Lowering the amount of ethanol needed to create gelatin type A nanoparticle crosslinked with EDC and NHS by the addition of NaCl and changing the pH

Thomas Jan Noordman

Supervised by:

Dr. Rong Wang (RadboudUMC)

Lucas Joziasse (RadboudUMC)

Prof. Dr. Willem Kegel (University of Utrecht)

Abstract

Gelatin is a promising material for the creation of colloidal gel, due to it being cheap and biocompatible. For these colloidal gels, gelatin nanoparticles are needed, which can be made from gelatin A or gelatin B. One of these methods to create gelatin nanoparticle is the desolvation method, were gelatin is first dissolved in water and then ethanol is added. The ratio between ethanol and water needed to create the gelatin nanoparticle is 7.2 to 1. Here the desolvation method for gelatin type A is further developed with a focus on reducing the amount of ethanol needed. To stabilize the gelatin nanoparticles 1-(3-dimethylaminopropyl)-3-ethyl-carbodiimide hydrochloride (EDC) and (N-hydroxysuccinimide) NHS are added as crosslinkers.

It is shown that using salt (NaCl) could reduce the amount of EtOH significantly, however too much salt will result in sedimentation. It is also shown that increasing the pH from 3 to 3.5 would also save a lot of ethanol. However, adding both salt and changing the pH doesn't further decrease the ethanol needed. In the end a reduction of 56% the volume of ethanol was possible. This lowers the ratio of ethanol and water to 3.2 to 1. This allows two times as much gelatin nanoparticle to be created in the same total volume of solvent, making it possible to create 45 g of gelatin nanoparticle in one 5 L batch.

Contents

Chapter 1

Introduction

1.1 Colloidal gels

Colloidal gels are a promising biomaterial. These gels are build up by micro- or even nanoparticles which allow for a "bottom-up" strategy for designing shape-specific bulk materials. These bulk materials can be used in a lot of fields such as drug delivery [1], food products [2], biological sensors [3], tissue engineering [4] and much more [5]. Colloidal gels can be made from many materials depending on the use and properties of the materials [1]. An interesting source for material is the use of protein, especially in the medical field. The use of protein is excellent as they are easy to modify for targeted delivery and they are biocompatible [6]. One of the most used natural protein sources is gelatin [1, 7]. It is cheap, readily available, very biocompatible and biodegradable [1]. It has a very low antigenicity and it doesn't produce harmful byproducts [1]. Because gelatin has a high number of accessible functional groups, chemical modification is also widely available. All these thing makes gelatin a very interesting material for drug delivery and tissue engineering [1]

1.2 Gelatin

Gelatin is a natural polymer consisting of 300 till 4000 amino acids [7]. It consists of repeating amino acids. The basis for the repetition is Gly-X-Pro with *Gly* being glycine, *X* being all different kinds of amino acids, but mostly lysine, arginine, methionine and valine and *Pro* being proline and hydroxyproline. This triblock is responsible for the triple helical structure of gelatin (see also figure 1.1) [1].

It is created by the hydrolysis of collagen [6]. Collagen functions in maintaining the integrity tissue and interacts with cell surfaces, extracellular matrices and growth factors. It is one of the most abundant proteins in mammals [8]. By treating the collagen with partial hydrolysis, gelatin can be formed. This can be done with acid to create gelatin A and with base to create gelatin B. The different types of gelatin differ in IEP (7-9 for A and 4-5 for B). They will also show a difference in drug release potentials [9].

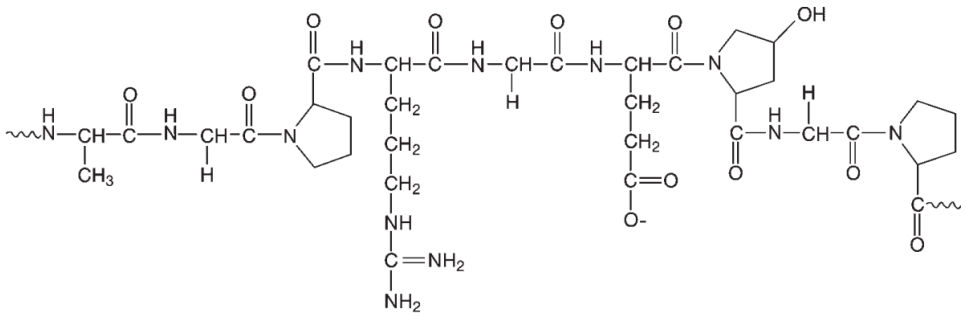

Figure 1.1: The basic chemical structure of gelatin. Source [1]

Since gelatin is such an abandoned polymer, there are many sources to get it from, but mostly the waste products, such as bovine and porcine sources are used [1, 9].

1.3 Gelatin nanoparticles

There are many ways to form gelatin nanoparticles (GNP), such as with micro emulsions or solvent evaporation [1, 9]. In this report, the synthesis for the creating of the gelatin nanoparticles is based on the desolation method (see figure 1.2). Gelatin is soluble in water. It is not soluble in ethanol. Thus by dissolving gelatin in water and then adding ethanol the gelatin will aggregate. What happens is that the gelatin polymers go from a stretched conformation in water to a coil conformation in the ethanol [10]. If this is done fast, then all the gelatin will clump together and sedimentate. If it is done slowly and controlled, small pieces of gelatin will form small coils. The addition of crosslinkers will stabilize the outer layer of these coils, resulting in stable nanoparticles [1, 7].

1.4 Crosslinkers

One of the most used crosslinkers is glutaraldehyde (GA) as it leads to long stability over time [1]. However, since GA is a non-zero length crosslinker (thus part of the molecules becomes part of the GNP), it influences the biocompatibility. Also GA is toxic, which isn't ideal for medical applications [1]. An alternative is the combination of 1-(3-dimethylaminopropyl)-3-ethyl-carbodiimide hydrochloride (EDC) and (N-hydroxysuccinimide) NHS. The GNP became more smooth and more homogeneous than the particles crosslinked with GA. Also, EDC and NHS aren't toxic. They are also a zero-length crosslinker [1], thus they can be washed away later.

The way the mixture of EDC and NHS works is the following: EDC will react with the carboxylic acid groups of glutamic and aspartic acid of the gelatin chains. It activates these groups causing them to hydrolyze, form O-acylisourea groups or react with the free amine groups. Of these three reactions only the reaction with the free amine groups is actually desirable. By adding NHS, the activated acid groups are less susceptible to hydrolyze or the formation of O-acylisourea groups [11]. The free amine groups come mostly from lysine and hydroxylysine [1].

It is important that the concentration of the crosslinkers isn't too high. The reaction between the carboxylic acid groups and the free amine groups isn't very selective. The idea is that this reaction happens within the GNP itself, as this will lead to more stability (so called intramolecular crosslinking). However, the carboxylic acid groups could also react with amine groups from a different GNP (intermolecular crosslinking). Intermolecular crosslinking could lead to sedimentation of the GNP as all the gelatin strains will fuse together. The higher the concentration of crosslinkers, the higher the change of intermolecular crosslinking. However, if the concentration of crosslinkers is too low, then not enough intermolecular crosslinking has happened to stabilize the GNP.

1.5 Goal of the research

The gelatin synthesis is widely used within the R&B group. Right now this is done at a low scale ($\pm 1.25\,\mathrm{g}$). This low scale results in a lot of man-hours spend on creating the gelatin nanoparticles. Also,

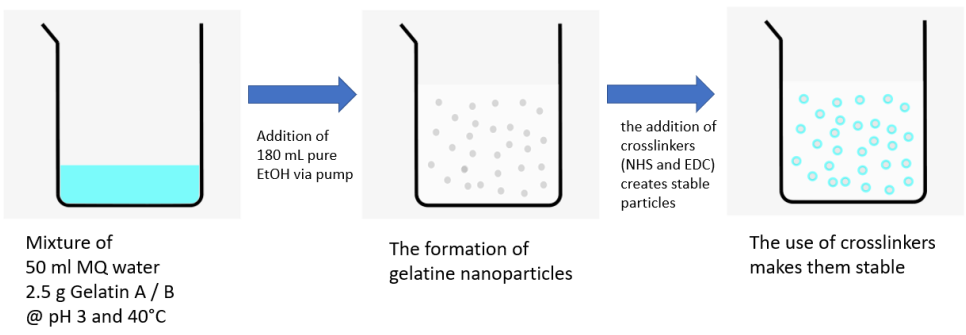

Figure 1.2: A scheme showing the general desolvation method used to create the gelatin nanoparticles

every batch has a slightly different size and polydispersity, which could have its effect on the research. Scaling it up to a higher scale (±10 g or even 50 g) would be ideal. This would not only result in more gel available for research, but also help the overall consistency of the gel as less batches needed to be made. The biggest problem for scaling up is the high amount of EtOH needed; for 1.25 g is 180 mL EtOH needed. To perform this synthesis at a research lab like in the R&B group, the total volume should not exceed 5 L. First of all, a higher volume would not be ideal for a research lab scale. Further, since the EtOH could not be recovered, it would take a lot of EtOH per batch to work at a higher scale. Currently this would mean that around 30 mg of gelatin could be produced per batch. By investigation if EtOH could be saved, more GNP could be made in one batch. However, it is also important to see if the way to reduce the EtOH (such as changing the pH) would not lead to gelatin nanoparticles with different properties.

Chapter 2

Synthesis

2.1 General gelatin synthesis

1.25 g gelatin A is dissolved in 25 mL MQ water while heated at 40 °C and stirring at 700 rmp. After the gelatin is dissolved, the pH is adjusted to 3.0 by adding a few drops of HCl (6.0 M) while measuring with a pH-meter. The mixture is heated back to 40 °C, increasing the stirring to 1000 rpm. Using a pump, set at 4 mL/min, 180 mL EtOH (100%) is added. A milky solution is formed. The solution is cooled to room temperature (20 °C). 50 mg of EDC and 6.0 mg NHS are added to the mixture via a 5 mL solution. The solution is left stirring overnight (15 h). The next day, the solution is cleaned using Crossflow filtration; all of the solution is poured through the crossflow, until about 25 mL is left. 500 mL MQ water is added. The solution is again filtered through the Crossflow until a 25 mL solution is left. Again 500 mL MQ water is added. The solution is again filtered through the Crossflow until around 50 mL solution is left. Then 50 mL of MQ water was poured to the crossflow to get all the nanoparticles out of the crossflow. This total volume of around 100 mL solution is stored in the fridge.

2.2 The effect of salt

To investigate the effect of salt on the gelatin nanoparticle formation, different salt concentrations were used. These salt concentrations should influence the Debye length of the gelatin nanoparticles 2.1.

6.5 g Gelatin A is dissolved in 130 mL MQ water while heated at 40 °C and stirring at 700 rmp. After the gelatin is dissolved, the pH is adjusted to 3.0 by adding a few drops of HCl (6.0 M) while measuring with a pH-meter. 125 mL of the gelatin solution is distributed over 5 beakers (thus 25 mL per beaker). The salt concentration of these 5 beakers were adjusted to 1, 10, 64 and 1000 mM. One batch had no salt added to it. Via a pump, EtOH (100%) was pumped at 4 mL/min until the solutions turned cloudy. The exact volumes can be found in table 3.1. The solutions were cooled to room temperature (20 °C). A mixture of 250 mg EDC and 30 mg NHS was dissolved in 10 mL MQ water. 2 mL of this mixture was added to each gelatin solution. The solutions were left to stir overnight. The particles were cleaned with the use of dialysis over the course of two days. The MQ water was changed multiple times per day. At the end a DLS and zeta potential was measured of the 5 gelatin solutions. The solutions were stored in the fridge.

2.3 The effect of pH

2.3.1 small scale experiment

To look at the effect of pH in a wide range, an experiment at a small scale (125 mg-scale) was set up with 8 different batches. This was done in the following way: 2.8 g gelatin A is dissolved in 56.7 mL MQ water while heated to 50 °C and stirring at 300 rpm. After the gelatin was properly dissolved, 1.25 mL of the gelatin solution was transferred to 8 falcon tubes (thus in total 20 mL of the gelatin solution was used). In each of the tubes various amounts of HCl (1.0 M) were added (0, 10, 15, 20, 25, 30, 35 or 40 µL). This corresponds with a pH of 5.4, 4.5, 4.2, 4.0, 3.7, 3.4, 3.2, 2.8. After stirring for 40 min 100 µL of every vial

Added salt concentration	real salt concentration[1]	Debye length in water	Debye length in ethanol
Salt free	1 mM	9.5 nm	5.4 nm
1 mM	2 mM	6.7 nm	5.1 nm
10 mM	11 mM	2.9 nm	1.7 nm
64 mM	61 mM	1.2 nm	0.68 nm
1 M	1 M	0.3 nm	0.17 nm

[1]the real salt concentration is based on the addition of HCl to lower the pH. The real concentration of charged molecules is difficult to calculate as the gelatin itself will have some desalting effects. This value of 1 mM should then be seen more as an estimated guess to get the order of size rather than the true exact value.

Table 2.1: The calculated Debye length for the various salt concentration for both water and ethanol

was taken out as a sample and transferred to a 96 wells plate (filling up one column of the wells plate). Then 1 mL EtOH (100%) was added to every falcon tube. After 1 min another sample (100 µL) was taken of every falcon tube, filling up another column of the wells plate. This was repeated with every mL of EtOH that was added until 11 mL of ethanol was added and the wells plate was filled. After this, the absorption of the 96 well plate was measured using an absorption meter at various wavelengths. DLS measurements of various wells was performed by dissolving the 100 µL in 900 µL MQ water.

2.3.2 larger scale experiment

Based on the previous experiment, three pH values (4.1, 3.8 and 3.5) were chosen to further investigate the effect at the bigger scale (625 mg scale). This was done in the following way: Three 12.5 mL solutions of 5 w/v% were created while stirring at 40 °C and stirring at 700 rpm. To each batch was 200, 250 or 300 µL of HCl (1.0 M) added, resulting in a pH of 4.1, 3.8 and 3.5 resp. After 15 min is 50 mL EtOH added to the sample via a pump with a speed of 4 mL/min. This resulted in a white smear in the middle of the sample for the batches at a pH of 5.1 and 5.0 (see figure in the appendix, figure 4) , while the sample at 3.1 turned turbid white. Another 10 mL of EtOH was added, however this didn't change anything about the color. DLS samples were taken by diluting the samples 5 times.

2.3.3 pH and Salt

To investigate if adding salt to a higher pH would save more EtOH, the following experiment was set up, similar to the small salt experiment: 1.25 mL of 5 w/v% was transferred to eight different vials. To each vial a different amount of salt and water was added to create different salt concentrations (0, 5, 10, 20, 50, 100, 200 and 500 mM). To each vial was 30 µL HCl (1.0 M) added to lower the pH to 3.5. After stirring for 40 min 100 µL of every vial was taken out as sample and transferred to a 96 wells plate (filling up one column of the wells plate). Then 1 mL EtOH (100%) was added to every falcon tube. After 1 min another sample (100 µL) was taken of every falcon tube, filling up another column of the wells plate. The same was after adding another mL of ethanol and another, all the way till 9 mL of Ethanol was added and the wells plate was filled. After this, the absorption of the 96 well plate was measured using an absorption meter at various wavelengths. DLS measurements of various wells was performed by dissolving the 100 µL in 900 µL MQ water.

2.4 Crosslinkers

To investigate the effect of different amounts of crosslinkers, 3 batches of 1.25 g gelatin A is dissolved in 25 mL MQ water while heated at 40 °C and stirring at 700 rmp. After the gelatin is dissolved, 550 µL HCl (1.0M) was added, lowering the pH to 3.4. Using a pump, set at 4 mL/min, 120 mL EtOH (100%) is added. A milky solution is formed. The solution is cooled to room temperature (20 °C). 50, 80 or 100 mg EDC with 6.0, 9.6 or 12 mg NHS resp. are added to the mixture via a 5 mL solution. The solution is left stirring overnight (15 h). The next day, the solution is cleaned using Crossflow filtration; all of the solution is poured through the crossflow, until about 25 mL is left. 500 mL MQ water is added. The solution is again filtered through the Crossflow until a 25 mL solution is left. Again 500 mL MQ water is added. The solution is again filtered through the Crossflow until around 50 mL solution is left. Then 50 mL of MQ water was poured to the crossflow to get all the nanoparticles out of the crossflow. This total volume of around 100 mL solution is stored in the fridge. The same was repeated with 2 batches of

1.25 g of gelatin, but 100 mL of EtOH (100%) was used. Here 80 or 100 mg EDC with 9.6 or 12 mg NHS resp. are added to the mixture via a 5 mL solution. The same was repeated with 2 batches of 1.25 g of gelatin, but 80 mL of EtOH (100%) was used. Here also 80 or 100 mg EDC with 9.6 or 12 mg NHS resp. are added to the mixture via a 5 mL solution. The DLS was measured via a 5 times dilution from the gelatin solution. For the Zeta potentials the gelatin solutions were 100 times diluted. All measurements were taken thrice.

2.4.1 Crosslinking density

To investigate the crosslinking density of the different gelatin nanoparticles, an assay with TNBS was performed based on the paper by Farbod et al [12]. 5 mg of dried gelatin nanoparticles were dissolved in 1 mL $NaHCO_3$ solution (4% w/v, pH 8.5) and freshly made 0.5% (w/v) TNBS solution. The solution was heated to 40 °C for 2 h. Then 2 mL of HCl (6 M) was added, while the temperature was raised to 60 °C. After 90 min, 4 mL of MQ was added. Of the resulting solution, 100 µL was diluted by 900 µL of MQ water in a 48 wells plate. The samples were measured by an absorption measurement at 346 nm. Each sample was done twice and measured three times (thus six times in total).

2.4.2 Rheology

To create the gel needed for the rheology, a 20% (W/W) solution with water was created. The mixture was put in a 40 °C oven over the day. The mixtures were vortexed a few times during the day. This was done until a homogeneous gel was formed. At the end, the sample was centrifuged for 5 min at 10.000 rcf. The gels were left overnight in fridge before they were measured with an AR 2000 ex Rheometer. For the measurements, an oscillation of 300 s at a strain of 0.5% was done fourth times. After the first oscillation measurement, a frequency sweep was performed going from 0.1% strain to 1000% strain logarithmic (5 points per decade). In between the second and third and third and fourth oscillation time measurement, an amplitude sweep was performed with an angular frequency of 1.0 rad/s from 0.1% strain to 1000% strain logarithmic (5 points per decade. After that a temperature ramp was measured from 20 till 40 °C at 0.5% strain and 1 rad/s

Chapter 3

Results and discussion

3.1 General gelatin synthesis

A batch of GNP was created using the original synthesis. The ratio EtOH and MQ water is 7.2:1. The size was 551 nm (±12.92 nm) with a polydispersity of 0.2213 (±0.03321). The particles had a zeta potential of 14.61 (±1.56).

3.2 The effect of salt

The sizes and zeta potential of five gelatin solutions can be found in table 3.1. This table also shows how much ethanol was added to form the GNP. These values are also plotted as ratio EtOH / MQ water in figure 3.1. These values show that adding more salt decreased the need for ethanol. This corresponds with literature [6]. As mentioned in table 2.1, an increase of salt concentration increases the Debye length. This means that the electrostatic repulsion between the charged gelatin species is less. The gelatin nanoparticles form because they dissolve badly in ethanol, but good in water. Thus by increasing the EtOH concentration, the solution becomes more and more apolar, therefore the gelatin molecules are more likely to stick together. In the end, they will form stable drops of gelatin (the gelatin nanoparticle) which has a good enough volume/surface ratio to become stable in EtOH. Increasing the salt concentration makes the gelatin species less repulsive to each other, thus they will sooner aggregate, therefore less ethanol is needed. Table 3.1 also shows that a higher salt concentration results in bigger

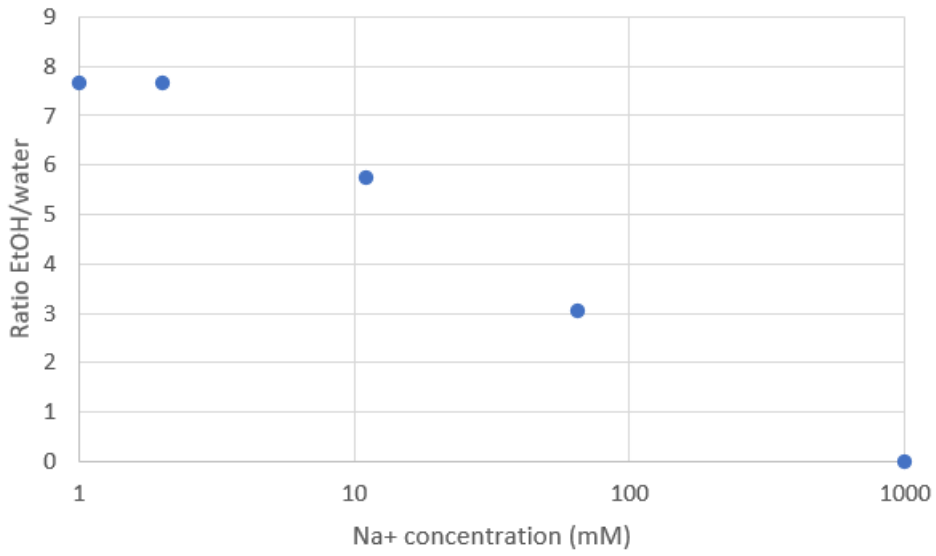

Figure 3.1: The ratio of EtOH and MQ water needed for the gelatin nanoparticles to aggregate, starting from a 5% (w/v) solution of gelatin and water at 40°C

	Volume EtOH (100%) added for precipitation (mL)	size (nm)	zeta potential (mV)
Salt free	240	245.5 (±0.257)	28.87
1 mM	240	265.5 (±0.245)	30.65
10 mM	159	329.1 (±0.186)	31.16
64 mM	99	541.6 (±0.157)	28.87
1 M	0	aggregated	aggregated

Table 3.1: The size and zeta potential for the five different samples at five different salt concentrations. The amount of EtOH added before precipitation is also given

particles. This has to do with polarity. Due to the charged groups attached to the gelatin polymer, the more polar the solvent is where gelatin is dissolved, the bigger the radius of gyration will be. The more salt in the solution, the more polar the solvent will be. Thus the gelatin has less energy gain by attracting to itself, resulting in a less dense ball of gelatin. When the GNP's form, these will then result in bigger particles in more polar solvent.

The 1 M solution was aggregated. This is clearly too much salt. This was expected looking at the calculated Debye lengths (table 2.1). The sample seems to be already aggregated as soon as the salt was added. Here the salt concentration is so high that the repulsion of the gelatin species seem completely gone. Because of this, the nanoparticles themselves are no longer stable in EtOH and will cluster together, resulting in aggregation. It seems that adding only 1 mM of salt doesn't have much of an effect on the ethanol needed, as was expected. The difference between the added salt and the salt already added to change the pH (NaOH) isn't much (both are around 1 mM).

3.3 The effect of pH

The raw data from the absorption measurements can be found in the appendix (fig 2). Figure 3.2 shows the ratio at which the gelatin solutions precipitate for the various pH values. It shows that the lower the pH, the more ethanol is needed. Since the pI of gelatin A is between 7 and 9 [6], a lower pH will result in more negative charged gelatin nanoparticles. The more negative charged gelatin repulse each other more, thus making them less likely to precipitate. Another reason more ethanol is needed to create the gelatin nanoparticles at lower pH has a higher water holding capacity [13]. Because the lower pH leads to the hydrophilic segments of the gelatin (mainly the CO and NH groups in the backbone) more hydrophilic,

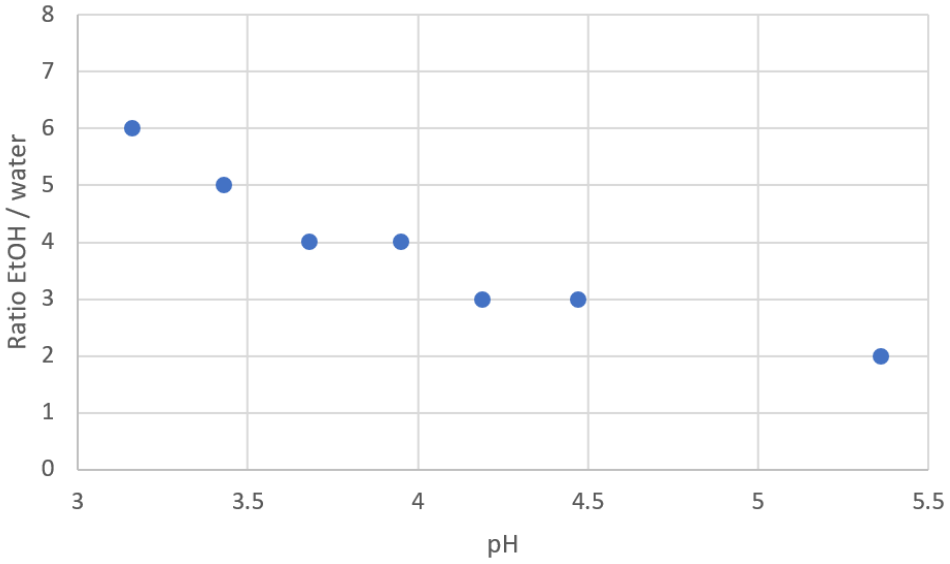

Figure 3.2: The ratio EtOH and MQ water needed to precipitate the gelatin solution (5 w/v%) at different pH values.

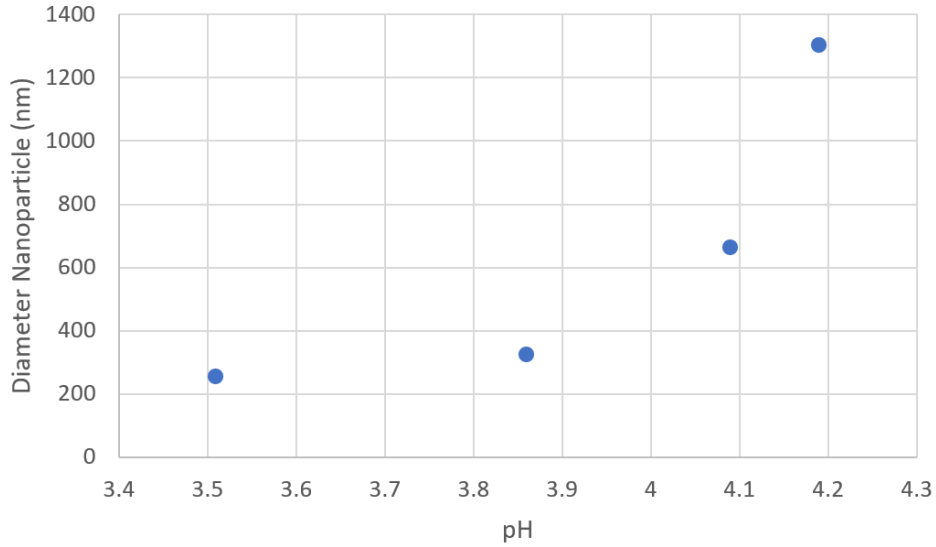

Figure 3.3: The correlation of pH and size of the gelatin nanoparticles. The nanoparticles were not crosslinked and measured without dilution via DLS

which makes the hydration network stronger. As the water retention capacity of gelatin is increased, more ethanol is needed to initiate phase separation [6]. Figure 3 shows that for the higher pH values (above 4) the gelatin particles are rather big. Also, sedimentation was visible in these batches (see also fig 1). The higher pH results in less negative charged gelatin, thus less electrostatic repulsion between the formed gelatin nanoparticles. Because of this, these particles are less stable, due to the Vanderwaals interaction still attracting them to each other. This would explain the sedimentation. It might be worth pointing out that the Pk_a of aspartic acid and glutamic acid are around this point (3.9 and 4.3 resp [6]). This could explain why the tipping point between aggregation (stable particles) and sedimentation (not stable particles) is around this point.

When 40 µL 1.0 M HCl was added, no particles formed. Literature shows that gelatin nanoparticle formation at very low pH is more difficult and only possible at higher gelatin concentrations. Therefore this result was expected.

The result of the DLS measurements can be found in figure 3. The measurements show that adding more acid seems to generate smaller particles (see figure 3.3). The reason for this could be similar to the one from the salt experiment. As mentioned earlier, at a lower pH, the gelatin becomes more hydrophilic. This would increase the radius of Gyration if the gelatin is in water. When the gelatin particles form, the gelatin polymers would be more stretched, resulting in bigger particles.

To have a more precise look on how much EtOH could be saved by changing the pH, three batches (pH, 4.1, 3.8 and 3.44) were scaled up to a higher scale (see also 2.3.2). These pH values were chosen as, based on the previous experiment, a higher pH would lead to sedimentation of the particle, while a lower pH doesn't save much EtOH. After a ratio of 1:4.1 of MQ and EtOH, the batch with a pH of 3.44 turned completely white, while the batches with pH 4.1 and 3.8 showed some whiteness, but not fully. Even adding some more ethanol (turning the ratio to 1:5) didn't make the solution more white (see also figure 4). Since the whiteness of the solution directly correlates to how much gelatin nanoparticles are created, it was decided than a pH of 3.44 would probably be the best pH for the gelatin nanoparticle synthesis.

Figure 3.3 shows the effect of different pH values on the size of the nanoparticles. These batches were made at the 1.25 g scale. The gelatin nanoparticles are uncrosslinked. This graph shows that tuning the pH could also be used to tune the size.

Since it turned out that by changing the pH to 3.5, it is possible to create gelatin nanoparticle with a EtOH/water ratio of 3.2, a lot of EtOH could be save compared to the old synthesis were a EtOH/water ratio of 7.2:1. This is a save of 56%. As stated in the introduction, the largest scale the gelatin nanoparticle could be made is with a total volume of 5 L. As the gelatin needs to be dissolved in a 5% (w/v) solution with water, this would allow for around 45 g of gelatin nanoparticle to be made in one batch (assume a yield of 75%, which is based on measurements in the lab), which would be an increase of 200%.

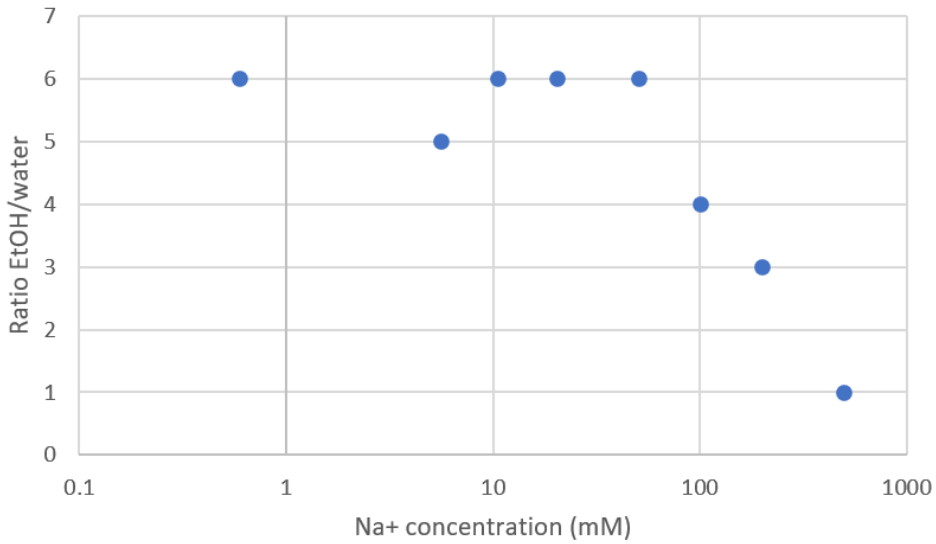

Figure 3.4: The volume percent EtOH needed to precipitate the gelatin solution (5 w/v%) at different salt (NaCl) concentrations at pH 3.4

3.3.1 pH and Salt

Figure 3.4 shows the volume percent of EtOH needed to precipitate gelatin. It shows that a low salt concentration (50 mM and lower), the ratio of EtOH and water is constant (6 to 1). Above 50 mM there seem to be a lot of EtOH saved. However, there is also sedimentation visible (see figure 1). It seems that at a pH of 3.5, the influence of salt doesn't result in more ethanol saved, while creating stable particles. It would make sense that the gelatin nanoparticle aggregate at a lower salt concentration, than with the lower pH of 3, discussed at the beginning of this chapter. The higher pH results in less negative charged gelatin, thus less electrostatic repulsion between the gelatin nanoparticles. Because the salt influences the gelatin stability, aggregation happens therefore at a lower salt concentration than before.

3.3.2 pH of gelatin batches

As the structure of gelatin isn't very consistent, one could question if adding the same amount of HCl to different batches of gelatin would result in a consistent pH. Table 3.2 shows however that that is indeed the case. The table shows that the difference between the highest and the lowest pH values are around 0.13. This seems high, however, this could easily be attributed to deviation of the pH meter itself. By simply cleaning the pH meter an extra time, a difference of 0.10 was measured on the same sample (even though the pH meter was properly cleaned beforehand). Further, there are two things to note here. First, the pH values don't correlate directly to the concentration of HCl (table 3.2 shows for 24 mM HCl an average value of 3.44, while -log(0.024) $\approx$ 1.61). This is due to the salting effect of the gelatin itself. Second, the gelatin referenced in this report was all from the same bottle. It could still be possible that the pH would be slightly different for different bottles of gelatin, since gelatin is a heterogeneous product after all. However table 3.2 shows that within one bottle, the pH would be consistent with a certain concentration of HCl.

3.4 Crosslinkers

Based on these experiments, we can conclude that the setup for saving EtOH is changing the pH to around 3.5. The addition of salt doesn't seem to save (much) ethanol. As it is important that proper gels can be made with these gelatin nanoparticles, it is interesting to see how the lower EtOH concentration and new pH influences the crosslinkers.

To investigate if the concentration of EtOH effects the crosslinking density, the effect of adding Ethanol was further investigated combined with different concentrations of crosslinkers. Different batches were made with 80, 100 and 120 mL EtOH. Here also different amounts of EDC and NHS were added (see also section 2.4). These 80, 100 and 120 mL correspond with a ratio 3.2, 4 and 4.8 to 1 between EtOH and

water.

3.4.1 Size and zeta potential

The size and zeta potentials of the gelatin nanoparticle were investigated via DLS. Table 3.3 shows the DLS measurements for different volumes of EtOH and crosslinkers. For the gelatin batches created with 120 mL EtOH, the gelatin particles seem to get smaller when more crosslinker is added. This probably has to do with more intramolecular crosslinking. For the samples created with 100 and 80 mL it seems that adding more crosslinkers results in bigger particles. However, the difference isn't much (around 10 nm). Thus it is entirely possible that this difference is just because of the variation between batches and not the amount of crosslinker added. Based on repeated experiments a difference of around 30 nm between batches was found. The Zeta potentials seem to be around the same values for all the samples.

3.4.2 TNBSA Assay

The results of the TNBSA assay can be found in figure 3.5. As can be seen, the crosslinking density for these nanoparticles seems to be very low. Only a maximum of 14% of the available amine groups have reacted with the crosslinkers. Because of the low amount, the crosslinkers don't completely stabilize the nanoparticles. Another thing that is interesting, is the fact that adding more crosslinkers doesn't necessarily seem to result in a higher crosslinking density. It is unclear why this is the case.

3.4.3 Rheology

Of the dried gelatin gels (20% (w/w)) the average storage and loss modules were measured (see table 3.4). The values of the storage modulus are high and the values of the loss modulus are low. Further than that, there doesn't seem to be a trend between the values and the difference in the parameters (i.e. the amount of EtOH or crosslinkers used).

The temperature dependent study also shows no clear trend between the gels. For all the batches, the storage modulus drops with temperature and the loss modulus stays more or less the same. However, the 50 mg EDC sample of the 120 mL batch, and the two 100 mL batches show great similarity in their plot (see figure 5). The same goes for the 100 mg of the 80 mL batch and the 80 mg batch of the 120 mL samples (see figure 6). However, the differences in parameters don't seem to explain why these plots look so familiar to each other.

Since the rheology experiments show a great variation in values, which don't seem to correspond with change in parameters - for the temperature dependent study, the gels with no parameters in common e.g. had a more similar results than gels with similar parameters, while for the values for the time dependent study almost all values look random). Based on this, one could conclude that there is a lot of variation between the different gels, but that these variations are not the result of the difference in crosslinkers or the volume of EtOH. It is possible that something in the gel formation process is responsible for these differences. However, it is unclear what is the case. Most of these gels were created at the same time with the same steps. One would expect that the pairs seen in the temperature experiment correspond to, for examples, the pairs in which the gels were created [1] , however, this is not the case. It is unclear

[1] not all gels were created at the same day, due to time constraints. The gels of three 120 mL EtOH batches were made together, the gel of 80 mg EDC and 100 mL EtOH batch was made together with the 100 mg EDC with 80 mL and the gel of 80 mg EDC with 80 mL EtOH batch was made together with the 100 mg EDC with 100 mL

	0 mM HCl	8 mM HCl	16 mM HCl	24 mM HCl
Average	5.41	4.51	4.02	3.44
Median	5.39	4.50	4.01	3.44
Standard Deviation	0.061	0.033	0.057	0.048
Max value	5.51	4.55	4.09	3.51
Min value	5.36	4.47	3.95	3.38
Difference min max	0.15	0.08	0.14	0.13
Data points	4	3	3	6

Table 3.2: The spread of the pH values of gelatin dissolved in water (5w/v%) by 40° by different concentrations of HCl

Volume EtOH used [ml]	Amount of EDC used [mg]	Size [nm]	Polydispersity [-]	Zeta potential [mV]
120	50	415.8 (±3.9)	0.1380 (±0.010)	27.52 (±0.66)
120	80	304.3 (±4.7)	0.1509 (±0.006)	17.91 (±0.70)
120	100	293.6 (±4.6)	0.05422 (±0.030)	32.55 (±0.70)
100	80	321.1 (±3.6)	0.06845 (±0.018)	31.48 (±0.90)
100	100	326.1 (±4.5)	0.09546 (±0.027)	36.12 (±0.55)
80	80	367.3 (±3.2)	0.08138 (±0.028)	36.26 (±0.65)
80	100	380.9 (±6.9)	0.05340 (±0.056)	33.94 (±0.69)

Table 3.3: The DLS measurements for 7 different batches were the amount of EtOH (100%) and amount of crosslinkers (EDC/NHS) were varied. The table gives the size, polydispersity and zeta potential. For the DLS measurements the sample was diluted 5 times for size and polydispersity and 100 times for zeta potential)

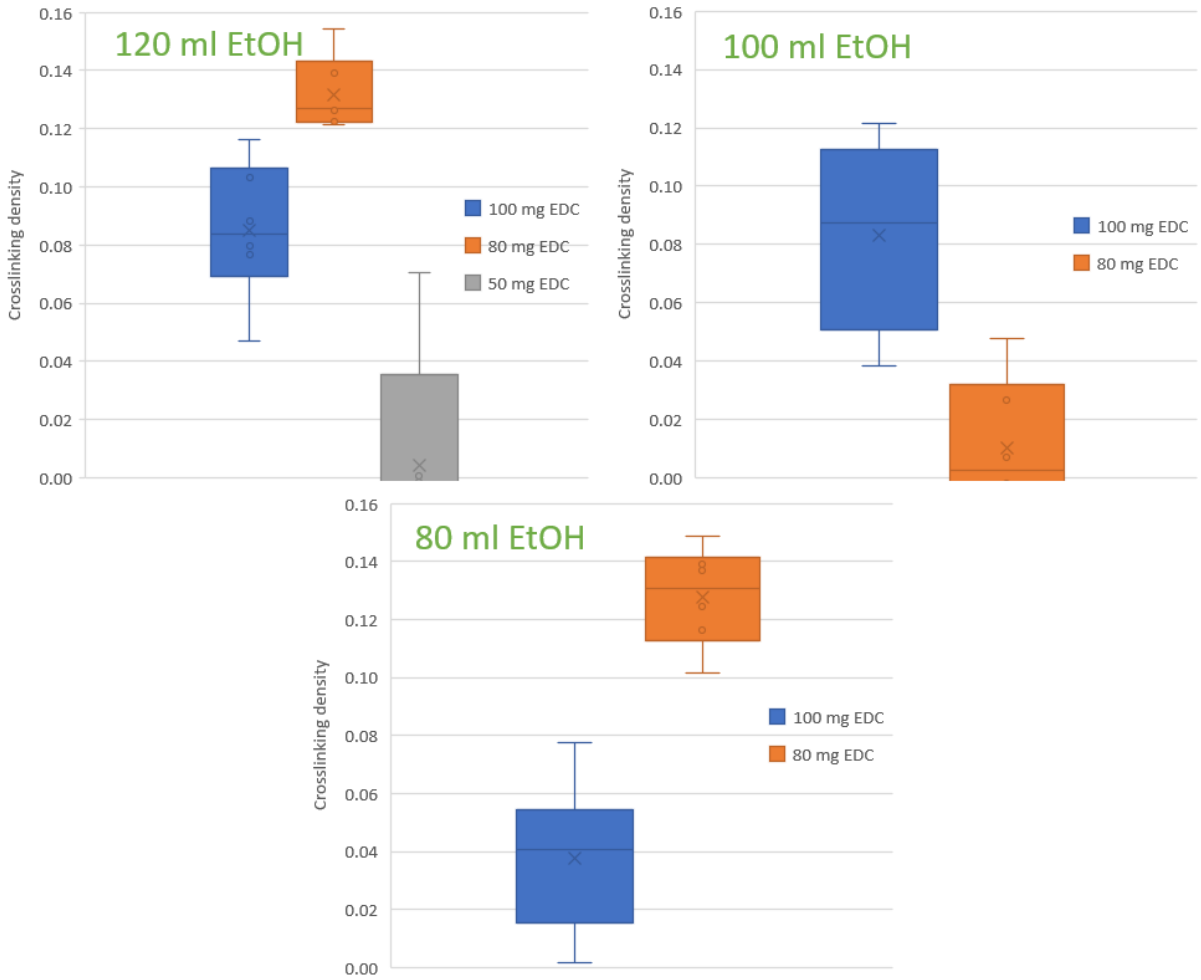

Figure 3.5: The crosslinking density of gelatin nanoparticles crosslinking with different amounts of EDC and NHS while the nanoparticles were synthesized with different amounts of EtOH (100%) . The ratio EDC and NHS is always 25:3

why there is such a large difference between the gels.

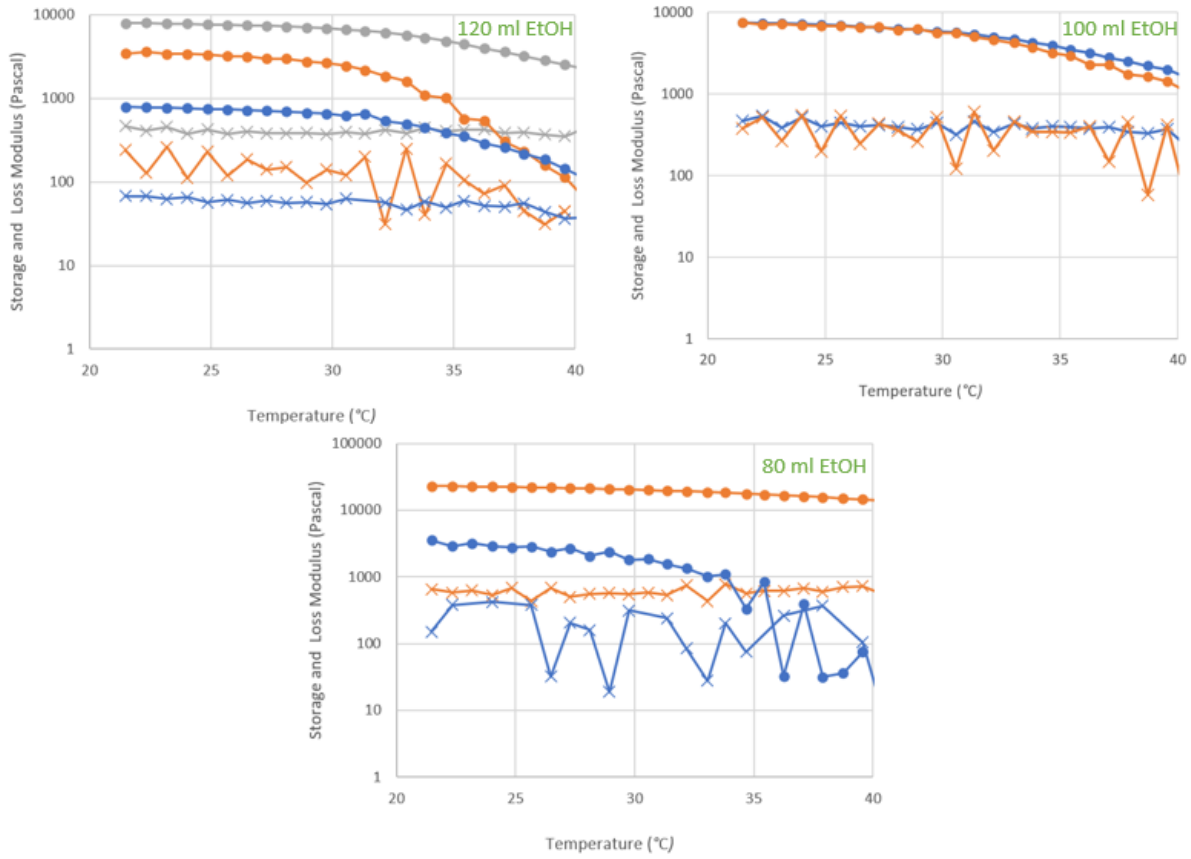

Figure 3.6: The storage (•) and loss modulus (X) for different temperatures with different concentrations of crosslinkers with different volumes of EtOH. Blue corresponds with 100 mg EDC and 12 mg NHS, orange with 80 mg EDC and 9.6 mg NHS and grey 50 mg EDC and 6 mg NHS

Volume EtOH used [ml]	Amount of EDC used [mg]	Average storage modulus [Pa]	Average loss modulus [Pa]
120	50	10 608 (±3.28)	556 (±12.7)
120	80	6 261 (±197)	293 (±17.8)
120	100	2 836 (±5.28)	197 (±8.23)
100	80	21 114 (±185)	1 055 (±48.0)
100	100	7 285 (±56.7)	538 (±31.2)
80	80	20 190 (±261)	575 (±46.0)
80	100	1 942 (±26.0)	174 (±4.00)

Table 3.4: The average loss and storage modules measured for 150 s at a strain of 0.5%

Chapter 4

Conclusion

The goal of this research was to find a way to reduce the amount of ethanol needed to form the gelatin nanoparticles. This report shows that this can be done by adding salt (NaCl). However, adding too much salt results in sedimentation. Also, adding salt influences the size of the gelatin nanoparticle, which might not be ideal. Another way of reducing EtOH is by changing the pH. The higher the pH the less EtOH is needed. However, if the pH becomes higher than 4, then sedimentation occurs. By changing the pH to 3.5 only a EtOH : Water ratio of 3.2 to 1 is needed to form stable nanoparticles. Here nanoparticles of around 300 nm could be made. It is also shown that increasing the pH will increase the size of the nanoparticles. Adding both salt and increasing the pH doesn't reduce the amount of EtOH needed any further.

Based on the experiments with the crosslinkers it was found that the amount of EtOH used doesn't affect the size of the particle. The concentration of crosslinkers does influence the size. It was further shown that the amount of crosslinkers and EtOH doesn't influence the gel formation, but rather something else that is yet unknown. Further research should be done toward discovering the important parameters for the formation of gel. It could also be useful to look if a further increase of the concentrations of crosslinkers would increase the stability of the particles. Right now the crosslinking density seems to be low.

In the end, nice stable gelatin nanoparticles could be made by changing the pH to 3.5. This rather simple change in protocol would save around 56% of EtOH compared to the old protocol. This would allow to scale up the gelatin nanoparticle synthesis to around 45 g, which is an increase of more than 200%.

Chapter 5

Acknowledgements

As science is never done alone, I would like to thank a few people for their help during my internship at the RadboudUMC. First of all, I would like to thank my supervisors from the RadboudUMC, Dr. Rong Wang and Lucas Joziasse for their supervision over the project. Their technical and biological knowledge surrounding the topic helped the project immensely. Also, their quick response to my questions and the regular meeting were an important asset to my research.

I would further like to thank all the people at the R&B group for helping me with the use of the equipment. I would also like to thank my supervisor from the UU Prof. Dr. Willem Kegel. Lastly I would like to thank my girlfriend Margo for spell checking my report and keeping me motivated.

Appendix

List of chemicals

- Gelatin A
- milliQ pure water (MQ)
- Ethanol (100%) (EtOH)
- HCl (6 M)
- HCl (1 M)
- HCl (0.1 M)
- 1-(3-dimethylaminopropyl)-3-ethyl-carbodiimide hydrochloride (EDC)
- N-hydroxysuccinimide (NHS)

Additional figures

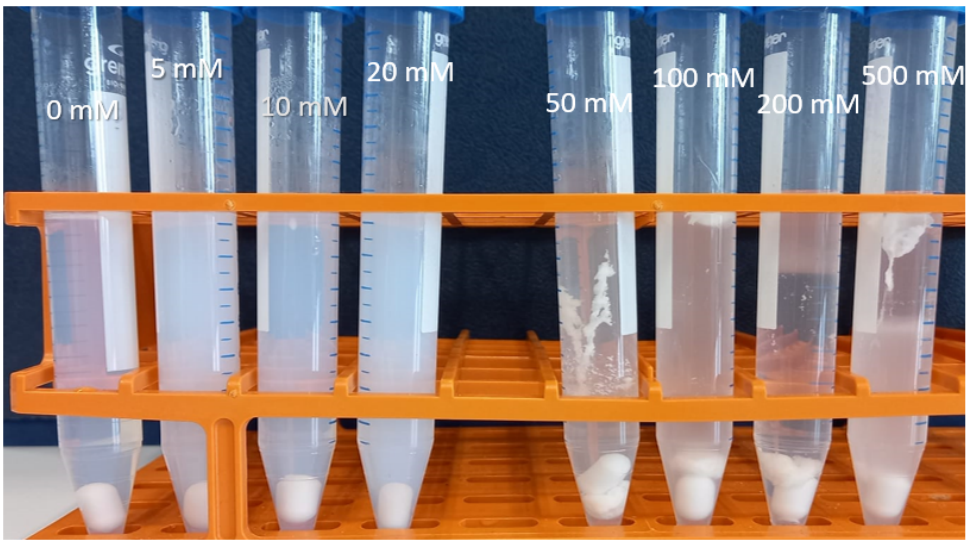

Figure 1: A photo of a small salt experiment after all the EtOH was added. In the figure the different concentrations of NaCl are shown. It can be seen that sedimentation happens after 50 mM NaCl

	0	1	2	3	4	5	6	7	8	9	10	11	mL EtOH
0	0.040	0.040	0.878	0.104	0.094	0.063	0.313	0.126	0.103	0.099	0.080	0.069	
10	0.061	0.040	0.037	1.855	1.780	1.823	1.797	1.441	0.690	1.917	0.297	0.346	
15	0.038	0.036	0.036	0.994	1.532	1.768	1.740	1.834	0.186	0.167	0.130	0.118	
20	0.036	0.037	0.036	0.055	0.270	0.447	0.532	0.577	0.125	0.078	0.063	0.060	
25	0.036	0.039	0.038	0.036	0.084	0.194	0.342	0.336	0.330	0.387	0.458	0.356	
30	0.038	0.037	0.041	0.034	0.047	0.090	0.147	0.190	0.218	0.205	0.222	0.187	
35	0.037	0.036	0.039	0.035	0.038	0.051	0.082	0.121	0.133	0.131	0.133	0.126	
40	0.037	0.035	0.036	0.036	0.035	0.070	0.056	0.053	0.059	0.058	0.040	0.053	
microL													
HCl													

Figure 2: The absorption measurements of the different samples taken. The values are of arbitrary value, through the higher means more absorption. The coloring is done in such a way that the more dark correspond to a higher value. On the top it shows how much ethanol was added per column and left is shows how much acid was added per row

	0	1	2	3	4	5	6	7	8	9	10	11	ml Ethanol
0	NP	NP	1324				98.25			80.52		ERR	
10	NP	NP	NP	978.4			934.4			1061		874.7	
15	NP	NP	NP	1189			604.2			532.4		415.8	
20	NP	NP	NP	NP	NP	375.6	347.1			387.4		529.1	
25	NP	NP	NP	NP	NP	443.1	409.2			455.2		516.7	
30	NP	NP	NP	NP	NP	NP	340.8			360.6		435.4	
35	NP	NP	NP	NP	NP	NP	NP	348.6		334.9		444.6	
40	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP	
microL													Size (nm)
HCl		one peak					multiple peaks		in the DLS measurements				

Figure 3: DLS measurement of various of the vials. The sizes of the nanoparticles are given in nanometers. The green color corresponds to one peak, the red corresponds to two or more peaks in the DLS measurements. NP stands for no particle formation and ERR stands for Error, meaning that the DLS apparatus wasn't able to measure that sample, Probably due to aggregation.

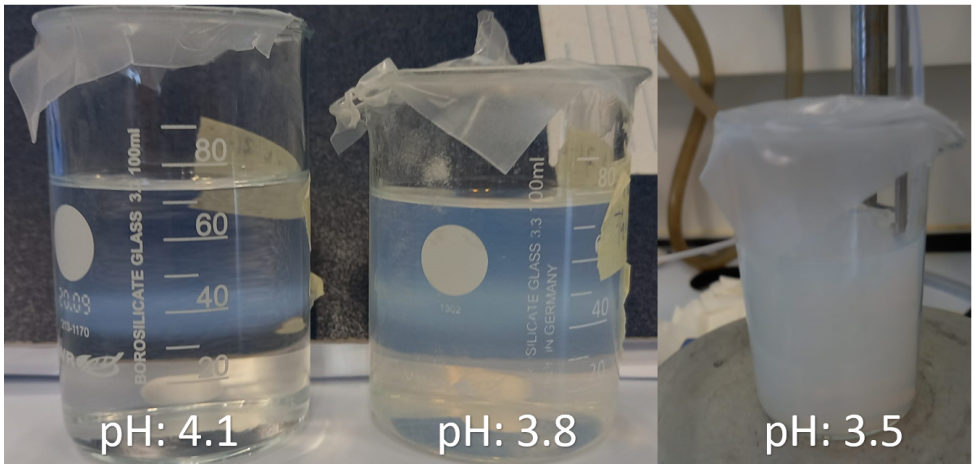

Figure 4: A photo of the gelatin solutions of the experiment described in 2.3.2 after 60 mL of EtOH (100%) was added

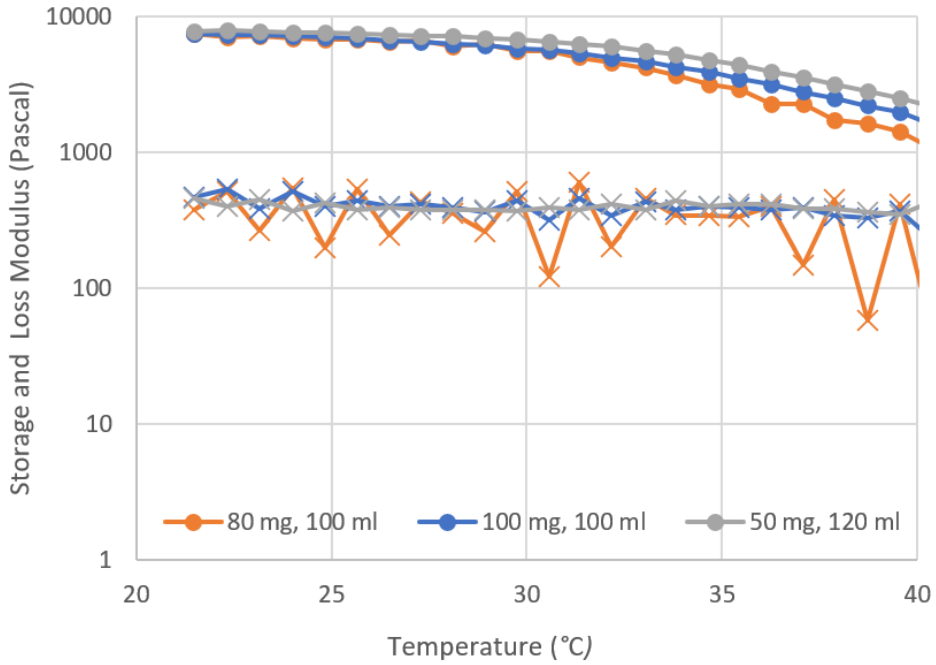

Figure 5: The storage (•) and loss modulus (X) of three different batches of gelatin gels (20% (w/w)) which show very similar results. These three batches are: the batch with 100 mg EDC, 12 mg NHS and 100 mL EtOH; the batch with 80 mg EDC, 9.6 mg NHS and 100 mL EtOH; and the batch with 50 mg EDC, 6 mg NHS and 120 mL EtOH.

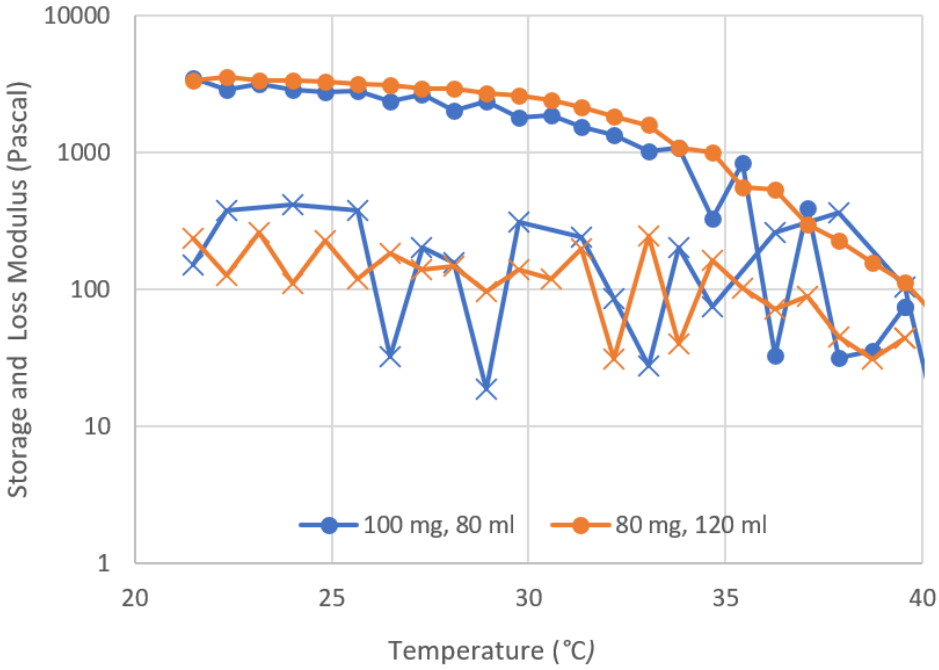

Figure 6: The storage (•) and loss modulus (X) of two different batches of gelatin gels (20% (w/w)) which show very similar results. These two batches are: the batch with 100 mg EDC, 12 mg NHS and 80 mL EtOH; the batch with 80 mg EDC, 9.6 mg NHS and 120 mL EtOH.

Bibliography

[1] A. O. Elzoghby, "Gelatin-based nanoparticles as drug and gene delivery systems: Reviewing three decades of research," *J. Control. Release*, vol. 172, no. 3, pp. 1075–1091, 2013.

[2] S. Banerjee and S. Bhattacharya, "Food Gels: Gelling Process and New Applications," *Crit. Rev. Food Sci. Nutr.*, vol. 52, pp. 334–346, apr 2012.

[3] L. V. Sigolaeva, S. Y. Gladyr, O. Mergel, A. P. H. Gelissen, M. Noyong, U. Simon, D. V. Pergushov, I. N. Kurochkin, F. A. Plamper, and W. Richtering, "Easy-Preparable Butyrylcholinesterase/Microgel Construct for Facilitated Organophosphate Biosensing," *Anal. Chem.*, vol. 89, pp. 6091–6098, jun 2017.

[4] W. Zhou, J. M. Stukel, H. L. Cebull, and R. K. Willits, "Tuning the Mechanical Properties of Poly(Ethylene Glycol) Microgel-Based Scaffolds to Increase 3D Schwann Cell Proliferation," *Macromol. Biosci.*, vol. 16, pp. 535–544, apr 2016.

[5] M. Karg, A. Pich, T. Hellweg, T. Hoare, L. A. Lyon, J. J. Crassous, D. Suzuki, R. A. Gumerov, S. Schneider, I. I. Potemkin, and W. Richtering, "Nanogels and Microgels: From Model Colloids to Applications, Recent Developments, and Future Trends," *Langmuir*, vol. 35, pp. 6231–6255, may 2019.

[6] S. M. Ahsan and C. M. Rao, "The role of surface charge in the desolvation process of gelatin: Implications in nanoparticle synthesis and modulation of drug release," *Int. J. Nanomedicine*, vol. 12, no. January, pp. 795–808, 2017.

[7] S. Kommareddy, D. B. Shenoy, and M. M. Amiji, "Gelatin Nanoparticles and Their Biofunctionalization," in *Nanotechnologies Life Sci.*, vol. 1, pp. 330–352, Weinheim, Germany: Wiley-VCH Verlag GmbH & Co. KGaA, sep 2007.

[8] G. A. Di Lullo, S. M. Sweeney, J. Körkkö, L. Ala-Kokko, and J. D. San Antonio, "Mapping the Ligand-binding Sites and Disease-associated Mutations on the Most Abundant Protein in the Human, Type I Collagen," *J. Biol. Chem.*, vol. 277, pp. 4223–4231, feb 2002.

[9] R. Yasmin, M. Shah, S. A. Khan, and R. Ali, "Gelatin nanoparticles: A potential candidate for medical applications," *Nanotechnol. Rev.*, vol. 6, no. 2, pp. 191–207, 2017.

[10] N. Taheri Qazvini and S. Zinatloo, "Synthesis and characterization of gelatin nanoparticles using CDI/NHS as a non-toxic cross-linking system," *J. Mater. Sci. Mater. Med.*, vol. 22, no. 1, pp. 63–69, 2011.

[11] A. J. Kuijpers, G. H. Engbers, J. Feijen, S. C. De Smedt, T. K. Meyvis, J. Demeester, J. Krijgsveld, S. A. Zaat, and J. Dankert, "Characterization of the network structure of carbodiimide cross-linked gelatin gels," *Macromolecules*, vol. 32, no. 10, pp. 3325–3333, 1999.

[12] K. Farbod, M. Diba, T. Zinkevich, S. Schmidt, M. J. Harrington, A. P. Kentgens, and S. C. Leeuwenburgh, "Gelatin Nanoparticles with Enhanced Affinity for Calcium Phosphate," *Macromol. Biosci.*, vol. 16, no. 5, pp. 717–729, 2016.

[13] J. M. Koli, S. Basu, N. Kannuchamy, and V. Gudipati, "Effect of pH and Ionic Strength on Functional Properties of Fish Gelatin in Comparison to Mammalian Gelatin," *Fish. Technolgy*, vol. 50, no. January, pp. 126–132, 2013.