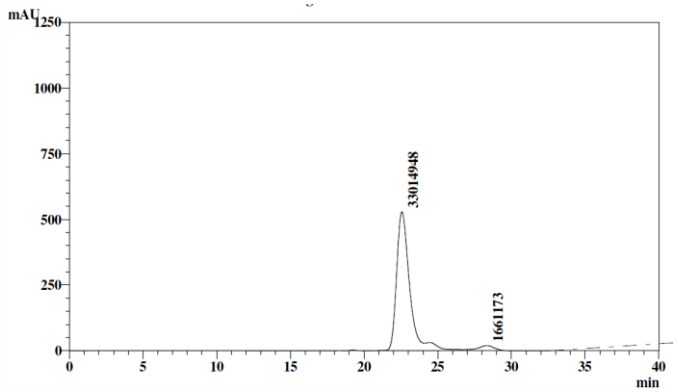


Figure 38 Chromatogram of DMTMM in phosphate buffer approximately 10 minutes after dissolution (before placed in 70 ºC water bath).

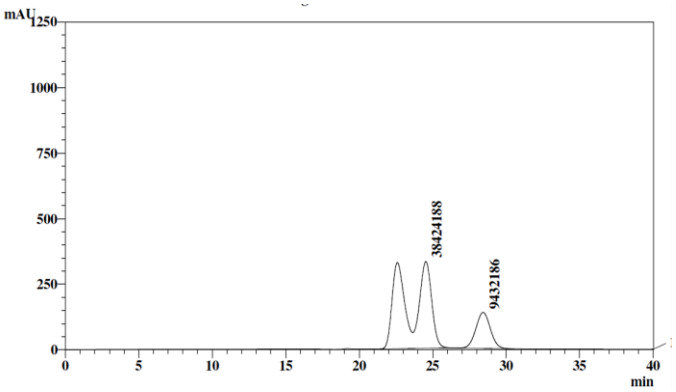


Figure 39 Chromatogram of DMTMM in phosphate buffer after 1 hour

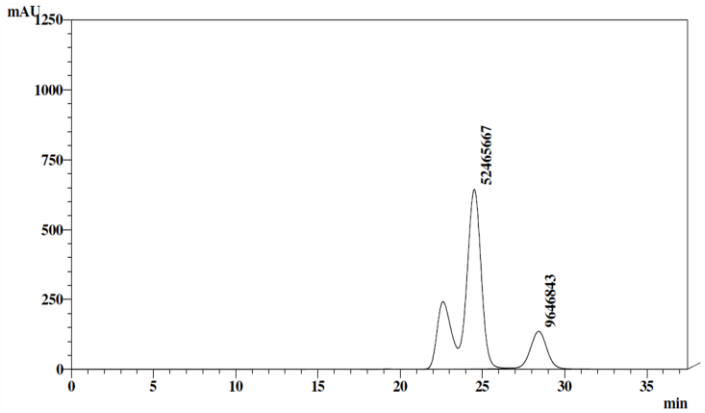


Figure 40 Chromatogram of DMTMM in phosphate buffer after 2 hours.

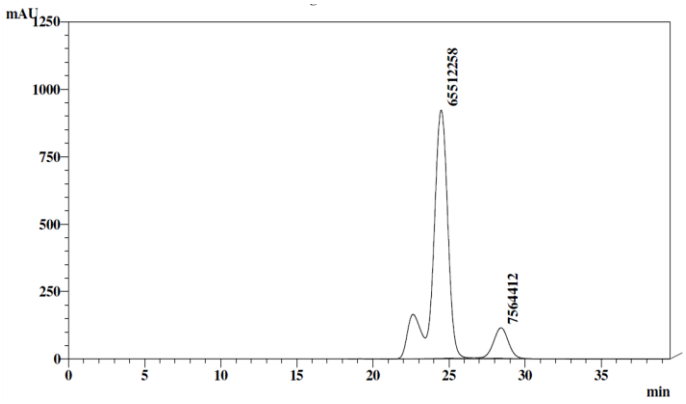


Figure 41 Chromatogram of DMTMM in phosphate buffer after 3 hours.

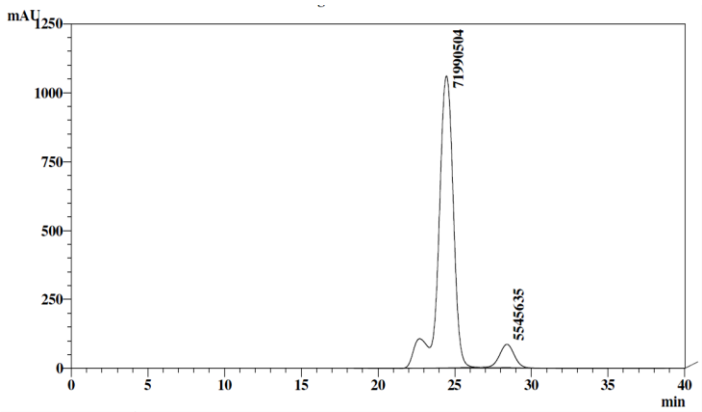


Figure 42 Chromatogram of DMTMM in phosphate buffer after 4 hours.

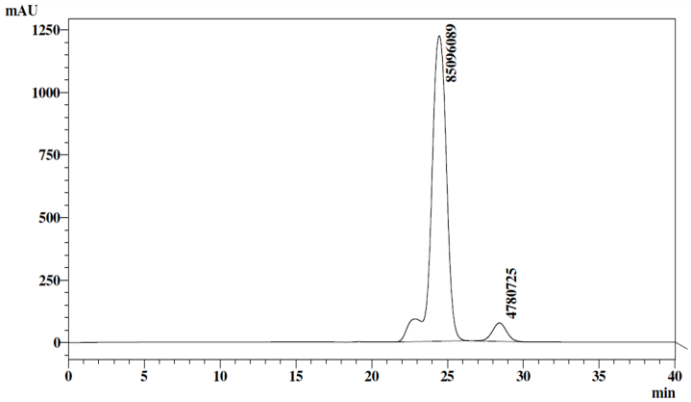


Figure 43 Chromatogram of DMTMM in phosphate buffer after 5 hours.

11.5 Appendix V: NMR stability study DMTMM

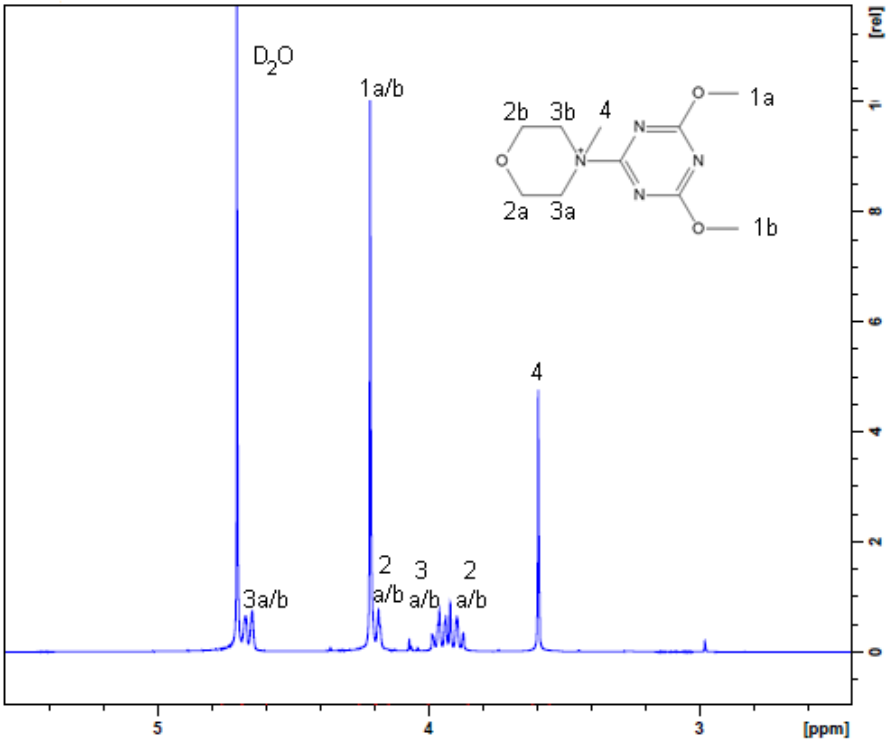


Figure 44 ^{1}H NMR spectrum of DMTMM in D_2O before degradation. The signals have been assigned to hydrogens in the molecule, but several of the peaks were indistinguishable with the experiments performed.

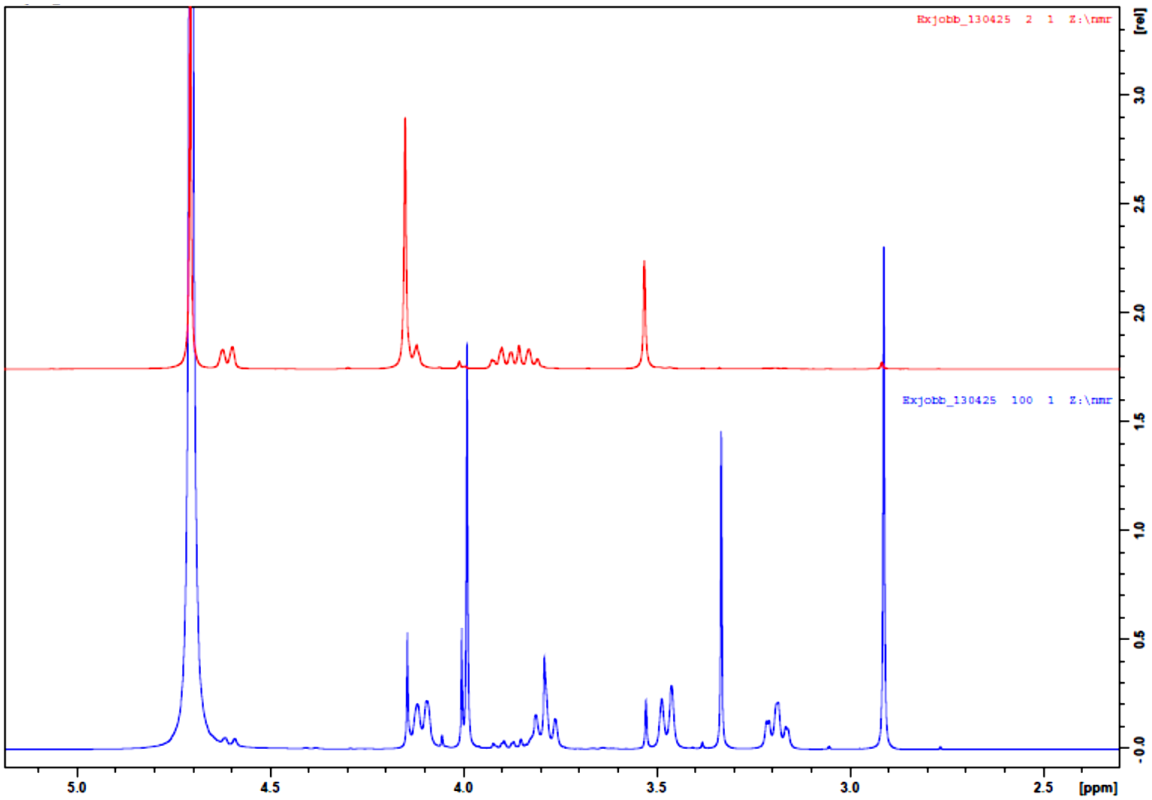


Figure 45 ^{1}H NMR spectrum of DMTMM. Top: Spectrum before degradation (after approximately 15 min). Bottom: Spectrum after degradation for 48 hours in 50ºC. Several new peaks have appeared and the original peaks from the starting material have shrunk.