Robustness testing of lab bench procedures for studying degradation of hyaluronic acid (HA) hydrogels

Bachelor thesis

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### 1 Abstract

In this report the already existing method in the report called “Development of the peroxyl radical degradation method” is supplemented to understand the critical factors that decide the degradation rate of hyaluronic acid (HA) in Galdermas products in vitro using radicals. The conclusion of the
report is that the method is relatively robust. It seems to be that the degradation rate of the hyaluronic acid is dependent on the amount of gel that is weighed in and the free amount of HA that is in the sample. The filtration step after degradation of softer gels might give misleading results. A survey over some of Galdermas products is done.

2 Introduction

Hyaluronic acid (HA, hyaluronan) is a linear, non-branched polymer of a disaccharide unit containing D-glucuronic acid and N-acetyl-D-glucosamine.[1] The polymer is a high molecular substance with a molecular weight of up to 10 million Da. It is also highly hydrophilic and plays an important role in the extra cellular matrix (ECM) for composition and structure, among other important functions.[2,3] The fact that the polymer has a lot of negative charges is the reason for that hyaluronic acid can keep large amounts of water, and therefore act as filler and osmotic buffer, which is one of the causes for why it is used in products for dermal fillers, osteoarthritis treatment, and other treatments and applications.[4] The half life of free hyaluronic acid is in vivo about 1 day, which implies that an injection of free hyaluronic acid in the body only lasts for about a week before it is fully degraded. The degradation rate of hyaluronic acid is in tissues dependent on degrading enzymes (hyaluronidases), radicals, pH and temperature.[5] To prevent the rapid decomposition of the gel products in the body, a cross linker called 1,4-butanediol diglycidyl ether (BDDE) is used. BDDE creates a crosslink between the polymers to form a gel-cluster, which hinders the degradation and hence slows down the degradation rate. It has been proposed that the degradation rate of hyaluronic acid with BDDE is dependent on the swelling ratio of the gel, e. g. the ability to let water in to the gel.[5] Another measurement available for gels is the degree of modification (MoD), which is the number of cross links divided by the number of disaccharides.[6,7]

In this radical degradation method the degradation rate of hyaluronic acid gels in vitro is determined by using peroxy radicals to degrade the gels and the thereby released free HA content in samples are measured with size exclusion chromatography.

The radical generator 2,2'-Azobis(2-methylpropionamidine) dihydrochloride (AAPH) used in this degradation method is also dependent on pH, which has been shown in vitro. It is therefore very important the AAPH-solution has the same pH for every measurement.[8] AAPH is a good substance for creating peroxy radicals, since it is not very much affected by the solvent or transition metals.[9] The half-life of AAPH is about 175 hrs at 37° C and pH 7. The first hours of decomposition of AAPH hence peroxy radical formation is almost constant due to the low degradation rate.

The reactions of AAPH are shown in following equations:

\[ R - N = N - R' \rightarrow R' + R'' + N_2 \quad \text{(Equation 1)} \]
\[ R' + O_2 \rightarrow ROO' \quad \text{(Equation 2)} \]
\[ ROO' + X - H \rightarrow ROOH + X' \quad \text{(Equation 3) [9]} \]

As seen in eq. 1, the AAPH molecule breaks down in to two radicalized carbon chains and nitrogen gas. The carbon chains in eq. 2 reacts with oxygen gas to form peroxy radicals, which in eq. 3
picks up a proton from the polysaccharide; and thereafter the degradation of the HA-gel is starting. It is not, however, known where the radicals attack the polymer.

In this report gels may be referred to as “firm” or “soft” gels. The firm gels have a relatively low degree of swelling, and a higher concentration of cross links, both chemical (that comes from BDDE) and mechanical (natural entanglements within the polymer), while softer gels have a relatively high degree of swelling, because of a lower concentration of BDDE and less natural entanglements.[6] Some of the gels contain lidocaine as painkiller.

3 Purpose

The purpose of this report was to supplement the already existing report on radical degradation of hyaluronic acid to get a greater understanding of some of the factors that affects the degradation rate of the HA gels using radicals.

4 Method

4.1 Radical degradation

An outline of the radical degradation method can be seen in Figure 1.

A 20 mM phosphate reaction buffer in 0.9 % aqueous sodium chloride with adjusted pH (7.50 ± 0.05) is first prepared. 0.25 g of the gel to be analyzed is then mixed over night with 8.75 g of the reaction buffer. The AAPH is dissolved in the same buffer solution to a concentration of 100 mM. 1 g of the AAPH-solution is then added to the falcon tubes containing the gel for a final AAPH concentration of 10 mM, and the tubes are vortexed. The tubes are centrifuged to get the free HA in the supernatant and the remaining gel at the bottom of the falcon tube. Every gel that is analyzed has two duplicates which are independent from each other despite the fact that they have about the same amount of gel, buffer solution and AAPH solution.

At time zero, 0.5 ml of the supernatant is moved to an eppendorf tube and filtered through a 0.2 µm syringe filter (13 mm diameter) and transferred to a HPLC-vial. The tubes are then put into a water bath at a temperature of 37 °C to start the degradation, and the timing is started. The same routine is then done every time the sampling is done, at t =1.5 h, 2.5 h, 3.5 h and 4.5 h. After every sampling is done, the tubes are vortexed before immersed to the water bath. Every time the supernatant is removed from the falcon tubes, the time is noted.
4.2 Calculations

In the calculations in this method, the amount of free HA at each sampling time is compared to what is theoretically possible, considering the initial amount of sample and its concentration.

To be able to calculate the residual gel at each sampling time, the HA concentration in the filtrate must first be corrected for the initial extractable HA component to give $C_{\text{deg}(0)}$ as shown in Eq. 4. The part of residual gel at each sampling, $R_{\text{deg}(n)}$, is calculated as $C_{\text{deg}}$ divided by the maximum of gel that can be degraded, $C_{\text{max}}$, see Eq. 8. The residual gel (%) is then calculated according to Eq. 9. It is assumed that the density of the gel and reaction mixture is $1 \text{ g/cm}^3$.

\[
C_{\text{deg}(t)} = C_f(t) - C_f(0) \quad \text{(Equation 4)}
\]
\[
V_{\text{tot}} = m_g + m_b + m_{\text{AAPH}} \quad \text{(Equation 5)}
\]
\[
C_{\text{tot}} = \frac{m_g \cdot C_g \cdot 1000}{V_t} \quad \text{(Equation 6)}
\]
\[
C_{\text{max}} = C_{\text{tot}} - C_f(0) \quad \text{(Equation 7)}
\]
\[
R_{\text{deg}}(t) = \frac{C_{\text{deg}(t)}}{C_{\text{max}}} \quad \text{(Equation 8)}
\]
\[
\text{Residual gel}_{\text{t}}(\%) = (1 - R_{\text{deg}(t)}) \times 100 \quad \text{(Equation 9)}
\]

$C_{\text{deg}(t)} = \text{Concentration of degraded HA in the filtrate at time } t \ (\mu g/mL)$

$t = \text{Sampling time}$

$C_f(0) = \text{Concentration of HA in the filtrate at sampling time } t \text{ given by the SEC analysis } (\mu g/mL)$

$V_{\text{tot}}(t) = \text{Total volume in the Falcon tube prior to the first sampling (mL). The density is assumed to be } 1.0 \text{ g/cm}^3$

$m_g = \text{Weighed amount of gel (g)}$
\( m_b \) = Weighed amount of reaction buffer (g)
\( m_{AAPH} \) = Weighed amount of 100 mM AAPH (g)
\( C_{tot} \) = Total concentration of HA in the sample (gel + extractable HA) (\( \mu g/mL \))
\( R_{deg}(t) \) = Part of degraded HA at sampling time t
\( C_g \) = The total sodium hyaluronat concentration of the weighed gel (mg/mL)
\( C_{max} \) = Concentration of HA in the gel part of the sample, that is the total amount of gel that can be degraded (\( \mu g/mL \))
Residual gel, (\%) = Part of residual gel from time zero at sampling time t (\%)

4.3 Chemicals

- NaCl - Merck
- Sodium sulphate - Merck
- Sodium dihydrogen phosphate dihydrate - Merck
- AAPH – Aldrich
- Acetonitril - Merck

4.4 Instrumentation

HPLC – Waters Alliance 2695 separations module with dual absorbance detector, Waters 2487
Column – Size exclusion Shodex SB402,5-4E, x \( \mu m \), 4.6 * 250 mm
Software – Empower ver. 3

4.5 Liquid chromatography

The HA-content in the solution is measured on a HPLC with size exclusion chromatography and detected with a UV detector. The mobile phase is 90 % SEC-UV buffer (with 80 mM sodium sulfate in 10 mM sodium phosphate buffer with pH 5.5) and 10 % acetonitrile. Samples that contain lidocaine are analyzed for 32 minutes; samples without lidocaine are analyzed for 18 minutes. The HA-content of the samples can be calculated against HA standard solutions of known concentration, from the area of the peak which occurs after 5 min of analysis.

5 Experimental

5.1 Effect of sample weight

To see if the degradation is affected by the weight of the sample, an experiment was carried out with three different amounts of the same gel. 0.15 g, 0.25 g and 0.35 g of the same gel were weighed in respectively, but with the same amount of buffer and AAPH solution to every gel.

5.2 Effect of gel content and filtration

5.2.1 Effect of variation of gel content and amount of HA

2 gels with different degree of swelling (one firm and one soft) were prepared to get 3 levels of gel content by adding different amount of HA to each initial gel. The test was carried out to see if the gels with same degree of swelling are degraded differently depending on the free amount of HA in the sample. The degradation for each gel should look about the same if the degradation of the gels are independent of the free HA that is in the sample.
5.2.2 Effect of filtration on gels with only HA-solution
Effect of the filtration step with gel with only free HA was analyzed by compare filtrated gel with non filtrated gel.

5.3 Extension of degradation time to 6.5 h
Degradation for an extended period of time was carried out to see how the gel is affected after longer time of degradation.

5.4 Repeatability and intermediate precision of the method
The repeatability and intermediate precision of the method was determined of the duplicates from the 10 measurements that have been done.

5.5 Effect of aging of filtrated sample solution
In the method it says that the analysis should be done within three days after reaction. An experiment was carried out to see if this is necessary by analyzing samples 2 days after reaction and 8 days after reaction.

5.6 Analyzing samples in order of gel instead of degradation time and effect of sampling technique

5.6.1 Order of gel instead of degradation time for analysis
When samples are being analyzed in the method, they are analyzed in order of degradation time and gel, and the duplicates are analyzed as pairs after each other. When samples have been analyzed in order of gel and degradation time there seems to be similarity in between duplicates, even though the duplicates are independent from each other, which give an indication of that the chromatography might have an impact of the result. In this test the order of analysis is changed to order of gel, and the duplicates are not analyzed two by two.

5.6.2 Effect of one tube per run or one tube per time point
An experiment was done to see how different sampling techniques affect the result of degradation of the gels. The “normal” technique, in which the same falcon tubes for all of the time where sampling is done are used, is compared to when separate falcon tubes with same amount of gel are used at separate time when sampling is done. One falcon tube is therefore prepared for every time sampling is done, which means that for 2 batches of gel, there are 2 * 2 * 5 test tubes that are prepared and going through radical degradation, instead of 4 (2 batches times the duplicates times test tube for every sampling time).

5.7 Survey of the degradation rate of Galdermas gels
A survey of the degradation rate for some of Galdermas products is done to see how the different properties of the gel affect the degradation rate.
6 Results and discussions

6.1 Effect of sample weight

Figure 2 shows the results from the sampling and the difference in the residual gel after each degradation time for each weighing. The 0.35 g has the slowest degradation, 0.25 g a little bit faster, and 0.15 g had the highest degradation rate. It seems that a smaller amount of gel gives a higher degradation rate. The difference in gel concentration for the samples increases with longer degradation time. At the last sampling time the biggest difference in residual gel between the different samples is about 9 percentage units. The difference between 0.35 g and 0.15 g are greater than for 0.25 g and 0.15 g.

Figure 2 Results from analysis with different sample weights

6.2 Effect of gel content and filtration

6.2.1 Effect of variation of gel content and amount of HA

Figure 3 represents the degradation of the firmer type of gels with lower degree of swelling. The gels with high gel content have lower degradation rate than the gels with lower gel content. The degradation rate in Figure 3 follows the order of increasing degradation with decreasing gel content, which implies that the gel content has an impact on the ability for the radicals to degrade the gel. The firmer gels do anyhow follow a somewhat similar degradation.

The results for the soft gel are shown in Figure 4. The filter came off the syringe during the filtration of the last sample of 82.1% gel content and gave a strange result at the time 4.5 h. That measuring point was therefore removed from the figure. What can be seen overall, though, is that the gels don’t have similar degradation rates, and therefore it appears that various amount of free HA in the sample affect the degradation rate for softer gels.
6.2.2 Effect of filtration on gels with only HA-solution

Since the added free HA was of high molecular weight, it was suggested that the 0.22 µm filter might capture some of the free HA for the early time points. At later time points, this free HA will be degraded, possibly allowing more of the added HA through.

Therefore a degradation of the solution of free HA with and without the filtration step was carried out to see if the filtration affects the result on the residual gel (Figure 5). The gel that had gone through filtration shows a gel content of about 9 %, and the one that did not go shows a gel content of circa 2 %. There is a clear difference between the results, which implies that the filtration has an impact, at least for HA of high molecular weight. It is reasonable to say that the filtration step in
this case gives a misleading result, and that the sample that did not go through the filtration step gives a more accurate result.

![Comparison of gel concentration with gel that have gone through filtration and gel without filtration](image)

**Figure 5** Comparison of gel concentration with gel that have gone through filtration and gel without filtration

### 6.3 Extension of degradation time to 6.5 h

Degradation according to the method was carried out on the reference gel, but extended to 6.5 h instead of 4.5 h to see how the gel acts during an extended degradation. That means that 7 samplings were done, instead of 5. The residual gel concentration after a longer degradation time is shown in Figure 6, compared to all of the reference gel analysis that has been made. The degradation rate seems to increase over the whole degradation time, despite the fact that the amount of gel decreases during the degradation. As seen from the figure, the reference gel is probably fully degraded after about 7 h of degradation.
6.4 Repeatability and intermediate precision of the method

From duplicate analysis of the reference gel at 10 occasions, the repeatability and the intermediate precision could be estimated.

An ANOVA test was carried out to see if there is a significant difference in between days measurements and within measurements on the reference gel, at the last filtration (after 4.5 h of degradation) and after 2.5 h of degradation.

The result from the ANOVA test that was done for the last filtration corresponds to the results that are shown in Figure 7. The ANOVA gave a p-value of 0.005, which shows that there is a significant difference of results between day-to-day measurements of the degraded gels.

Another ANOVA was done on samples that had been degraded for 2.5 h, and also had a p-value of 0.005, which means that there is a significant difference between days at degradation time 2.5 h as well. The variation in between the occasions is (at t =4.5 h) 3.2, and the intermediate precision 3.7 residual gel percentage units.

![Figure 6 Comparison of residual gel concentration with gels that have been degraded for 4.5 h and 6.5 h](image)
6.5 Effect of aging of filtrated sample solution

Reference samples that had gone through radical degradation and filtration were analyzed 2 and more than 8 days after the degradation, to verify if it is necessary to do the analysis within 3 days after the reaction or not (stated in the existing report). The measurement on the samples analyzed 8 days after reaction was done on the same solution as the samples that were analyzed 2 days after filtration. The results are shown in Figure 8.

The difference in residual gel of the 8d samples compared to the 2d samples slightly increases with degradation time. The differences increase to 5.3 percentage units at 4.5 h. The difference is greater than the intermediate precision, and therefore it seems appropriate to keep the analysis within 3 days after filtration.
6.6 Analyzing samples in order of gel instead of degradation time and effect of sampling technique

6.6.1 Order of gel instead of degradation time for analysis

An example of the possibility of that chromatography has an impact of the result is shown in Figure 11 where the both duplicates of gel no. 1 increases in residual gel concentration from time 1.5 h to 2.5 h. The same thing appears to happen in Figure 10 at time 3.5 h on the 0.25 g-gel. It seems strange that both of the duplicates of the samples follow the same pattern when the result theoretically should show a decrease in residual gel instead of an increase. The duplicates are independent from each other, except for that they have the same amount of gel, and the possibility of that both duplicates shows an increase in residual gel seems highly unlikely. It is suggested that these results is dependent on that the chromatography affects the result more than only the degradation.

Figure 9 shows the results from when the analysis was made in order of the gel instead of time. The first 5 duplicates have about the same values and show no difference in between samples. The last two measuring points shows a slight difference between the duplicates. Since there was no other visible increase of the residual gel in any of the samples in Figure 9, maybe more than sample b in the last two points differs a little bit from the curve, it is difficult to see if an analysis with a different order can give a more reasonable result.

![Figure 9](image)

*Figure 9* Results from analysis in different order than the other tests performed

6.6.2 Effect of one tube per run or one tube per time point

The result from the analysis is seen in Figure 10. The results were hard to interpret, compared to the result with same test tubes that were used for every sampling time is seen in Figure 2. The method was also slightly more complicated to do since a larger amount of not only gel, but also reaction buffer and AAPH-solution was needed. It was therefore decided that the method was unnecessary to continue using.
6.7 Survey of the degradation rate of Galdermas gels

7 of Galdermas products were analyzed to see how they behave under degradation with radicals (Figure 11). It appears that tested products cover a wide range of degradation rates. One gel is completely degraded after 3.5h, one gel is above 90 residual gel % after 4.5 h, and the other gels somewhere in between. Since the tested gels are very different in the way they are produced, a large difference in degradation rates may be expected. The large difference in estimated degradation rates suggests that this method may be very useful in testing the durability of new gels that are developed.
7 Conclusions

The radical degradation method is a relatively robust method to measure the degradation of HA-gels, with a repeatability of 3.2 percentage units and an intermediate precision of 3.7 percentage units of residual gel.

The method also seems to be able to distinguish a wide range of gels, which suggests that this method may be very useful in testing the durability of new gels that are developed. If needed, the degradation time could be extended to cover even more durable gels.

The degradation rate is dependent on the amount of gel that is analyzed, where larger amount of gel has a slower degradation rate than small amounts, which means that 0.25 g ± 0.05 g is a good requirement. Testing of aged samples shows that also the requirement to analyze the filtered samples within 2 days is reasonable.

Investigations of degradation rates with various gel content and free HA shows that degradation for firmer gels follows the same pattern where gels with higher gel content has slower degradation rate than gels with lower gel content. The softer gels did not quite follow the same pattern, but the results from the analysis of the gel with 0 % gel content shows that it can be because of that the filtrations of the samples give a misleading results, probably because of that bigger HA-pieces cannot go through the filter or that it prevents residual HA to pass through the filter. Degradation for a longer time does not appear to be necessary, since the degradation rate follows the same pattern as for the shorter degradation times.

To further develop the method one can try to use a filter with larger pores to let the major pieces through the filter to see if it adopts better to analysis of the softer gels. Another part to continue investigating in the method is to do a more comprehensive overview of the impact from the chromatography, not only for order of analysis, but also investigate more in what creates the fronting in some chromatograms of different gels.

8 References


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