A kinetic study of the degradation of hyaluronic acid at high concentrations of sodium hydroxide

Jonathan Fagerström Troncoso
Abdelrahman Idjbara
Ida Karlsson
Maria Lekander
Teresia Lindgren, Simon Ström
Abstract

A kinetic study of the degradation of hyaluronic acid at high concentrations of sodium hydroxide

Jonathan Fagerström Troncoso, Abdelrahman Idjbara, Ida Karlsson, Maria Lekander, Teresia Lindgren, Simon Ström

During production of different Hyaluronic acid (HA) products Galderma use a high concentration of NaOH in the crosslinking process of HA. HA’s degradation kinetics are widely unknown at higher pH’s and is interesting for the future of product development of HA. Static Light Scattering (SLS) was used to determine the weight average molecular weight (MW) of samples with four different NaOH concentrations, four temperatures, three reaction times and one t0 sample. The results were evaluated in forms of Arrhenius graphs for different NaOH concentrations, the activation energy (EA) with respect to pH and the reaction rate for each temperature with respect to NaOH concentration. It was found that the degradation of HA was more strongly affected by temperature than by NaOH concentration and that the EA did not show any significant signs of change between higher concentrations of NaOH.
A kinetic study of the degradation of hyaluronic acid at high concentrations of sodium hydroxide

Jonathan Fagerström Troncoso
Abdelrahman Idjbara
Ida Karlsson
Maria Lekander
Teresia Lindgren
Simon Ström

Abstract: During production of different Hyaluronic acid (HA) products Galderma use a high concentration of NaOH in the crosslinking process of HA. HA’s degradation kinetics are widely unknown at higher pH’s and is interesting for the future of product development of HA. Static Light Scattering (SLS) was used to determine the weight average molecular weight ($M_W$) of samples with four different NaOH concentrations, four temperatures, three reaction times and one $t_0$ sample. The results were evaluated in forms of Arrhenius-graphs for different NaOH concentrations, the activation energy ($E_A$) with respect to pH and the reaction rate for each temperature with respect to NaOH concentration. It was found that the degradation of HA was more strongly affected by temperature than by NaOH concentration and that the $E_A$ did not show any significant signs of change between higher concentrations of NaOH.

Uppsala University
August 19, 2016
Foreword

We would like to thank Galderma:
Thank you Åke Örlund for being so kind and quick to answer.
Thank you Katarina Hägestam and Morgan Larsson for, without any formal obligations, getting yourselves involved in the project and spreading positive energy.

We also would like to thank Uppsala University:
Thanks to Gunnar Johansson for being supportive and always believing in us, even in hard times.
Thanks to Isak Öhrlund for always coming with good advice.
Thanks to Mats Bohman for lending us space at your laboratory, and showing great interest in the project.

Finally, we would like to thank Jan Bohlin for informing us about capillary viscometry, though we never used it, and Sven Norén for helping us with the laboratory equipment.
Contents

1 Introduction .................................................. 2
  1.1 Background ............................................. 2
    1.1.1 Galderma AB ...................................... 2
    1.1.2 Hyaluronic acid .................................. 2
    1.1.3 Applications of Hyaluronic Acid .................. 2
    1.1.4 Prior studies of degradation of Hyaluronic Acid .. 3
  1.2 Aim .................................................... 4

2 Theory ...................................................... 5
  2.1 The Arrhenius Equation ................................. 5
  2.2 Different Methods of Analysis .......................... 6
    2.2.1 Size Exclusion Chromatography .................... 6
    2.2.2 Light-scatter ..................................... 7
    2.2.3 Refractive Index detector ......................... 7
  2.3 Capillary Viscometry ................................... 8
  2.4 Limitations ............................................. 8

3 Method .................................................... 9
  3.1 Choice of method ....................................... 9
  3.2 Procedure of method .................................... 9
    3.2.1 Preparation of NaOH and Phosphoric acid stock solutions .... 9
    3.2.2 Preparation of Phosphate Buffer (150mM NaCl and 50mM Na₂HPO₄) .. 9
    3.2.3 Preparation of Samples ............................. 9
  3.3 Analysis ................................................. 12
    3.3.1 Analytical equipment ............................... 12
    3.3.2 Equipment settings ................................ 13
    3.3.3 Analysis of samples ............................... 13

4 Results ..................................................... 14
  4.1 Preliminary Experiment ................................. 14
  4.2 Main Experiment ....................................... 14

5 Discussion ................................................ 21
  5.1 Preliminary Results .................................... 21
  5.2 Main Experiment ....................................... 21
  5.3 Conclusions ........................................... 22

Bibliography .................................................. 23

6 Appendix ................................................... 24
  6.0.1 Material ............................................. 24
  6.0.2 Lab Materials ....................................... 24
  6.1 Graphs over inverted molecular weight vs. time that was used to create the arrhenius plot. 25
  6.2 Examples of chromatograms ............................. 29
1 Introduction

1.1 Background

1.1.1 Galderma AB

Galderma was founded in 1981 by Nestlé and L’Oréal. The company has for over 30 years been dedicated to deliver innovative medical solutions to meet people’s dermatological needs. Galderma is a global brand with a broad product portfolio available in 80 countries.

Galderma Nordic was founded as Q-Med by Bengt Ågerup. Ågerup graduated from Uppsala University with a Ph.D in the physiology of kidneys. According to Birgitta Forsberg, economic journalist, Ågerup came in contact with hyaluronic acid while working for Pharmacia (Forsberg, 2012). In 1979, Pharmacia launched Healon, a sodium hyaluronate preparation for intraocular surgery (Henderson et al., 2nd ed. 2014). From this, he could draw his own conclusions on the potential of HA on osteoarthritis and non-surgical aesthetic procedures. Restylane was launched in 1994 and, in 2003, the first HA filler approved by the US FDA (Dayan and Bassichis, 2008). 20 million treatments have been performed since the premiere.

Galderma acquired Q-Med in 2011.

1.1.2 Hyaluronic acid

Hyaluronic acid (HA) is not actually an acid, but rather a naturally occurring glycosaminoglycan produced in the body. The HA molecules are unstable and degrades when exposed to heat and sunlight. It is one of the main components in the skin and other tissues and is, for instance, known for being a major component of the synovial fluid, being involved in wound healing and cell migration (Fakhari and Berkland, 2013).

HA is also an important component of the extracellular matrix, which is a collection of molecules that provides structural and biochemical support to the surrounding cells. The appearance of the outer layer of the skin, the epidermis, is primarily supported by the dermis, the middle layer of the skin and the extracellular matrix it secretes (C. et al., 2010). One can say that the dermis acts as a construction foundation for strength and support to the skin, in which HA is an important component.

Hyaluronic acid was discovered by Karl Meyer and John Palmer in 1934. They discovered and isolated HA from the vitreous body of cows’ eyes but its structure was not completely determined until 20 years later where it showed that the HA molecule consist two monosaccharide units, D-N-acetyl glucosamine (GlcNAc) and D-glucuronic acid (GlcA) connected by β(1→3) and β(1→4) glycosidic bonds. HA was first identified as an acid but under physiological conditions it acts as a salt. Ender Balazs was the first man to patent an application of HA, this as a substitute for egg white in bakery products.

In the late 1950s the first biochemical application of HA took place where it was used during eye surgery as a vitreous substitution. (Garg and Hales, 2004)

1.1.3 Applications of Hyaluronic Acid

For many people, their looks play an important part in their self-esteem and for some, changes in skin texture and appearance such as wrinkles and reduced skin plumpness are not welcomed.

Wrinkles are folds, creases or ridges in the skin that usually appear as a person ages. Why skin-changes appear may be because of various reasons such as extrinsic aging, i.e. dehydration, facial expressions, sun exposure, smoking etc. (Nordquist, 2009).

Wrinkle formation, as a result of intrinsic aging, the natural aging process that takes place over the years regardless of outside influences is unavoidably. As a person ages the body produces a smaller amount of substances, for example hyaluronic acid, which are needed to keep the skin plump (American, 2005).

However there are means, beauty products, to help hide, prevent and reduce the unwanted age signs such as makeup, fillers, Botox, creams etc. Beauty products are globally a billion dollar industry and there are many different companies specializing in developing and deliver different solutions to meet people’s dermatological needs. HA is used in many beauty products because of its beneficial properties, which is that it can both bind and retain a large amount of water i.e. it has hydrating properties. Due to its hydrating
and skin caring properties the HA molecule is ideal to use in creams and makeup against wrinkles and aging (Gupta, 2016).

HA is also used in fillers, a cosmetic treatment used to smooth wrinkles or pitted scars in the skin. It is used partly due to its hydrating properties and because it is a natural occurring substance in the body, as well as the fact that it is biodegradable, which minimizes the risk of getting an inflammation. But also because it has a better durability compared to the other available biodegradable fillers and since the antidote hyaluronidase to HA is available (de Maio and Rzany, 2006). Another reason why HA is commonly found in fillers is due to the fact that the viscosity can change widely and imitate different types of tissue.

The molecular weight of hyaluronic acid ranges from 5 kDa (5000 g/mol) and up to 20,000 kDa and it can form very viscous solutions (Gupta, 2016) (Farwick et al., 2008). Both cellular and physiological effects are directly dependent on the molecular weight, hence when using HA in creams and gels it is important that the HA has an appropriate molecular weight. HA of high molecular weight cannot penetrate into the tissue, while very low molecular weight effectively penetrates into the tissue. Studies show that solutions containing HA with a molecular weight as low as 50 kDa are best suited for general use in skincare products since it effectively penetrates into the skin without causing any inflammation. However, when the molecule weight is as low as 50 kDa it does not create a thick gel.

When used in fillers, the HA is in gel form and when producing these fillers they undergo a process that include NaOH. When making fillers, cross-links between the HA chains needs to be made in order for the gel to get the right viscosity and increased stability/duration.

Since fillers are used for imitating different types of tissues it also needs to have different viscosities. Hence the degradation rate during different circumstances is important to keep in mind when making fillers or any other tissue imitation. The degradation rate is also interesting when making creams and other beauty products since the molecular size is of importance for its ability to penetrate the skin. Therefore it is of interest to know the extent to which the degradation of HA depends on the NaOH concentration as well as on temperature. The higher the temperature is the faster the degradation, and the more the molecules break apart, the smaller the molecules (and the molecular weight) becomes. This will affect the HA’s physical and chemical properties such as tissue volume, viscosity, and elasticity (Fakhari and Berkland, 2013).

1.1.4 Prior studies of degradation of Hyaluronic Acid

Very little has been investigated in terms of the degradation of HA at different pH’s and temperatures. The degradation of HA has been investigated at different pH-values using kinetics measurements in order to determine the ratio of the hydrolytic degradation of HA (Tokita and Okamoto, 1995). The pH-values studied were at 3, 7 and 11 pH. The temperature range included 40, 50, 60, 70, 80 and 100°C. Here the weight average molecular weight, \( M_w \), was determined by gel-permeation chromatography. It was proved that the HA molecule was degraded by random chain scission at the glycoside bonds at given experimental conditions since a linear correlation between \( \frac{1}{M_w} \) versus time was found, meaning that the reaction is a first order reaction. Tokita and Okamoto conclude that the rate of hydrolytic degradation of HA in aqueous solution is strongly dependent on pH.

The degradation of HA in an low pH environment has also been studied (Tommeraas and Melander, 2008). In this study, concentrations of 0.0010, 0.010, 0.10, 0.50, 1.0, and 2.0 M of HCl were used, which correspond to pH 5 and lower. HA was hydrolysed at 40, 60 and 80°C. For hydrolysis at 60 and 80°C it was shown that the rate constant for degradations was linearly dependent on the HCl concentration.

It was also shown that acid hydrolysis of HA is a random process at all pH-values studied and that there was no detectable side reaction. By constructing kinetic plots of \( \frac{1}{M_w} \) versus time, the reaction was shown to follow first order kinetics. At pH -0.3 to 3 it was found that the rate constant was linearly dependent on pH, but above this pH it was show that the rate constant decreased more slowly. Most polysaccharides have a linear dependency where the rate constant is proportional to the concentrations of hydrogen (in \( H_3O^+ \) state). This probably depends on HA and its increasing affinity for protons. The glycoside bond \( \beta (1 \rightarrow 4) \) was determined to be the breaking bond in HA with an activation energy of 137 kJ/mol for the 0.1 M HCl.
Studies of other polysaccharides are of interest, since the glycoside bond $\beta (1 \rightarrow 4)$ appears in many of them, for example cellulose derivative. Cellulose from corn fibre has been studied and the kinetics of cellobiose (a disaccharide) and glucose were examined (Mosier et al., 2002). For cellobiose, the compound of interest for the project, sulfuric and maleic acid were used for hydrolysis with a concentration at 50 mM and with a temperature range from 135 to 175°C. In the study, they concluded that the activation energy of hydrolysis was $114 \pm 9.3 \text{ kJ/mol}$ for maleic acid and $110 \pm 29.6\text{kJ/mol}$ for sulfuric acid, both with a 95 % confidence interval. It is confirmed that the hydrolysis depends on the hydrogen ion concentration and not on the source of acid.

1.2 Aim

While the degradation of HA in acid environments has been studied, it is unknown how the degradation of HA and high NaOH concentrations correlate and this information can be used to make products of specific properties. Therefore, the degradation-rate of hyaluronic acid at different high NaOH concentrations that has not been looked at closely before was investigated. By finding the Arrhenius equations for specific concentrations of NaOH, the impact that NaOH has on the degradation of HA was determined.
2 Theory

2.1 The Arrhenius Equation

The Arrhenius equation, see equation 1, is an empirical relationship that describes how the rate of a reaction is dependant on the temperature (Atkins and de Paula, 2014). The relationship between molecular weight and the rate constant can be seen in equation 2.

\[ k = Ae^{-\frac{E_a}{RT}} \]  

(1)

\[ \frac{1}{M_w(t)} = \frac{1}{M_{w,t=0}} + kt \]  

(2)

By plotting \( \frac{1}{M_w} \) against \( t \), the rate constant can be determined by the resulting slope of the curve, Figure 1.

![Figure 1: Determining the rate constant \( k \).](image)

Taking the natural logarithm of both sides of equation 1 gives equation 3:

\[ \ln k = \ln A - \frac{E_a}{R} * \frac{1}{T} \]  

(3)

Using the rate constant \( k \) obtained from Figure 1, and equation 3, an Arrhenius plot can be constructed, Figure 2. The resulting slope of the Arrhenius plot is \( -\frac{E_a}{R} \).

![Figure 2: An example of an Arrhenius plot. The slope of the plot is equal to \( -\frac{E_a}{R} \).](image)

The Arrhenius equation only describes a reactions dependency on temperature, meaning that if another factor that affect the reaction rate is changed a new Arrhenius plot needs to be determined.
The activation energy $E_a$ of a reaction is the energy required to reach the transition state for a reaction, in other words, it is the energy needed to start the reaction, see Figure 3. As such, the activation energy will be constant for a specific reaction pathway even though the reaction rate might differ - a higher energy input results in a larger number of molecules reaching the activation energy, giving a higher rate of reaction.

![Activation energy diagram](image)

Figure 3: Example of an energy profile of an exothermic reaction. The height of the energy barrier between reactants and products is the activation energy.

2.2 Different Methods of Analysis

In order to determine the rate constant $k$, the molecular weight for the different samples had to be determined. In this section some instrument that could be used to measure the weight average molecular weight will be described.

2.2.1 Size Exclusion Chromatography

Size Exclusion Chromatography (SEC) is a chromatographic method for the measurement of the molecular weight. The results given by SEC are often misinterpreted, as the method does not actually measure any physical properties directly related to the molar mass or the molecular weight; SEC simply separates the molecules based on their volume, not molecular weight, in order to obtain average molar masses.

A separating column separates the different sized HA molecules from each other and since the salt particles are so small they will elute at a much later time and thus be completely separated from the HA sample. The sample passes through a column that contains a gel-packing matrix and diffuses into and out of the pores inside the gel. The smaller molecules (the salt) diffuse more frequently and will have a longer distance to travel and will therefore be the last molecules that exit the column while the larger molecules diffuse less frequently and will have a shorter distance to travel, hence the larger molecules exit first. (Harris, 2010)
The SEC-column is coupled with light scattering and a concentrations detector is used for molecular weight characterization of polysaccharides and other water-soluble polymers.

2.2.2 Light-scatter

Light scatter is a technique used in physical chemistry where light is sent out, and after hitting a molecule or a particle some of the light will be absorbed and re-emitted in all directions (Limited, 2013). There are two different types of light scatter techniques: dynamic and static, and some times both are used for analysis at the same time. Dynamic light scattering (DLS) is a technique that measures the size and size distribution for molecules and particles and is especially applied for particles less than 1 nm.

Static light scattering (SLS) is a technique that measures the absolute molecule weight through the intensity of the scattered light by a molecule and its molecular weight and size described by the Rayleigh theory (Instruments, 2016). The Rayleigh theory states that large molecules spread more light than smaller molecules from a given light source and that the intensity of the scattered light is proportional to the molecular weight of the molecule. From this, one can gain the weight average molecular weight (\(M_w\)) of a macromolecule, for example a polysaccharide.

The light scatter instrument can be of different types: either a multi-angle-light scattering (MALS) detector or a right angle light scattering (RALS) detector. RALS is measuring at a right angle, which is the only angle that is used in the measurements. A RALS can be a stand-alone instrument or a MALS can be used as a RALS. Since RALS is only measuring at 90° it is assumed that the intensity of the scattered light is the same at this angle as it is for 0° (Limited, 2013). This technique is not suitable for large molecules since they usually have an angle dependence.

A MALS detector measures at several different angles. The flow cell of a MALS photometer is surrounded by an array of photo diodes with each photo diode fixed at a specific angle (Podzimek, 2015). Depending on the specific refractive index increment \(dn/dc\) of the solvent, the angles changes. The specific refractive index increment \(dn/dc\) is important in order to collect any measurements from light scattering. The value of \(dn/dc\) decreases with increasing wavelength, though this dependence is less apparent at higher wavelengths meaning that the \(dn/dc\) will not differ much at higher wavelengths.

The \(dn/dc\) is also dependent of temperature and solvent, and it is connected to the sensitivity of the measurement, because at given concentrations and molar mass the intensity of the scattered light increases with \(dn/dc\) squared (Podzimek, 2015).

2.2.3 Refractive Index detector

Different concentration detectors can be connected to the light scatter device such as an ultra violet (UV) detector, mass spectrometry (MS) detector or a refractive index (RI) detector. The MS-detector have become more and more popular since it is the most selective and sensitive detector, though this device can only be used if the analyte can be ionized. The UV-detector is close behind the MS-detector in terms of selectivity and sensitivity but is based on the absorbance of ultraviolet (UV) light and can only be used if the compound is fluorescent (Dolan, 2012).

The RI-detector, or as usually referred to as differential refractive index detector, is as the name reveals, based on \(dn/dc\). RI-detectors are less sensitive and less selective than a UV-detector and can also be affected by factors that affect \(dn/dc\), for example temperature. It measures the difference in \(dn/dc\), which means that as long as only solvent (mobile phase) flows through the detector no peak will appear. But as the analyte passes the difference in \(dn/dc\) is detectable and a signal is recorded (Dolan, 2012).

There are two ways to detect the light of the lamp in an RI-detector; either by light refraction or the changes of angles as it passes through the fluid of the flow cell. Some detectors are able to detect absolute refractive index and some, which is more common, measures the difference in \(dn/dc\). As the light is being sent through the fluid it must be detected, usually this is done by photo diodes, and as the refractive index changes so does the light shining on the diode. This change is detected and compared to the relative intensity of the signal produced by the photo diodes (Dolan, 2012).
2.3 Capillary Viscometry

One other method that could be used to analyse the samples of HA is capillary viscometry, which means that the time taken for a defined quantity of fluid to flow through a capillary with a known diameter and known length is measured. The method could be performed by hand with a glass capillary viscometry or with an automated viscometer. By knowing the time it takes for the fluid to flow through the capillary, the molecular weight can be calculated with a series of equations.

2.4 Limitations

Parts of the analysis equipment, for example the UV-detector that are a part of the instrument but not used or of interest for the results, will not be described or explained.
3 Method

3.1 Choice of method

The project group chose to use the analysis method Size Exclusion Chromatography (SEC) with static light scattering. This method is an absolute method that measures the weight average molecular weight i.e. it does not measure against any other standard value but measures an absolute (direct) value for that sample.

The choice of using this method was partly for economical reasons and for practicality, although a lot time could have been saved using Capillary Viscometry. The project group also had more knowledge about the SEC method with light scattering rather than the alternative method.

The analytical equipment, the SEC column, needed to be equilibrated before analysis with the correct buffer, but only if used earlier with another buffer. This would only be done for a short amount of time, maximum of two days at low flow to stabilize the column. The instrument was also available a limited amount of time.

Due to the time restraint it was decided, unless samples had to be remade, that only one sample each was to be made and analysed for each set of temperature/NaOH-concentration/reaction time. This would give 48 sample plus two time zero samples giving a total of 50 samples. It was decided that the following temperatures: 20, 40, 60 and 80°C would be used for the experiment. The temperatures were chosen with one temperature close to room temperature, 20°C, one high temperature well below the boiling point of water, 80°C, in order not to loose too much water. The remaining two temperatures were at even intervals between the two. The different concentrations of NaOH were chosen to be 1, 2, 4 and 10 %(W/W) which was new for this area of research. Covering a full order of magnitude of concentrations makes the comparison between samples easier.

The choice of neutralization agent was Phosphoric acid, which has a pKₐ = 7.2, close to a completely neutral solution. The molar relationship between NaOH and Phosphoric acid is 1:2/3 because of this buffer point. The choice was also facilitated by the fact that the analytical equipment could be run on a phosphate buffer without the need to filter the salt from the samples.

3.2 Procedure of method

3.2.1 Preparation of NaOH and Phosphoric acid stock solutions

Two stock solutions of 200g 20%(W/W) NaOH and 5M Phosphoric acid were prepared to facilitate the preparation of the amounts of NaOH and Phosphoric acid to each sample. They were prepared accordingly:

20%(W/W) NaOH
40.0g NaOH was added to 160.00g of DIW (De-Ionized Water) and dissolved.

39%(W/W) (5M) Phosphoric acid
138.5g 85%(W/W) H₃PO₄ was added to a 250ml measuring flask and diluted to 250ml with DIW.

3.2.2 Preparation of Phosphate Buffer (150mM NaCl and 50mM Na₂HPO₄)

One measuring flask (250ml) was filled to half with DIW. 3.3ml of concentrated H₃PO₄ and 3g NaOH (s) was then added to the flask and filled to 250ml with DIW. pH was measured with a pH meter and was slightly compensated with NaOH to bring the solution to a pH of 7.2. Another measuring flask (250ml) was mixed with 45.8g of NaCl and filled to 250ml with DIW. Both flasks were mixed in a 5L can and filled to 5L with DIW.

3.2.3 Preparation of Samples

The HA-samples were prepared in sets of three with each sample having the same concentration NaOH and the same temperature, with different reaction times (t₁, t₂ and t₃) according to Table 1 below. This is to
facilitate the handling and accuracy of the experiment by not being overwhelmed by the amount of samples at the same time. As well as two unreacted reference samples (two different batches of HA were used due to shortage of substance) representing \( t = 0 \) which is the same for all temperatures and NaOH-concentrations. All the measurements were done on an calibrated scale directly into 20ml sealable vials with an accuracy of milligrams \((10^{-3}g)\).

Table 1: Sample table at a specific temperature \( T \). The table is repeated four times for the different temperatures.

<table>
<thead>
<tr>
<th>Reaction time</th>
<th>1% (W/W) NaOH</th>
<th>2% (W/W) NaOH</th>
<th>4% (W/W) NaOH</th>
<th>10% (W/W) NaOH</th>
</tr>
</thead>
<tbody>
<tr>
<td>( t_1 )</td>
<td>1% (W/W) NaOH</td>
<td>2% (W/W) NaOH</td>
<td>4% (W/W) NaOH</td>
<td>10% (W/W) NaOH</td>
</tr>
<tr>
<td>( t_2 )</td>
<td>1% (W/W) NaOH</td>
<td>2% (W/W) NaOH</td>
<td>4% (W/W) NaOH</td>
<td>10% (W/W) NaOH</td>
</tr>
<tr>
<td>( t_3 )</td>
<td>1% (W/W) NaOH</td>
<td>2% (W/W) NaOH</td>
<td>4% (W/W) NaOH</td>
<td>10% (W/W) NaOH</td>
</tr>
</tbody>
</table>

The reaction time for each temperature was decided by preliminary samples. The preliminary samples used were 20\(^°\)C with the concentrations 1 and 10\% NaOH and 80\(^°\)C with NaOH concentrations of 1 and 10\% (W/W), these samples were prepared similarly as described below. These samples were used to determine the cornerstones of the timetable so that the reaction time for each temperature could be estimated. The times for the main experiment are shown in Table 2.

Table 2: Timetable for the different temperatures (For samples 40\(^°\)C, \( t_3 \) at 1 and 4\% NaOH was shortened to 25s and 200s respectively.)

<table>
<thead>
<tr>
<th>Temperature ((^°)C)</th>
<th>20</th>
<th>40</th>
<th>60</th>
<th>80</th>
</tr>
</thead>
<tbody>
<tr>
<td>( t_1 ) (s)</td>
<td>28</td>
<td>800</td>
<td>7</td>
<td>200</td>
</tr>
<tr>
<td>( t_2 ) (s)</td>
<td>86</td>
<td>400</td>
<td>14</td>
<td>400</td>
</tr>
<tr>
<td>( t_3 ) (s)</td>
<td>172</td>
<td>800</td>
<td>25</td>
<td>200</td>
</tr>
</tbody>
</table>

10
The 20%(W/W) NaOH solution was mixed with H₂O for each Sample# (see Table 3) in a separate vial and pre-heated at the designated temperature, this to ensure that when the reaction was started the sample quickly reached the wanted temperature. The 39%(W/W) phosphoric acid was also mixed with H₂O in a separate vial for each Sample# (see Table 4) and cooled in an ice bath, so that the heat generated from neutralizing the sample was minimal and cooled as fast as possible to stop the reaction.

Each sample was prepared with 3,750g 2%(W/W) HA in a third vial. The pre-heated NaOH vial was well mixed in the HA vial before being put into the thermostat. The reaction was stopped at time t (Table 2) by neutralizing the sample with the cooled phosphoric acid and immediately cooling the sample in an ice bath. 0.500g of the reacted sample was extracted and diluted with 12,000g DIW in a new vial to reach the desired HA concentration for the analysis (0.02%(W/W)).

Table 3: Reaction sample preparation measurements.

<table>
<thead>
<tr>
<th>Sample#</th>
<th>NaOH (%(W/W))</th>
<th>HA 2%(W/W) (g)</th>
<th>NaOH 20%(W/W) (g)</th>
<th>H₂O (g)</th>
<th>Total mass (g)</th>
<th>HA %(W/W)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1%</td>
<td>3.750</td>
<td>0.5</td>
<td>5.750</td>
<td>10.00</td>
<td>0.750%</td>
</tr>
<tr>
<td>2</td>
<td>2%</td>
<td>3.750</td>
<td>1</td>
<td>5.250</td>
<td>10.00</td>
<td>0.750%</td>
</tr>
<tr>
<td>3</td>
<td>4%</td>
<td>3.750</td>
<td>2</td>
<td>4.250</td>
<td>10.00</td>
<td>0.750%</td>
</tr>
<tr>
<td>4</td>
<td>10%</td>
<td>3.750</td>
<td>5</td>
<td>1.250</td>
<td>10.00</td>
<td>0.750%</td>
</tr>
</tbody>
</table>

Table 4: Neutralization sample preparation measurements.

<table>
<thead>
<tr>
<th>Sample#</th>
<th>Mass of sample (g)</th>
<th>Phosphoric acid 39%(W/W) (g)</th>
<th>H₂O (g)</th>
<th>Total mass (g)</th>
<th>HA %(W/W)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10.00</td>
<td>0.419</td>
<td>4.581</td>
<td>15.00</td>
<td>0.500%</td>
</tr>
<tr>
<td>2</td>
<td>10.00</td>
<td>0.838</td>
<td>4.162</td>
<td>15.00</td>
<td>0.500%</td>
</tr>
<tr>
<td>3</td>
<td>10.00</td>
<td>1.675</td>
<td>3.325</td>
<td>15.00</td>
<td>0.500%</td>
</tr>
<tr>
<td>4</td>
<td>10.00</td>
<td>4.188</td>
<td>0.812</td>
<td>15.00</td>
<td>0.500%</td>
</tr>
</tbody>
</table>
3.3 Analysis

3.3.1 Analytical equipment

The analytical equipment consisted of one SEC-column, a UV-detector, a MALS (Multi-Angled Light Scattering detector) and a Refractometer connected according to Figure 4.

The column used in this project is a superose-6 10/300 GL with particle size 11 to 15 $\mu$m with a molecular weight range of 5000 to 5 000 000.

![Analysis schematic](image)

Figure 4: Schematic figure of the analytical equipment

![Equipment used in the analysis](image)

Figure 5: Equipment used in the analysis. From top left, counter clockwise: Refractometer, MALS, UV-detector and SEC-column.

The system was equilibrated with phosphate buffer at least 48 hours before any analysis was done.
3.3.2 Equipment settings

The laser used in the light scatter had a wavelength of 690 nm and for hyaluronic acid with sodium chloride in water solution, dn/dc was 0.155 mL/g at room temperature (Podzimek, 2015). The flow in the system was set to 0.5ml/min during analysis.

3.3.3 Analysis of samples

A 0.3ml sample was injected into the injector with a syringe at the same time the data collection for the software was started. After the sample was detected and the baseline was back to normal (approximately 25 minutes) the software recording was stopped and the results were calculated. A period of at least 30 minutes was required between each analysis to stabilize the baseline and letting the salt from the previous analysis elute.

In order to determine the weight average molecular weight of the samples, integration intervals were required. These intervals were set to span two-thirds of the height of the dRI peak (The blue line seen in Figure 6).

![Figure 6: Example Chromatogram from sample 20°C, 1% NaOH, t₃. Lightscatter is shown in red, dRI in blue and UV is not shown, Y-axis is shown in a relative scale. The integration window cover approximately two-thirds of the dRI peak.](image)

During the preliminary experiment a suitable HA concentration for the analysis was also investigated. By diluting one sample into different HA concentrations and analyze those, their chromatograms could be examined in order to see which chromatogram gave the clearest data.
4 Results

4.1 Preliminary Experiment

The preliminary experiment gave information about suitable times that the samples should be in water bath for the different temperatures. They also gave information that the 0.02 %(W/W) HA concentration gave the clearest data during the analysis.

4.2 Main Experiment

Table 5: Molecular weight for reference samples

<table>
<thead>
<tr>
<th></th>
<th>Mw (g/mole)</th>
<th>Deviation (±%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>First Batch</td>
<td>0.748 × 10^6</td>
<td>2</td>
</tr>
<tr>
<td>Second Batch</td>
<td>0.769 × 10^6</td>
<td>2</td>
</tr>
</tbody>
</table>

Table 6: Raw data for samples at 20 and 40°C

<table>
<thead>
<tr>
<th>Temperature °C</th>
<th>NaOH Concentration (%(W/W))</th>
<th>Time</th>
<th>Mw (g/mol)</th>
<th>Deviation (±%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>1</td>
<td>4 h</td>
<td>0.6251 × 10^6</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>12 h</td>
<td>0.5241 × 10^6</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>24 h</td>
<td>0.4070 × 10^6</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>4 h</td>
<td>0.6070 × 10^6</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>12 h</td>
<td>0.5407 × 10^6</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>24 h</td>
<td>0.3453 × 10^6</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>4 h</td>
<td>0.7145 × 10^6</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>12 h</td>
<td>0.5629 × 10^6</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>24 h</td>
<td>0.3186 × 10^6</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>4 h</td>
<td>0.5411 × 10^6</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>12 h</td>
<td>0.5318 × 10^6</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>24 h</td>
<td>0.2624 × 10^6</td>
<td>1</td>
</tr>
<tr>
<td>40</td>
<td>1</td>
<td>2 h</td>
<td>0.3483 × 10^6</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4 h</td>
<td>0.2496 × 10^6</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>8 h</td>
<td>0.1687 × 10^6</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>2 h</td>
<td>0.2452 × 10^6</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4 h</td>
<td>0.2299 × 10^6</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>8 h</td>
<td>0.1707 × 10^6</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>2 h</td>
<td>0.3451 × 10^6</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4 h</td>
<td>0.2047 × 10^6</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>7 h</td>
<td>0.1201 × 10^6</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>2 h</td>
<td>0.2383 × 10^6</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4 h</td>
<td>0.1601 × 10^6</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>7 h</td>
<td>0.1169 × 10^6</td>
<td>1</td>
</tr>
</tbody>
</table>
Table 7: Raw data at 60 and 80°C

<table>
<thead>
<tr>
<th>Temperature °C</th>
<th>NaOH Concentration (%(W/W))</th>
<th>Time</th>
<th>$M_w$ (g/mol)</th>
<th>Deviation (±%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>60</td>
<td>1</td>
<td>10 min</td>
<td>$0.4345 \times 10^6$</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>20 min</td>
<td>$0.2913 \times 10^6$</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>40 min</td>
<td>$0.1801 \times 10^6$</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>10 min</td>
<td>$0.3491 \times 10^6$</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>20 min</td>
<td>$0.2164 \times 10^6$</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>40 min</td>
<td>$0.1215 \times 10^6$</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>10 min</td>
<td>$0.3278 \times 10^6$</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>20 min</td>
<td>$0.1985 \times 10^6$</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>40 min</td>
<td>$0.1129 \times 10^6$</td>
<td>2</td>
</tr>
<tr>
<td>80</td>
<td>1</td>
<td>10 min</td>
<td>$0.2794 \times 10^6$</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>20 min</td>
<td>$0.1441 \times 10^6$</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>40 min</td>
<td>$0.08815 \times 10^6$</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>2 min</td>
<td>$0.4745 \times 10^6$</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5 min</td>
<td>$0.3015 \times 10^6$</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>8 min</td>
<td>$0.1753 \times 10^6$</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>2 min</td>
<td>$0.3801 \times 10^6$</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5 min</td>
<td>$0.1810 \times 10^6$</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>8 min</td>
<td>$0.1036 \times 10^6$</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>2 min</td>
<td>$0.3299 \times 10^6$</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5 min</td>
<td>$0.1613 \times 10^6$</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>8 min</td>
<td>$0.1090 \times 10^6$</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5 min</td>
<td>$0.1243 \times 10^6$</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>8 min</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

The deviation is the uncertainty margin of the molecular weight given during the analysis. 80°C, 10 %(W/W) and 8 minutes gave no result. It is believed that the hyaluronic acid in this sample was broken down into segments too small for detection.

The data from Tables 6 and 7 were plotted as described in section 2.1.
Figure 7 shows the last step through which $E_A$ can be calculated. Other graphs can be found in the Appendix.

$E_A$ was calculated by multiplying the slopes in Figure 7 by $-R$ and plotted against NaOH concentration in Figure 8.
Table 8: Calculated activation energies at the different NaOH concentrations.

<table>
<thead>
<tr>
<th>Concentration NaOH (%(W/W))</th>
<th>Activation energy (J/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>95617</td>
</tr>
<tr>
<td>2</td>
<td>99768</td>
</tr>
<tr>
<td>4</td>
<td>96781</td>
</tr>
<tr>
<td>10</td>
<td>98937</td>
</tr>
</tbody>
</table>

Figure 8: Relationship between Activation Energy $E_A$ and concentration of NaOH (%(W/W))

Figures 9 through 12 show comparisons of reaction rates of the different NaOH at a specific temperature, in order to illustrate the effect of NaOH concentration on reaction rate.
Figure 9: Reaction rate comparison of samples at 20°C

Figure 10: Reaction rate comparison of samples at 40°C
Figure 11: Reaction rate comparison of samples at 60°C

Figure 12: Reaction rate comparison of samples at 80°C
Figure 13: The Reaction rate $k$ at different temperatures against the Concentration (%(W/W)) of NaOH.
5 Discussion

5.1 Preliminary Results

As presented in the results section, the signal at 30 minutes for the samples at 80°C gave no clear data. It is therefore believed that the HA had been broken down into segments too small for the MALS to detect. The heating times were shortened and the HA concentration doubled in order to give a stronger signal for small segments.

According to the analysis of sample 2, 3, 5 and 6, the HA decomposed slowly at 20°C after one hour. Therefore there was no significant change in molecular mass over time. The testing times were thus prolonged in order to get a greater change in molecular mass.

The conclusions from these preliminary results show how the suitable reaction times for the samples in the water bath at the different temperatures could be determined. The preliminary result did also give the information that the HA concentration needed to be higher when analysing the samples.

5.2 Main Experiment

At all temperatures, each concentration is degraded at a linear relationship if the difference in molecule weight and to Figures 9 through 12 is looked at which confirms that a hydrolytic reaction occurs. Some points are deviating: 20°C, 10% between time 1 (4h) and time 2 (12h), and at 40°C, 2% between time 1 (2h) and time 2 (4h) in Table 7, where the difference in molecular weight is not that great (0.0093 vs. 0.0153) compared to other values. In order to investigate these values, the samples should be reanalysed. In addition, the samples may need to be redone.

At all temperatures it can be seen that the higher the concentration of NaOH, the lower is the detected molecular weight. This means, as expected, that the concentrations of NaOH have an impact on the degradation of HA, illustrated in Figures 9 through 12. It could also be noted that contrary to the exponential trend of the temperature’s effect on the degradation rate, the NaOH concentration and degradation rate followed a linear tendency. Since much shorter time intervals were needed for 80°C compared to 20°C in order to detect any molecular weights with the used settings, the temperature plays a great part at the degradation. This is also seen at 80°C and 10% time 3: 8 min in Table 7, where the molecular weight is thought to be too low for detection with chosen detectors, meaning that the degradation occurs at a very high rate in this sample.

It was expected that the higher the concentration at each temperature, the lower should the molecular weight be, compared at the same point in time. For concentrations at 20 and 40°C in Table 7, this is not always the case and since the degradation depends of the temperature, a change in temperature would affect the results. At 20°C, the water bath had some trouble maintaining the temperature, due to the surrounding temperature being higher. Another problem was that in the lab where the analysis was made there was a big window just in front of the analytical equipment and sunrays were hitting the column. This might have increased the temperature of the column on a sunny day, which could have affected both sample and dn/dc since the incoming fluid would have had a higher temperature and the dn/dc used is for 25°C. This was unfortunately discovered too late and could simply have been avoided using aluminium foil, since aluminium foil could easily been wrapped around the exposed column in order to reflect the light of the sun. For stabilizing the temperature of the column, an oven can be used as this gives less unknown temperature fluctuations. However, this kind of instrument was not available for the project.

It seems to be that the difference in degraded HA, Table 7, is lower at higher temperatures than at the lower temperatures. This might depend on the fact that the degradation occurs so fast that the time intervals should have been shortened at the higher temperatures, which would be hard to do with the method used for the sample preparation in this project.

During the experiment the first batch of HA ran out, resulting in samples being prepared from two different batches of HA. However, as seen in Table 5, these two batches give similar results, indicating that using two different batches does not affect the final result in any significant way.
The activation energy for the samples can be seen in Table 8. As the values are so similar, it can be assumed that the reaction pathway at the different NaOH concentrations is the same. Calculating a mean from the values gives an activation energy of 97.8 ± 1.8 kJ/mol with a 95% confidence interval. This activation energy differs from the activation energy found by Tommeraas and Melander (2008) determined to be 137 kJ/mol. This indicates that another reaction pathway occurs during this investigation. It is thought that OH\(^{-}\), rather than acting as a base in an acid/base degradation reaction, is acting as a nucleophile in this experiment.

The physical properties of HA is important for the clinical function of fillers. By knowing the degradation rate at different crosslinking conditions, it is possible to predict the HA chain length of the final product. This can be used to create different types of gels, with different properties.

Since the pH is at elevated levels during the crosslinking process, possibly with an elevated temperature, the hyaluronic acid will degrade to a molecular weight lower than the original raw material during the process. This means that the weight of the final product will have a lower molecular weight than the starting HA, and therefore different physical properties.

If the degradation rates at different crosslinking conditions are known, it is possible to predict the length and weight of the HA chains in the final product. This means that it is also possible to predict how changes in the crosslinking process will affect the properties of the final product.

### 5.3 Conclusions

The activation energy of the degradation at high sodium hydroxide concentrations was determined to be 97.8 ± 1.8 kJ/mol. This activation energy is lower than what has been found in prior studies, meaning that hyaluronic acid degrade at a higher rate in a highly basic environment compared to in an acidic environment.

These results are useful for Galderma since the knowledge of the degradation rate of hyaluronic acid at different crosslinking conditions allow them to predict how changes to the crosslinking process will affect the physical properties of the final product.
Bibliography


6 Appendix

6.0.1 Material
- Sodium Hydroxide, NaOH (s)
- 2% Hyaluronic Acid (HA) (aq)
- Conc. Phosphoric Acid, H₃PO₄ (85 % (W/W)) (aq)
- De-ionized water (DIW)
- Ice bath

6.0.2 Lab Materials
- Thermostat
- 20ml Vials
- Accurate Thermometer
- 4 250ml Containers for stock solutions
- 5L can for buffer solution
- Automatic pipette
- pH-paper
- SEC-column (12µm)
- UV-Detector
- Refractive index detector (RID)
- Multi Angled Light Scatter (MALS)
6.1 Graphs over inverted molecular weight vs. time that was used to create the arrhenius plot.

Figure 14: Inverted Molecular Weight against Time at 20°C
Figure 15: Inverted Molecular Weight against Time at 40°C
Figure 16: Inverted Molecular Weight against Time at 60°C
Figure 17: Inverted Molecular Weight against Time at 80°C
6.2 Examples of chromatograms

Figure 18: Chromatogram of 2% NaOH sample that have been in a 20°C water bath for 4 hours.

Figure 19: Chromatogram of 2% NaOH sample that have been in a 20°C water bath for 24 hours.
Figure 20: Chromatogram of 1% NaOH sample that have been in a 40°C water bath for 2 hours.

Figure 21: Chromatogram of 1% NaOH sample that have been in a 40°C water bath for 8 hours.
Figure 22: Chromatogram of 4% NaOH sample that have been in a 40°C water bath for 10 minutes.

Figure 23: Chromatogram of 4% NaOH sample that have been in a 40°C water bath for 40 minutes.
Figure 24: Chromatogram of 10% NaOH sample that have been in a 80°C water bath for 2 minutes.

Figure 25: Chromatogram of 10% NaOH sample that have been in a 80°C water bath for 5 minutes.