Analysing and experimenting the preparation of Gelatin Methacryloyl for 3D bioprinting

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Alén, Niina Analysing and experimenting the preparation of Gelatin Methacryloyl for 3D bioprinting

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The process of preparing Gelatin Methacryloyl (GelMA) is analysed and tested. The thesis begins with an overview of 3D bioprinting, optimal properties of materials used in 3D bioprinting, and an introduction of GelMA. The steps of preparing GelMA are then analysed based on literature and new ways of analysing the protocol are discussed.

The preparation of GelMA consists of the synthesis, filtering, freeze-drying, and the measurement of the degree of substitution. The synthesis is examined for technique and transparency. Two methods of filtering GelMA are then being analysed and tested. Dialysis is a widely used method of filtering GelMA but its duration lacks standardization. A method of determining when the dialysis is ready is being developed. Another filtering method, preparative cromatography, is tested. Chromatography is tested because it could be a faster filtering method compared to dialysis. The theory of freeze-drying is analysed next and the method is tested in action in the experimental section. Lastly, the method of measuring the degree of substitution is being analysed and tested.

As a result, all of the steps in the process of preparing GelMA are examined and an optimal method for the process is suggested. The analysis of preparing GelMA raised discussion and new ideas for further development and research.

Keywords: Gelatin Methacryloyl, quality control, synthesis, dialysis, freeze-drying, degree of substitution

Contents

1 Preface

3D bioprinting (bioprinting) technologies have developed fast and the possibilities of 3-dimensional printing of biological objects are nearly endless. Bioprinting can be used in tissue engineering to test drug delivery mechanisms and disease progression and bioprinting could even be used to produce organs for transplantations [1]. The technology for these applications already exists but the materials (bioinks) limit the achievement of the next milestones [2, 3, 4]. The optimal bioink should have specific rheological and mechanical properties, it should be biocompatible and have appropriate rate of degradation, and the optimal bioink should have effective gelation properties [2]. The list of required properties is long and the range of materials fulfilling the criteria is narrow. One potential and widely used material is Gelatin Methacryloyl (GelMA).

The preparation of GelMA has been tested in many studies but the protocol lacks accuracy. When correctly prepared, GelMA could be used in many of the previously mentioned applications of bioprinting. The protocol for the synthesis of GelMA has been documented in multiple studies but they are usually a scratch of the surface and do not go into detail. The goal for this Master's thesis is to analyse the steps of the synthesis and post-processing of GelMA, and to enhance the interpretability, repeatability, and reliability of the protocol.

The process of preparing GelMA for bioprinting consists of the synthesis, filtering, freeze-drying, and the mixing of the printable bioink. This thesis does not focus on the mixing of the bioink as it is a rather simple step after the condensed material has been prepared. In the synthesis, the gelatin is diluted into a solvent and then methacrylated to become crosslinkable with light. The synthesis is examined for technique and repeatability. Methacrylation creates unwanted byproducts that need to be filtered out for the bioink to be compatible with cells. In this thesis, two filtering methods are tested and analysed. Dialysis is a widely used method for

filtering GelMA but the duration of the dialysis is reported to last different times without sufficient arguments [5, 6]. One goal of this thesis is to define a way of determining when the dialysis is ready and how it can be tested. After the filtering is done, the material is freeze-dried. The freeze-drying process is analysed and tested. After the process of preparing GelMA is ready, the degree of substitution can be calculated. The degree of substitution (DS) describes how many amino groups of Gelatin were methacrylated. DS indicates directly how reactive GelMA is to the light. The protocol for determining DS is analysed and tested.

Based on the literature search and training at the University of Tampere, the process of preparing GelMA is tested in the lab of Brinter Oy. The results of the steps in preparing GelMA are then being discussed and analysed to develop the protocol further. The method for determining the sufficient dialysis end point has even been used in a scientific article since it was developed during the tests of this thesis [7].

2 Theoretical Background

2.1 Bioprinting in general

The main idea of additive manufacturing (AM) is that an object is built layer-by-layer with computer aided design (CAD) in a specific predefined 3-dimensional (3D) form. The method of forming these layers depends on the AM technique. The history of 3D printing dates back to the invention of stereolithography in 1983 [8]. From there, multiple different techniques of additive manufacturing have been developed. Fused deposition modelling (FDM), selective laser sintering (SLS) and laminated object manufacturing (LOM) are a few examples of AM techniques that can print with polymers and metals [8]. In bioprinting, biology and additive manufacturing are combined to print biocompatible 3-dimensional parts [2]. Bioprinting can be used for example in tissue engineering, regenerative medicine, and in biological research [3].

There are two main approaches in 3D bioprinting based on the cell deposition method. The cells can be embedded in a hydrogel and printed after mixing the cells into the gel or the cells can be deposited into a pre-printed structure. These approaches are called scaffold-based and scaffold-free cell deposition methods. The scaffold-based bioprinting process is faster than the scaffold-free bioprinting but the maturation of cells can be much faster in scaffold-free approach. The scaffold-based cell deposition is more damaging to the cells but the cells can be more accurately deposited with this method. [2]

2.1.1 Bioprinting techniques

In addition to different approaches on the cell deposition, there are different technologies for bioprinting. The simplest bioprinting technology might be the extrusion-based bioprinting that originates from the Fused-Deposition Modelling developed in

the 1990's [3]. Extrusion based bioprinting includes pneumatic printing with pistons and using screws as seen in Figure 1A. Pneumatic pressure can be used to push down a piston in a cartridge that pushes down the material that is then extruded out of an nozzle. Pneumatic printing can also be done without a piston and the air pressure can be combined with a screw. The screw can also be used as the pressure source for the piston to be pushed down. The extrusion-based bioprinting is relatively affordable technique, the printing is fast, and the range of bioinks is wide [3]. The extrusion-based bioprinting also allows the use of different bioinks and cells in the same printing with coaxial printing tools and even high viscosity materials can be extruded with this method [2]. The goal of this thesis is to optimize the synthesis of Gelatin methacryloyl (GelMA) and GelMA is also usually printed with extrusion-based bioprinting, especially with the piston combined with pneumatic pressure.

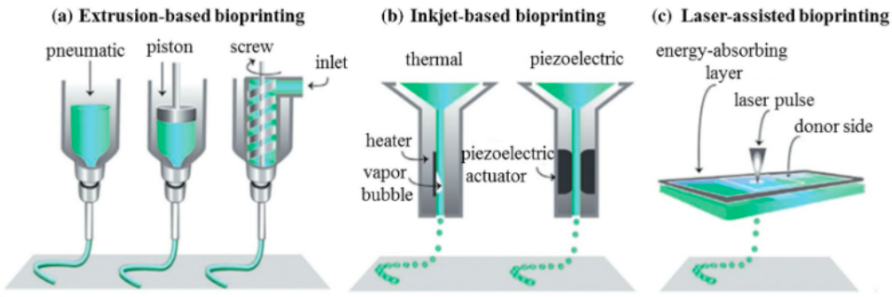

Figure 1. A schematic representation of different types of 3D bioprinting technologies. A) Extrusion-based bioprinting with pneumatic pressure, piston and a screw. B) Inkjet-based bioprinting with thermal and piezoelectric components and C) laser-assisted bioprinting. [2]

The second method of bioprinting is inkjet bioprinting. In inkjet bioprinting, small droplets of the material are deposited on a substrate [9]. The droplets are often formed with thermal or piezoelectric elements as seen in Figure 1B. The volume of the droplets vary in range of 10 to 150 pL depending on the droplet formation parameters and the viscosity of the ejected ink [9]. In thermal inkjet bioprinting the

droplets are created by air bubbles that are created with thermal energy [2]. These air bubbles then push down the material and create the ink droplets. Piezoelectric printing is more accurate with the volume of the droplets than thermal inkjet printing but the cells can be damaged in the piezoelectric frequencies [2].

A representation of the setup of laser-assisted bioprinting is in Figure 1C and a representation of the printing process is in Figure 2. Laser-assisted bioprinting consists of a ribbon surface on top of the bioink layer. A laser beam is focused on the surface between the ribbon and the bioink, as seen in Figure 2A. The vapour pressure of the absorbing ribbon layer creates bubbles towards the bioink film (Fig. 2B) [2]. As the bubble has expands, the inner pressure of it has decreases and it leads up to a collapse shown in 2C [10]. Once the bubble collapses, a jet is formed on the bottom of the bioink layer and a small droplet is applied on the substrate, Fig 2D [10]. The vaporising ribbon layer can contaminate the substrate and the printing process is rather slow compared to extrusion- and inkjet-based printing [2]. The printing has high accuracy and does not have a nozzle, which is an advantage considering the block ups and pressure changes in the nozzle [10].

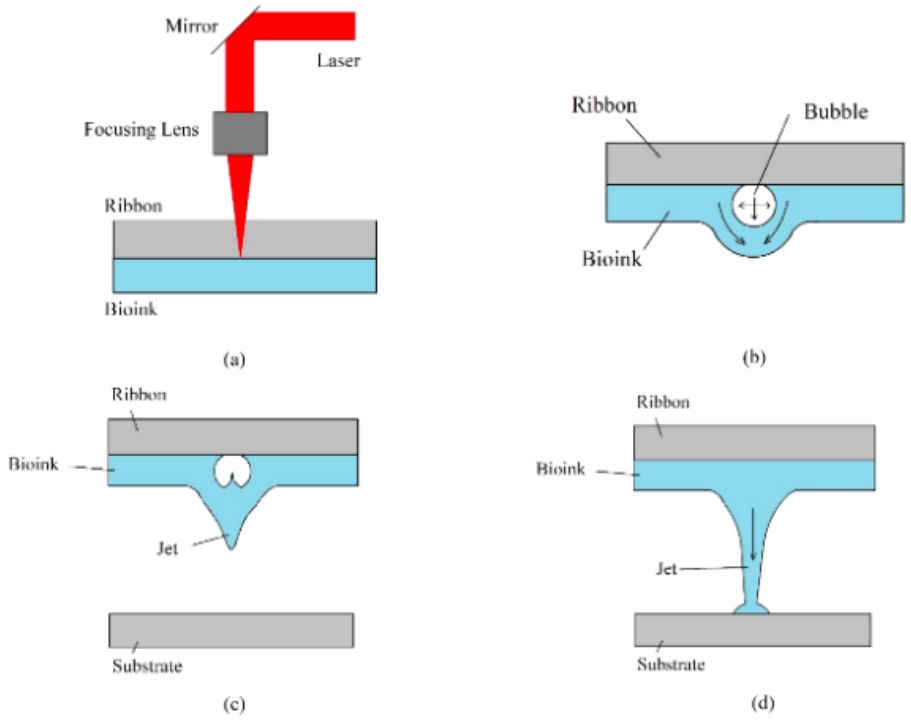

Figure 2. Laser-assisted printing steps. Step a) shows how the laser is focused on the surface between ribbon and bioink. In part b) the air bubble has been created and it pushes the bioink to expand downwards. In step c) the collapse of the bubble is illustrated as the inner pressure is decreased during expansion. Finally, in step d) the bioink creates a jet that drops a small amount of the bioink on the substrate. [10]

2.1.2 Optimal bioink

Bioprinting technologies have evolved fast. Current bioprinters are accurate with high resolution, they have customizable features for different bioinks, ability to do aseptic work, large variety of different nozzles and printheads and they are fully capable of printing complex structures, even organs. The problem of printing these complex structures lies in the bioinks [2, 3, 4]. The ideal bioink should have high printability, the ability of gelation during printing, printing fidelity, structural stiffness, biocompatibility, tissue regeneration properties, permeability for 0_2, nutrients, metabolic waste and controlled biodegradability [2]. The material should also support cell attachment, proliferation and function [4]. A schematic representation of

the requirements for bioinks is in Figure 3. Bioinks are the limiting factor in bioprinting because this list of requirements is difficult to implement. Decent printing can still be done with fewer properties than the optimal bioink requires.

Figure 3. Bioink quality requirements for optimal printability in 3D bioprinting [2].

Printability is the key of differentiation between 2D cell culture and 3D cell culture. For example, the cell culture medium has all of the other requirements of the optimal bioink but it lacks printability. If the material has low printability but it has all of the other qualities of an optimal bioink, the material can not be printed. The focus here is on the extrusion-based bioprinting because GelMA is printed with this method.

Printability can be modified by adjusting the print settings of a bioprinter, the scaffold design or tuning the mechanical properties of the bioink [11, 12]. The bioprinter settings include pressure and printing speed optimization that usually correlate to each other. With larger pressure more of the bioink is extruded and

the speed needs to be increased for the filament size to remain constant. In scaffold design, layer height of the CAD model is an important factor that might even need continuous modifications as the printing goes on and the layers collapse a little [11]. Layer height needs to be accurately set up for the layers to attach to each other. If the layer height is too high, the placement of a new layer will not succeed, and with an effective crosslinking method, high layer height will form clumps. There is more about crosslinking in a later part of this Chapter. On the other hand, if the layer height is too low, the nozzle will drag the previous layer destroying it. Both of these errors cumulate over time and therefore they need to be avoided.

The third factor in printability is the mechanical properties of the bioink that can be modified with the ink's composition. The mechanical properties include viscosity, shear thinning, liquid-air surface tension, loss and storage modulus, crosslinking mechanism, degradation behaviour, smoothness and shape fidelity [11, 12, 13]. Most of these physical parameters can be examined with rheology measurements. With sweep tests, the loss modulus and the storage modulus of the bioink can be measured. If the loss modulus is dominant under stress, the material behaves like a liquid and it will not hold its form after the printing [13]. Another physical measure is the changes in extrusion force that indicate the smoothness of the bioink. If the extrusion force stays constant, the material is more homogenous and the printing settings apply to the whole volume of the bioink [13]. The viscosity of the bioink is also an important physical quantity that can be examined with the rheological measurements. The viscosity range suitable for bioprinting is between 300 to 30 000 mPas [14] and the viscosity correlates directly to the printing pressure and the structural integrity after printing.

Crosslinking Hydrogels are commonly used in bioprinting because of their biocompatibility, degradation properties and the possibility to tune the mechanical properties with the ink composition. Yet, hydrogels often lack the structural in-

tegrity that is needed for complex 3-dimensional structures. To increase the structural integrity of hydrogels, a crosslinking method can be utilised. Crosslinking is a reaction where a chemical bond is formed between two molecules or two parts of one molecule making the material stiff [15, 16]. In bioprinting, crosslinking makes it possible to gain structural integrity for materials that would otherwise spread or collapse during or after the printing. In Figure 4, the methacrylation of Gelatin is illustrated along with the mechanism of crosslinking. In the methacrylation, the methacrylic groups react with the free amine groups of Gelatin. More about methacrylation is in Chapter 2.2.1.

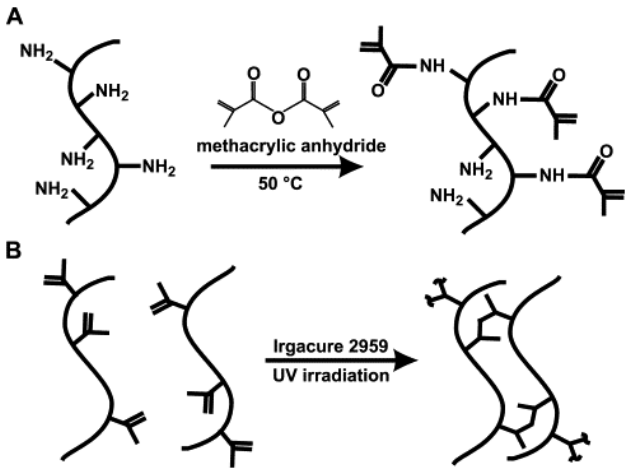

Figure 4. Methacrylation and crosslinking of Gelatin. [17]

Crosslinking can be initiated at least with light, thermal energy, some ions, enzymes and chemical reagents [15, 18]. Crosslinking with ions is mostly used with sodium alginate bioinks that can be crosslinked with calcium chloride [16]. Sodium alginate inks are usually very low viscosity inks that could not be 3D-printed without crosslinking. Thermal crosslinking involves the materials that change their viscosity by heating up or cooling down. This method is usually not cell-friendly and the crosslinking degree can not be precisely determined [16]. Agarose is an example of a

bioink that is liquid at above 40 °C and when it is cooled down to body temperature the crosslinking begins. The bonds that are formed with crosslinking can be H-bonds, hydrophopic interactions, electrostatic attraction, or ionic crosslinking [16].

With GelMA, the crosslinking method used is photocrosslinking. Photocrosslinking with light requires a photoinitiator that starts the crosslinking reaction and a matching light wavelength to activate it. Photocrosslinking is minimally invasive and cost-effective, the UV curing can be done locally, and it can be controlled remotely [15, 16]. Extensively used photoinitiator, 2-hydroxy-l-[4-(hydroxyethoxy)phenyl]-2-methyl-L-propanone (Irgacure 2959), has the advantage of slight water solubility up to certain degree and cell-friendliness. A more recently developed photoinitiator, Lithium phenyl-2,4,6-trimethylbenzoylphosphinate (LAP), is also widely used because of its more effective crosslinking. In Figure 5, the absorbance spectrums of Irgacure 2959 and LAP are presented along with their molar extinction coefficients. The molar extinction coefficient describes how much light the material absorbs at the specific wavelength. [18]

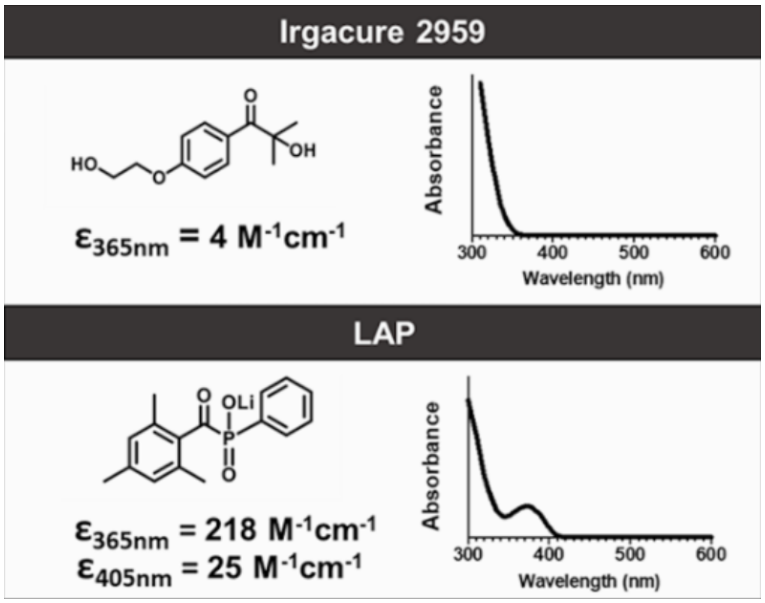

Figure 5. Absorption spectra of Irgacure 2959 and LAP and their molar extinction coefficients. [18]

High frequencies damage the cells [15] and therefore the molar extinction coefficient for Irgacure 2959 was calculated in Fig. 5 at 365 nm instead of the peak value 300 nm. For the same wavelength, LAP has molar extinction coefficient of $218\,\mathrm{M^{-1}cm^{-1}}$ and Irgacure 2959 has $4\,\mathrm{M^{-1}cm^{-1}}$. LAP even has another peak at 390 nm that causes the 405 nm light to have a molar extinction coefficient of $25\,\mathrm{M^{-1}cm^{-1}}$. LAP has multiple times more effective crosslinking than Irgacure 2959 even at more cell-friendly frequencies. Therefore, the use of LAP has increased significantly as the photinitiator for GelMA.

2.2 Gelatin methacryloyl

Gelatin methacryloyl (GelMA) is a photocurable hydrogel used in tissue engineering and regenerative medicine applications of 3D bioprinting [1]. GelMA is prepared by modifying Gelatin type A with Methacrylic anhydride (MA) to create a photocurable hydrogel when introduced to a photoinitiator and matching ultraviolet (UV) light [1]. The cured GelMA holds its structure in body temperature which is a property the original Gelatin type A does not have [1]. Gelatin methacryloyl contains many sequences that promote cell attachment and cell remodelling and it is highly biocompatible and broadly tunable [6, 19].

The ready bioink contains GelMA, buffer solution, and a photoinitiator. Gelatin methacryloyl bioink is prepared from a freeze-dried pellet that is diluted into a buffer solution. Generally, Phosphate buffered Saline (PBS) solution is used [6]. GelMA is diluted into the buffer with concentrations usually of 5 % w/v or 10 % w/v. The 5 % w/v GelMA is more temperature sensitive but it has favourable mechanical properties for cell attachment [20]. The 10 % w/v GelMA is more temperature stable and it has higher compressive modulus and lower swelling properties than 5 % w/v GelMA [20]. Higher concentrations are not usually used because of the dense molecular structure of the bioink that is not a good for cell movement [21]. A

photoinitiator is then added into the GelMA solution. Irgacure 2959 and LAP are common photoinitiators used with GelMA.

In bioprinting, the rheological properties of the bioink are the key for successful printing process and structural integrity after the printing, as discussed in Chapter 2.1.2. Viscosity of the bioink determines often how well the printed structure holds its form. The viscosity of Gelatin metharyloyl can be modified by adjusting the hydrogel solution concentration, the temperature, the degree of substitution (DS), or the curing settings during/after printing [19, 20, 21]. The concentration of GelMA plays a significant role in the viscosity of the bioink. The cells might favour the 5 % w/v because it has lower viscosity but from the printing point of view 10 % w/v GelMA is much easier to use [21]. The viscosity can also be increased by lowering the printing temperature or using crosslinking during the print [21]. Usually, the curing settings are not a sufficient enough way of increasing the structural fidelity of the bioink. Efficient curing combined with low (5-15 °C) and stable temperature can increase the viscosity enough for good printability even with 5 % w/v GelMA.

The applications of GelMA are in biomedical research. Gelatin methacryloyl can be widely used in tissue engineering, it can be utilized in drug delivery, organ-on-a-chips, and in biosensors. Tissue engineering with GelMA includes skin, tendon, bone, cartilage, and vascular regeneration, it can even be used in neural engineering. The extracellular matrix (ECM) of GelMA can be modified with other polymers, small molecule drugs, and growth factors to meet the requirements of tissue regeneration. GelMA can also be used as the bulk material of drug delivery devices for more controlled drug release. By modifying the hydrogel relationships, the release rate can be controlled. GelMA could be used as a raw material for artificial organs on chip that mimics organs, Organ-on-a-chip (OOAC). Biosensors detect a chemical or a biological change (allergens for example) of a component and GelMA could be used to enhance the detection sensitivity with its mechanical and electrical properties.

[22]

2.2.1 Synthesis

The synthesis of Gelatin methacryloyl was first introduced by Van Den Bulcke *et. al* in 2000 [23]. The synthesis has been further developed and repeated in multiple studies [20]. The original idea of the synthesis is shown in Figure 6. First, Gelatin type A is diluted in a buffer solution and then Methacrylic anhydride (MA) is added in dropwise under vigorous stirring [6, 20, 22]. There is variance in the amount of MA to be added but a common amount is between $0.1-1\,\mathrm{mL/g}$ [24]. The residual MA and the byproduct Methacrylic acid are then filtered out with dialysis for approximately 5 days with 10 kDa membrane [22].

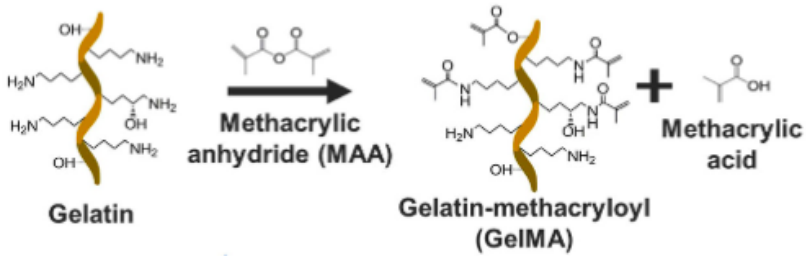

Figure 6. The methacrylation of Gelatin type A. Addition of Methacrylic anhydride, which reacts with lysine and hydroxyl lysine groups of Gelatin creating a byproduct of Methacrylic acid. [22]

2.2.2 Analysing the synthesis

The synthesis presented in the previous section is rather poorly quantified. Quality control is missing almost completely in most of the synthesis literature. The degree of substitution is determined in most of the sources but the used method is left out. The dialysis is usually trusted to be finalized after approximately 5 days [22] but it is often not confirmed. Here, the quality control protocols of quantifying that the steps in the synthesis of Gelatin methacryloyl have worked are being examined from the literature and further tested in Chapter 3.

UV/UV VIS spectroscopy The degree of substitution (DS) of Gelatin methacryloyl describes the ratio between free amine groups of the Methacrylated molecule compared to the free amine groups of pure Gelatin. In Figure 4A, the methacrylation is illustrated. In the methacrylation, the methacrylic groups react with the free amine groups of Gelatin. The amount of methacrylated groups is determined by the duration of the reaction and the volume of Methacrylic anhydride added. The degree of substitution is desired to be between $65 - 75\%$ for the cross-linking to be in the optimal range. [17]

DS can be determined by UV spectroscopy, where the absorbance spectrum of GelMA is compared to the absorbance spectrum of pure Gelatin. In the spectroscopy, GelMA and gelatin type A are diluted in a bicarbonate buffer and after dilution is complete, 2,4,6-Trinitrobenzenesulfonic acid (TNBSA) is added into them. TNBSA is left to react with the solutions at 37 °C for at least 30 minutes. Then, both solutions are scanned for their absorbance spectrum and the peak values are detected. By comparing these peak values, the degree of substitution is determined. [1]

The function of a UV spectroscope is based on the Bouguer-Lambert-Beer law: $\lg\left(\frac{\mathrm{I_0}}{\mathrm{I}}\right) = \lg\left(\frac{100}{\mathrm{T(100\%)}}\right) \equiv A = \epsilon \operatorname{cot} c \cdot d$, where A is the absorbance, T is the transmittance, ϵ is the molar decadic extinction coefficient, c is the concentration of the sample, d is the path length in cm, I_0 is the intensity of the entering light and I is the intensity of the light emerging from the sample. ϵ depends on the wavenumber or wavelength of the sample and the functional relationship between ϵ and wavenumber is called the absorbance spectrum that is observed to find the peak values. [25]

NMR spectroscopy ^{1}H nuclear magnetic resonance spectroscopy (H NMR) can also be used to determine the degree of substitution of GelMA [1, 5, 6, 24, 26]. H NMR determines the degree of substitution by calculating the peak area relationship of methacrylated groups to free amino groups. H NMR was not used in the

experimental section of this thesis because the degree of substitution is possible to be determined with UV spectroscopy and the UV spectroscope was more easily accessible resource than H NMR. H NMR is still widely used to determine the degree of substitution of GelMA and here the theory of it is introduced.

H NMR involves ^{1}H nuclei that have quantum mechanical spin of 1/2 and therefore they have a small dipole momentum. When these dipoles are placed in a magnetic field, B_0, the quantized spin aligns the dipole in certain orientations related to the $\mathbf{B_0}$. The orientations more closely aligned with $\mathbf{B_0}$ have the lowest energy level and the orientations further away from being aligned with $\mathbf{B_0}$ have high energy levels. Jumps in the energy levels are the transitions between the allowed orientations that require emission or absorption of energy related to Planck relation, $\mathrm{E} = \mathrm{h}\nu_0$. In the equation h is the Planck's constant ($h = 6.626 \cdot 10^{-34}\, Js$) and v_0 is the NMR frequency. The ν_0 is also proportional to the magnitude of $\mathbf{B_0}$. B_0, that can be derived from Larmor equation, $\omega = \gamma\, \mathrm{B}_0$, where $\omega = 2\pi\nu_0$ and γ is called the gyromagnetic ratio. Larmor equation is the key to NMR. [27]

The same NMR nuclei can have different resonance frequencies in different molecular compounds because of the local net field of $\mathbf{B_0}$ is different related to the shield of electrons around it. The effective field is then reduced by the shielding constant σ, and then $\mathbf{E_{eff}} = \mathbf{B_{eff}} = \mathbf{E_0}(\mathbf{1} - \sigma)$ and consequently the NMR frequency is shifted to $\nu = \mathrm{B}_{\mathrm{eff}}\gamma = \nu_0(1 - \sigma)$. The frequency shift is known as the chemical shift that is visualised in Figure 7 for common chemical moieties. Different chemical groups can therefore be detected and with the study of the degree of substitution, the free amino groups and modified amino groups can be detected and their ratio can be calculated. [27]

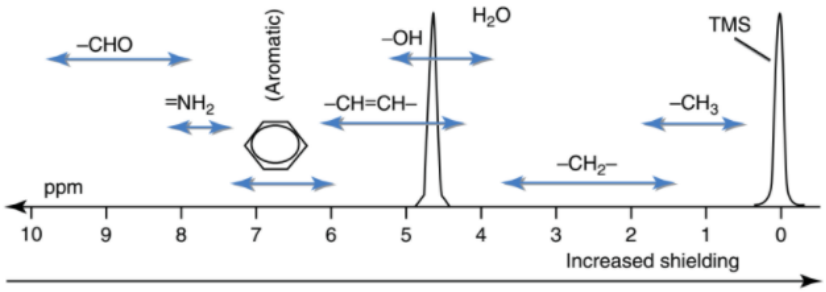

Figure 7. ^{1}H chemical shifts for different chemical moieties. [27]

Dialysis Dialysis purifies GelMA from unreacted MA and methacrylic acids created as a byproduct of the methacrylation (see Figure 6) [6, 22, 24]. The dialysis is conducted with MWCO 12-14 kDA dialysis membranes [5, 6] and it is carried out for 5-7 days [6, 22]. Not much else could be found about the dialysis of GelMA and nowhere was the dialysis examined for its effectiveness.

Inspired by the measurement of the degree of substitution, a method of taking samples from the dialysis water and analysing them on a UV spectroscope was invented in this Master's thesis. In dialysis, the impurities are being filtered out from inside the dialysis bags (GelMA) into the MQ water outside of the bag. Thus, the concentration of the impurities in the MQ water should theoretically decrease when the dialysis goes on and the water is frequently changed. Research about the amount of impurities in dialysis water would provide information about the effectiveness of the dialysis and the amount of impurities that cannot be filtered out. This study could also determine a more accurate way of knowing when the dialysis is ready instead of the 5 to 7 days rough duration estimate.

Preparative cromatography The dialysis was estimated to last 5 to 7 days, which is not efficient and it sets boundaries on the starting days of the synthesis. With dialysis, the synthesis should always be started on a Monday so there would be enough time to finish the dialysis during work week. The dialysis requires human

supervision and attention several times a day and that is difficult to implement during the weekends. Therefore, other filtering methods should be considered for removal of the unreacted MA and byproducts of the methacrylation.

Preparative cromatography is commonly used to separate and analyse different liquid mixtures [28]. It has also been used to analyse molecular weight fractions of gelatin [29] but it has not been used as a replacement of dialysis. Preparative cromatography could still possibly be used to filter GelMA with the method of changing the buffer solution of the gel to MQ water while filtering out particles with certain size [30]. The filtration of particles is determined by the filter specifics. Preparative cromatography includes high performance liquid cromatography (HPLC) that is used to separate compound mixtures and analyse or purify the components [28]. In Figure 8, the flow chart of HPLC is described and similar method is used in the experimental part of this thesis. The setup consist of a solvent, sample, and a detector. The solvent is fed to the system and combined with the sample. Then, filtration is carried out and the filtered liquid is analysed. In Fig. 8, the filtration is done with HPLC tube but this can be replaced with a membrane filter. The chromatography method can be further induced with heat and magnetic stirring.

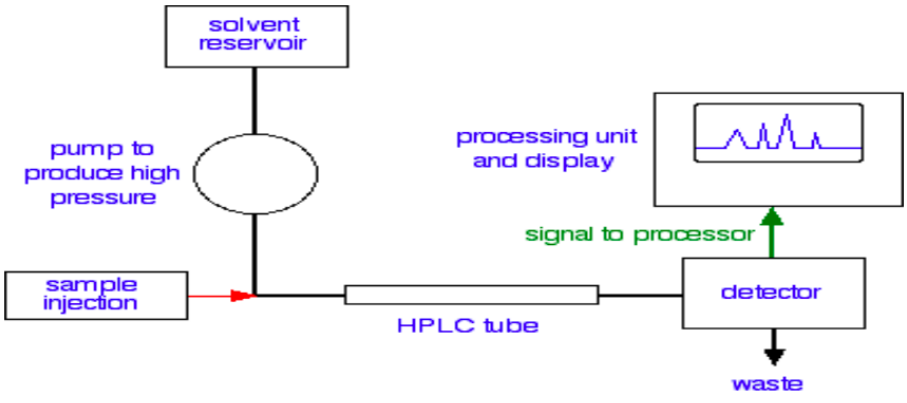

Figure 8. The flow chart of an HPLC system. [28]

In the process of preparative chromatography, the gel is heated up and mixed in a reservoir. MQ water is added into the reservoir and the mixture is filtered

through a selected filter into an analyser and out to a waste reservoir. This process is continued until the value observed on the analyser reaches a certain level. The certain level indicates the optimal state of the mixture when the buffer has been completely changed or the sample has been purified. The analysed quantity can be conductivity of the filtered solution indicating a change in the amount of ions in the filtered liquid. While analysing the composition of the mixture, the optimal level is reached when all compounds have been analysed. If the optimal level is never reached or it would take more time than is available, the filtration can also be stopped earlier. [30]

Freeze-drying Freeze-drying is a process where the liquid is vaporized from the material by sublimation or desorption. A specific program with optimized shelf and chamber temperatures and the level of vacuum is used to vaporize the liquid. The removal of liquid allows the concentration of GelMA bioink to be adjusted to any specific value desired. The commonly utilized concentrations of GelMA are 5 % w/v and 10 % w/v diluted into PBS. The concentration of non-freeze-dried GelMA would be difficult to estimate because of the volume changes during dialysis. The expiration of GelMA is also prolonged with freeze-drying.

For water to get sublimated from the gel, the pressure and temperature needs to be lower than the triple point in the phase diagram. The phase diagram of water is shown in Figure 9. The triple point of water is at 0.61 kPa pressure and 273.16 K. The regions in the phase diagram describe the thermodynamic states of solid, liquid, and gaseous phases. Transitions from one thermodynamic state to another can be done by adjusting pressure and temperature accordingly. In the Fig. 9, four methods of thermodynamic state transitions are illustrated. Hot air drying can be done in high pressure and high temperature, vacuum drying in a vacuum and medium temperature, freezing in high pressures and low temperatures, and freeze-drying in low pressure and low temperature. Freeze-drying is the method of getting

from solid phase to gaseous phase without going through liquid phase, without heat damage on the material, and maximal structure preservation. Gelatin's protein structure could easily be damaged in other drying methods. [31]

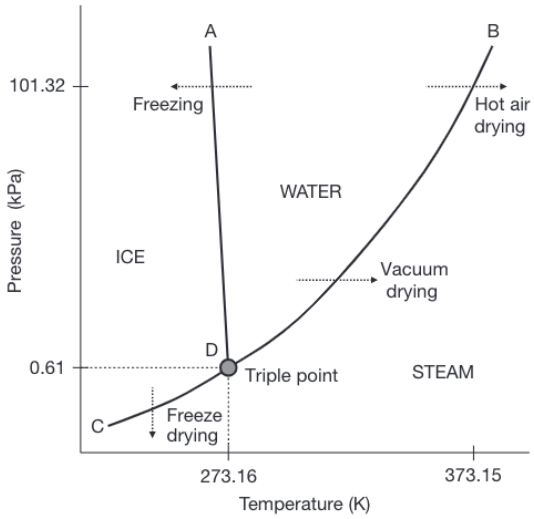

Figure 9. The phase diagram of water. [31]

Freeze-dryer's basic components are a drying chamber, condenser, vacuum pump and a heat source, as displayed in Figure 10. The drying chamber is where the material is stored during the freeze-drying process. Once the water has been sublimated, it is the condenser's task to collect the gaseous water and freeze it to ice. A vacuum pump is required to create low enough pressure in the chamber and heat source to control the temperature of the system. [31]

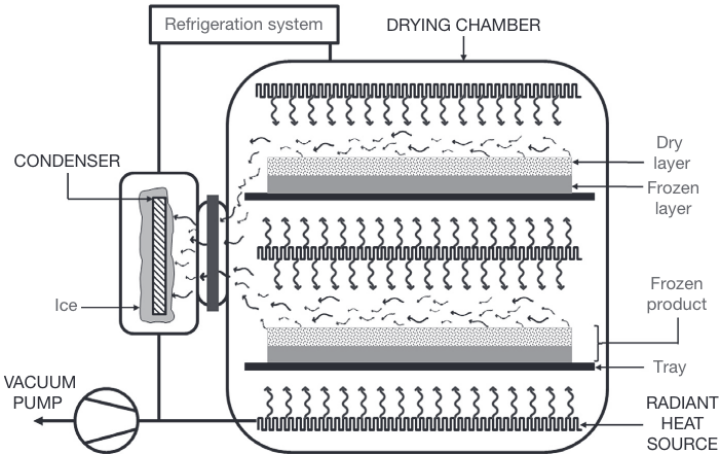

Figure 10. The basic components of a freeze-dryer. [31]

The freeze-drying process is carried out in three steps: initial freezing, primary drying and secondary drying. In initial freezing, the product is freezed below the eutectic point of the material where all mobile water is freezed. In primary drying, temperature is increased under vacuum conditions to remove water from the material by sublimation. The secondary drying removes the bound water when the vapour pressure is decreased and the temperature is increased. As a result, a freeze-dried pellet with 1-3 % moisture content is achieved. [31]

3 Materials and methods

The quality control protocols and the protocol for synthesis were tested. Two complete rounds of synthesis were carried out before finding out the effects of small changes in the protocol. These changes were observed with quality control protocols and printing tests. The quality control protocols confirm the differences between printing with different batches of GelMA and this also confirms that the quality control protocols detects the differences.

3.1 Synthesis

The initial protocol was provided by Hatai Jongprasitkul from University of Tampere (TUNI). This protocol was implemented first in TUNI together with M.Sc. Jongprasitkul and then repeated in Turku at the Brinter premises. Modifications needed to be made to the protocol because of different set of equipment available and because of environmental factors. The synthesis protocol from Chapter 2.2.1 was utilized in the tests made in Turku.

The initial protocol from TUNI begun by adding 1 g of Gelatin type A (Sigma-Aldrich, Germany) and 10 ml of Phosphate Buffered Saline (PBS, Gibco, USA) into a 50 ml round-bottom flask. The mixture was heated up to 50 °C and mixed with a magnetic stirrer for an hour with stirring speed of 1000 rpm. After the Gelatin had dissolved, Methacrylic anhydride was added dropwise in 400 μl cycles for two rounds and by waiting 10 minutes in between. The solution was kept on the magnetic stirrer for 1,5 h to reach the degree of substitution of 65−75 %. The methacrylation was then stopped by adding 80 ml of PBS into it. At this point, the synthesis was ready but the solution contained unreacted Anhydrids and acidic byproducts that needed to be filtered out. The filtering was carried out by dialysis.

In Turku, the portion size of the prepared GelMA was increased in the first round of synthesis and every component of the synthesis was increased proportionally. The

portion size was now 6 grams of gelatin type A into 60 ml of PBS in a 1000 ml beaker. The stirring rate was increased to 2000 rpm. The addition of Methacrylic Anhydride was executed in the same way as previously described. Now, the addition cycle was repeated 12 times instead of just two and the wait between cycles was decreased to 1 minute from 10 minutes. The mixture was then stirred as previously and the dialysis was carried out like in the protocol from TUNI.

In the next round of synthesis, the stirring was carried out in a 200 ml glass bottle because the first synthesis had problems with dissolving the gelatin. Because the first synthesis had problems right in the beginning, it was not counted as the first complete round. In the actual first round of synthesis, the gelatin dissolved into PBS well when the stirring was carried out in this smaller bottle. The addition of Methacrylic Anhydride took approximately 40 minutes. The 1,5 hour waiting of methacrylation began when the pipetting was started. Because the container was now only 200 ml, the addition of PBS needed to be done in two parts. This was carried out by warming up most of the PBS on a separate magnetic stirrer and then the GelMA was added into it. The GelMA container was flushed with the rest of the PBS and added into the other container.

3.2 Filtering

The filtering is an important step in the process of preparing Gelatin methacryloyl for it to be used safely with living cells. The applications of GelMA are related in tissue engineering and regenerative medicine and therefore the product needs to be non-toxic. The unreacted Anhydrids and the byproduct of the methacrylation reaction are toxic to cells and they need to be filtered out. Here, two filtering methods described in section 2.2.2 were tested out.

3.2.1 Dialysis

Dialysis is a method of filtering out particles with a specific size. The selection of molecular weight cut-off determines the particle sizes that are filtered out. The dialysis was carried out with 12−14 kDa molecular weight-cutoff (MWCO) dialysis bags for 4 days changing the dialysis water 3 times a day. A foil was placed over the dialysis beaker for the heat to distribute evenly. The dialysis was carried out in 50 °C Milli-Q water with magnetic stirring speed of 1000 rpm. After the 4-day-dialysis, GelMA was ready to be freeze-dried.

A study was made to test the purity of the dialysis water before each water change. This idea came from the method of determining the degree of substitution described in Chapter 2.2.2. In the protocol for determination of the degree of substitution, the absorption spectrum of a control sample is compared to a pure material sample and a modified material sample. Based on this approach, a sample from the dialysis water was taken at every water change to be compared to each other. In theory, the absorption peak of the Anhydride should decrease in every measurement. A sample was taken from the water with a 1 ml pipet and put into a 1 ml Eppendorf tube before every water change and compared to each other with a UV spectroscope (Mettler Toledo, UV5NANO) after 4 days of dialysis.

3.2.2 Preparative cromatography

In the second round of synthesis, 3 g of gelatin was used for the synthesis. Other components were scaled to match the original proportions. Instead of the dialysis, preparative cromatography was tested at the Turku University of Applied Sciences with Jani Pelkonen, *Senior Lecturer at Chemical Engineering.*

The GelMA was run through a preparative cromatograph that changed the buffer from PBS to water and filtered out everything smaller than 10 kDa out. This way, the unreacted MA should have been filtered out. The preparative chromatograph

(Bio Rad, BioLogic LP) in Figure 11 measured the conductivity of the filtered buffer and the decrease of the conductivity was an indication that the salt of PBS had been filtered out.

The cromatograph was first tested with Milli-Q water and PBS to see the reference points of conductivity. The MQ water had a conductivity of 0 mS/cm and PBS had a conductivity of 20 mS/cm. The 0 mS/cm was then set as the desired outcome of the filtering and when the 0 mS/cm was reached, all of the saline buffer had theoretically changed to MQ water.

Figure 11. The preparative cromatograph used for filtering out unreacted Anhydrids and byproducts of the synthesis of Methacrylated Gelatin.

The machine was filled up with 50 ml of GelMA and 70 ml of warm Milli-Q water. The MQ water's and GelMA's temperatures were adjusted to 50 °C to make GelMA liquid. A stirring bar was placed in the container that had GelMA inside and stirred with a speed of 3000 rpm.

The chromatograph absorbed the buffer from GelMA through a filter with 3 bar pressure created with N_2 gas tank. The filtered buffer was lead through a mixer and through a conductivity meter into a waste bin. The suction speed of the buffer

was set to 1, 50 ml/min. For comparison, the suction speed for PBS was 10 ml/min. When the buffer from GelMA was decreased down to 50 ml, more MQ water was added. If the volume of the added MQ water was equal to the sample volume, conductivity was cut in half.

3.3 Freeze-drying

After dialysis, the solution from the first round of synthesis was aspirated into 8 ml glass bottles filling them up with 5 ml of GelMA. The caps were left slightly open because the material expands in freeze-drying and the liquid evaporates through those holes, which can be seen in Figure 12. The glass bottles were freezed first at -80 °C and then transported into freeze-drying. The freeze-drier (Martin Christ LyoLog) with the bottles inside is shown in Figure 13.

Figure 12. The cap of the bottles have little holes to make the evaporation of the liquid easier during freeze drying.

Figure 13. The Gelatin methacryloyl bottles in the Lyophilizator being freeze-dried.

The Lyophilizator closed the caps by pressing the shelves together at the end of the freeze drying cycle. As seen in Figure 13, there were orange blocks on the other shelves to help close the bottles evenly. Two bottles were selected to have a meter inside to monitor the temperature and pressure of GelMA in addition to the freeze-drier's own shelf and chamber gauges.

The program used for lyophilizing GelMA is shown in Table I. The shelf temperature was first adjusted to -30 °C for the loading of the samples. After the program was started, vacuum increased to 0.280 mbar while temperature was increased to -5 °C. A safety pressure was activated after 2 hours of lyophilizing. After 8 hours at -5 °C and 0.280 mbar, the temperature was increased to 4 °C for 9 hours. The last phases of the freeze-drying consist of warming the bottles up to room temperature for 9 hours and after that, decreasing the vacuum down to 0.050 mbar for 3 hours.

Table I. The freeze-drying cycle used for lyophilizing GelMA.

Section number	1	2	3	4	5	6	7	8	9
Phase	load	dry	dry	dry	dry	dry	dry	dry	dry
Section time [h:min]	-	2:00	6:00	1:00	8:00	1:00	8:00	1:00	2:00
Shelf temperature [°C]	-30	-5	-5	4	4	22	22	22	22
Vacuum [mbar]	-	0.280	0.280	0.280	0.280	0.280	0.280	0.050	0.050
Safety pressure [mbar]	-	off	1.650	1.650	1.650	1.650	1.650	1.650	1.650

3.4 Degree of substitution

The amount of free amino groups can be determined by UV spectroscopy by comparing the amount of free amino groups of pure Gelatin and Gelatin metharyloyl. Pure Gelatin and Methacrylated Gelatin were both measured for their absorbance spectra with UV spectroscope after the GelMA from the first synthesis had been freeze-dried.

The samples were prepared by adding 2 mg of Gelatin type A in an Eppendorf tube and 2 mg of GelMA in another tube. A buffer solution was added in both tubes and also in a third tube for the blank sample. 4.41 μL of trinitrobenzene sulfonic acid (TNBSA) was added to all three tubes. The samples were pipetted into the UV

spectroscope and scanned in the range of wavelengths between 250 nm and 500 nm.

4 Results

The synthesis method had first problems at dissolving the gelatin into PBS. After an hour of stirring the mixture, it had formed a gel although it was supposed to be clear liquid. This problem was solved by using a mixing container with smaller volume because the problem seemed originate from the mismatch between the size of the stirring bar and the diameter of the container. If there was too much empty space at the ends of the stirring bar, the mixture did not mix efficiently enough. In conclusion, the stirring bar should be about the same length as the container is in diameter for vigorous and homogenous enough stirring. In the next round of synthesis, a smaller bottle was used with same sized stirring bar and the solution became homogenous.

4.1 Dialysis

In the first synthesis, the dialysis worked well and in the second one, the foil was left off. When there was not a foil over the dialysis beaker, the dialysis bags dried from the top side that did not touch the water. This confirms the need for the foil on top of the beaker.

The dialysis water was tested with UV spectroscope. The pure material sample in this case was a 10 % (w/v) Methacrylic Anhydride solution and the control sample was pure Milli-Q water. When the pure material sample was scanned with UV spectroscope, the absorbance spectrum in Figure 14 was formed. This was used as the reference to find out, where the Anhydride peak was on the wavelength scale. The peak seems to be between $200-250$ nm.

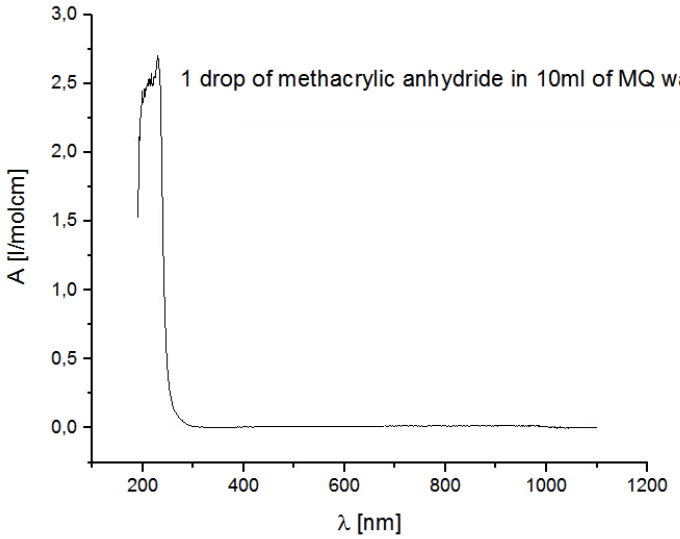

Figure 14. The reference graph of the absorption spectrum of 10 % (w/v) Methacrylic Anhydride.

After the reference was scanned, the rest of the samples were analyzed with the UV spectroscope. In Figure 15, graph the dialysis had been in action for 2 hours. The sample after 6 h of dialysis is in Figure 16, after 24 h in Figure 17, after 27 h in Figure 18, after 48 h in Figure 19, after 72 h in Figure 21 and after 96 h in Figure 20.

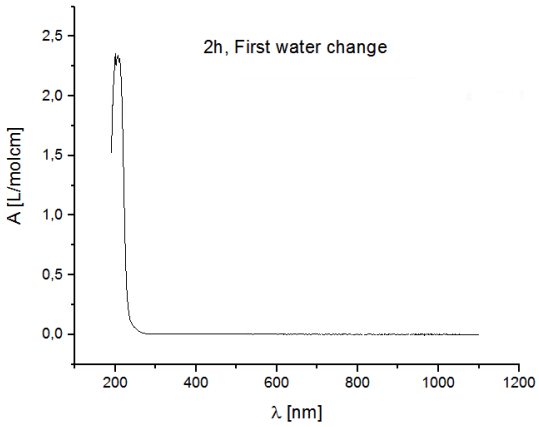

Figure 15. The absorption spectrum from UV spectroscope of the dialysis water of GelMA after 2 hours of dialysis.

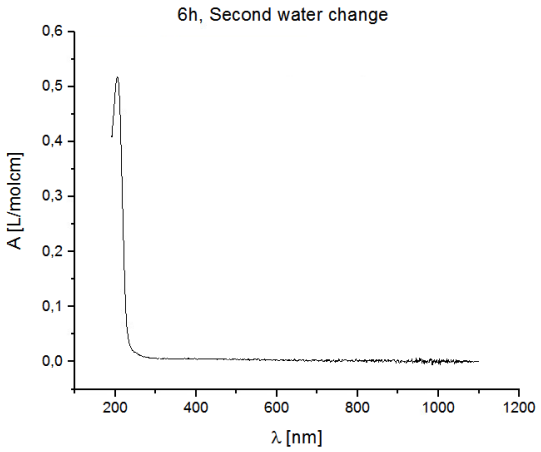

Figure 16. The absorption spectrum from UV spectroscope of the dialysis water of GelMA after 6 hours of dialysis.

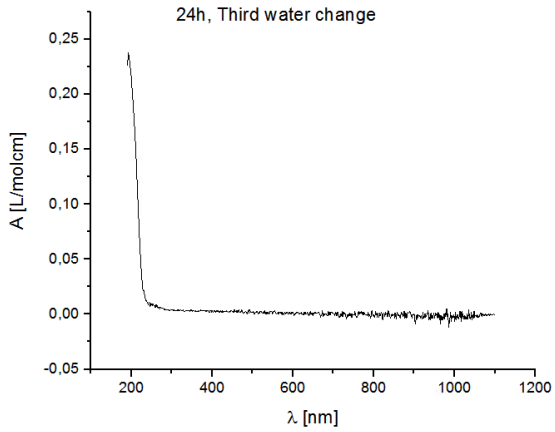

Figure 17. The absorption spectrum from UV spectroscope of the dialysis water of GelMA after 24 hours of dialysis.

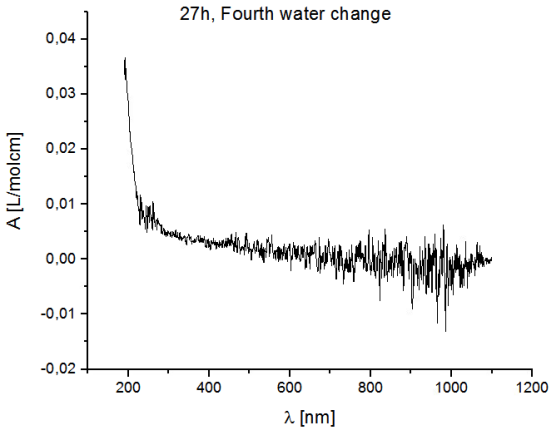

Figure 18. The absorption spectrum from UV spectroscope of the dialysis water of GelMA after 27 hours of dialysis.

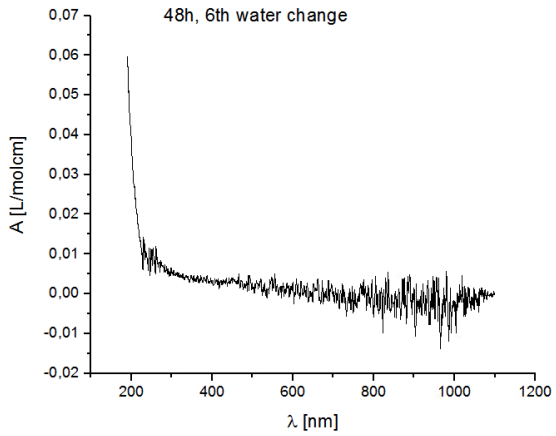

Figure 19. The absorption spectrum from UV spectroscope of the dialysis water of GelMA after 48 hours of dialysis.

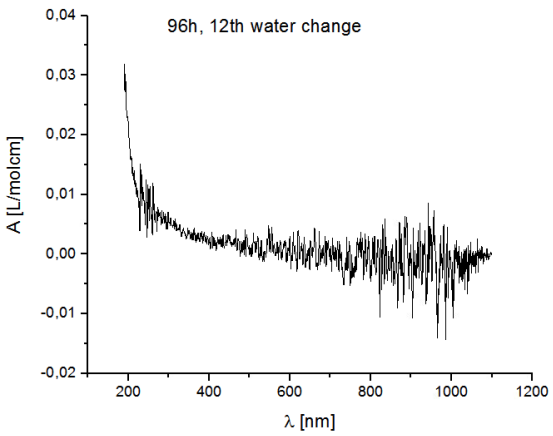

Figure 20. The absorption spectrum from UV spectroscope of the dialysis water of GelMA after 96 hours of dialysis.

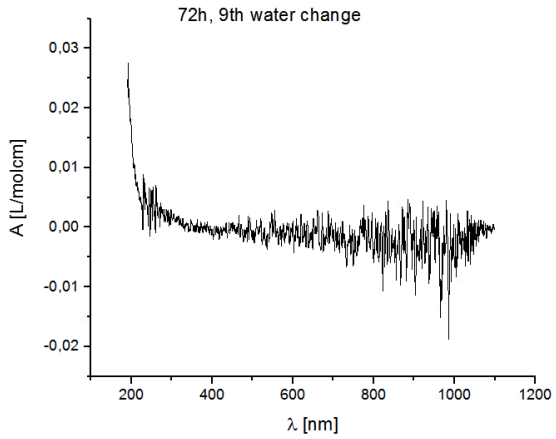

Figure 21. The absorption spectrum from UV spectroscope of the dialysis water of GelMA after 72 hours of dialysis.

From all of the graphs, the peak value was determined and the peak values were plotted as a function of time under dialysis in Figure 22. The circle mark in the Figure is the reference point where the absorption spectrum of 10 % (w/v)

Methacrylic Anhydride was measured. A curve of form $a \cdot x^b$ was fitted in the data based on trial and error. Exponential function seemed more logical and more easily connectable to the physics, but as it was tested, the curve did not seem to fit into the measurement points as well as the current one.

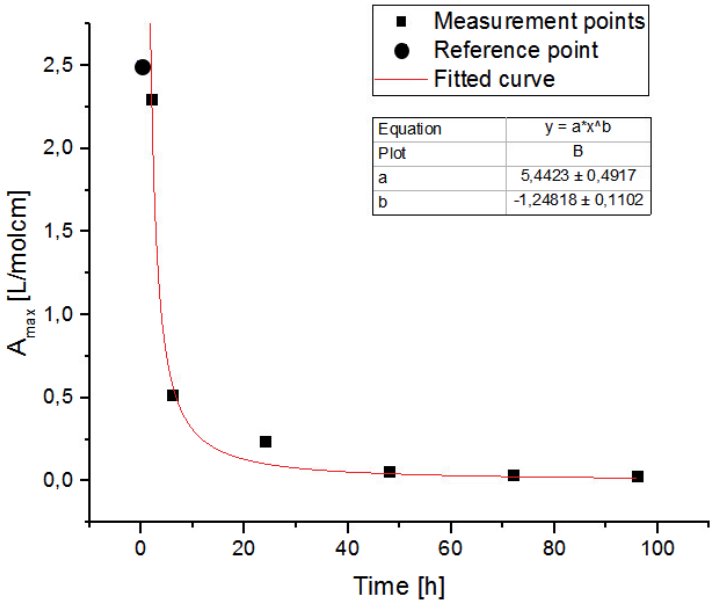

Figure 22. The reduction in the absorbance maximums over time.

This method has been thereafter used in another published study [7] to prove the effectiveness of the dialysis. The reviewers had asked to prove the success of dialysis and M.Sc. Jongprasitkul used this method to do so and the publication was accepted after this.

4.2 Preparative cromatography

As a result, the effectiveness of the buffer exchange is examined. The additions of MQ water and the changes in the volumes and conductivity are tabulated in Table II.

Table II. The additions of MQ water to a chromatograph that is filtering out saline buffer from GelMA and replacing it with Milli-Q water. The conductivity of the filtered liquid was also measured and tabulated here.

Phase	MQ water [ml]	GelMA [ml]	Volume [ml]	End volume [ml]	Conductivity [$\frac{\text{mS}}{\text{cm}}$]
1	70	50	120	60	9
2	50	60	110	60	5
3	50	60	110	50	3
4	50	50	100	50	1,6
5	50	50	100	30	0,9

The accurate values measured from the chromatograph were exported and Figure 23 was drafted from this data with *Origin 2016* -program. The conductivity decreased after every MQ water addition and reached a plateau after 5−10 minutes after the addition. When the plateau was reached, more MQ water was added. The addition of MQ water was repeated four times to achieve a conductivity of 0,90 mS/cm. The conductivity could have been closer to a zero if the process would have been repeated a few more times. In the end of the process some air bubbles got in to the system and that is why in the Figure 23 there are some downward peaks at 140-150 minutes.

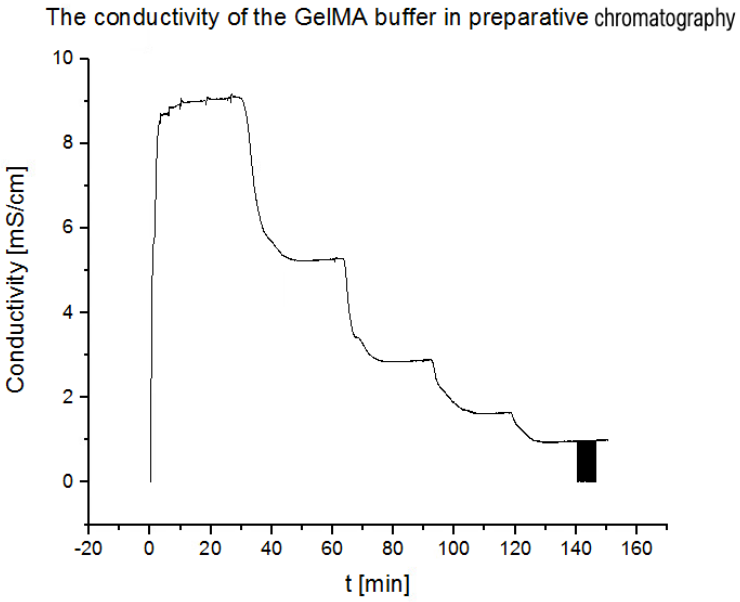

Figure 23. The conductivity of the GelMA buffer in preparative chromatograph.

Because the process of getting 0,90 mS/cm conductivity took almost 3 hours of constant human supervision, this method was not further explored. The overall human supervision time of dialysis is also approximately 3 hours and the amount of impurities decreased is $1-\frac{0.03}{2.5} = 0.988 \approx 99\,\%$ compared to the $1-\frac{0.90}{9} = 0.9 \approx 90\,\%$ decrease of conductivity in cromatography. The values might not correlate this directly but they give an understanding of the changes. Cromatography has not been used in literature for GelMA and the purified GelMA from this experiment was never lyophilized and tested in printing. Therefore, it is not clear if the cromatography changed the mechanical or chemical properties of GelMA and if it can be used in the future.

4.3 Freeze-drying

The freezing at -80 °C broke 32 % of the bottles as shown in the Figure 24. In the future, the bottles should be first freezed at -20 % for overnight and at -80 °C only for 30−60 minutes before freeze-drying. The goal temperature of the material before

freeze-drying was -30 °C because that is the initial shelf temperature.

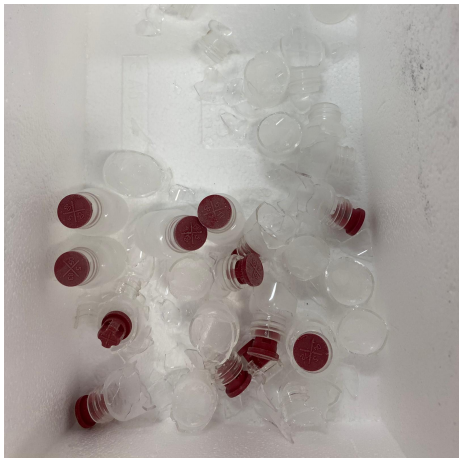

Figure 24. 32 % of the freeze-drying bottles of GelMA broke at the −80 °C freezer.

The ready product from the freeze-drying is shown in Figure 25. The product turned out to be a light weight substance with a consistency of styrofoam. This product combined with a solvent and a photoinitiator produces the printable bioink.

Figure 25. The freeze-dried GelMA.

Freeze-drying process kept records on the temperatures of the shelves and the samples with the meter inside. The freeze-dryer tracked the vacuum magnitude

as well. The report from freeze-drying is in Figure 26. In the figure, the red line describes the temperature of the shelves, blue and green lines describe the temperature of the samples and the purple line describes the pressure inside the chamber. When the samples reach the temperature of the shelves, there is no more liquid to be evaporated from the samples and then the products are ready. The freeze-drying continued in total for 29,5 hours.

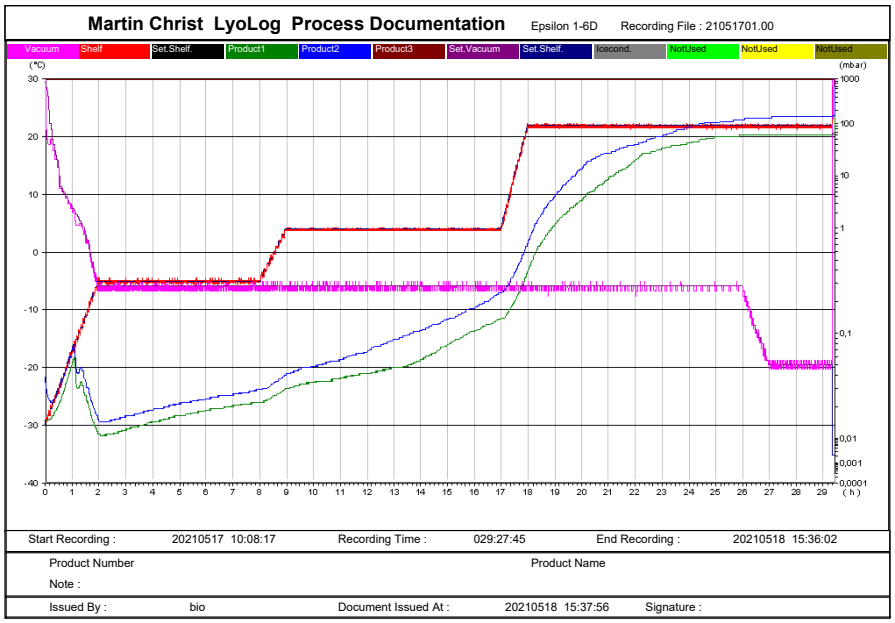

Figure 26. The graph from freeze drying.

4.4 Degree of substitution

As a result from the UV spectroscope, the graphs 27 and 28 were drawn with *Origin 2016* -program. The graphs describe the absorbance spectrums of the pure Gelatin and Gelatin methacryloyl.

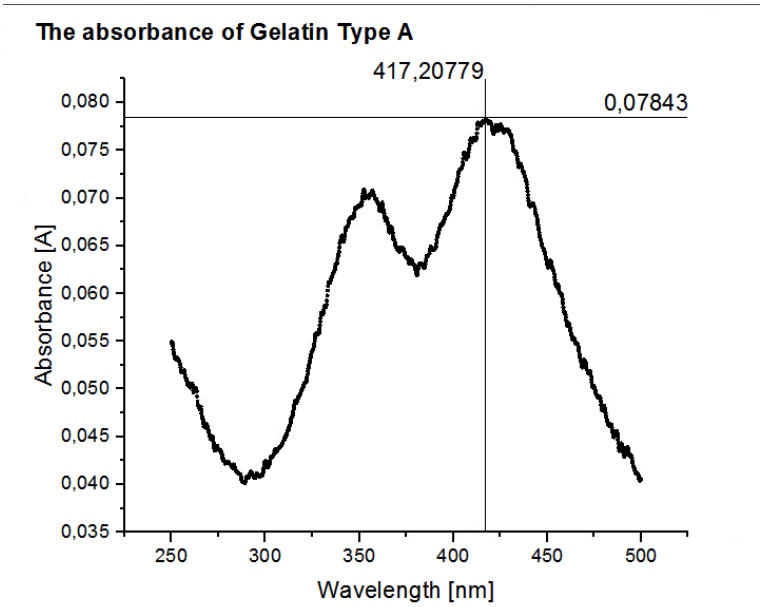

Figure 27. The absorbance of Gelatin Type A.

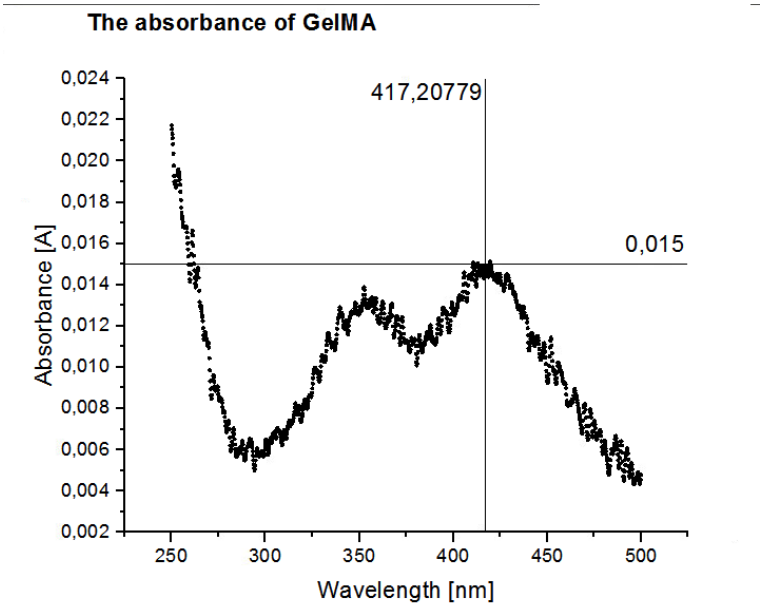

Figure 28. The absorbance of GelMA.

The peak values from both graphs were detected and their ratio was calculated to determine the degree of substitution. The results are in Table III.

Table III. The peak values of Gelatin and GelMA.

	Gelatin Type A	GelMA
Peak value [A]	0,07843	0,0150

First the ratio was calculated between the peak absorbances $\frac{0{,}0150\,\mathrm{A}}{0{,}07843\,\mathrm{A}} = 0,19125$. Then the modification degree was calculated by subtracting the ratio from one, $\mathrm{Mod}\% = 1 - 0,19125 = 0,80875 \approx 81\,\%$. The resulting degree of modification is 81 %.

4.5 The optimized method

As a result, here is the suggested method for preparing Methacrylated Gelatin based on the previously described experiments.

Heat up PBS on a magnetic stirring plate to 50 °C by putting the plate to 70 °C. Dilute Gelatin into PBS corresponding to 10 % w/v ratio. The dilution should take place in a beaker or bottle where mixing is effective for the whole volume of the solution. The mixing should continue for 30−45 minutes or until the solution becomes clear. Put on magnetic stirring at 1000−2000 rpm depending on the volume prepared. The whole solution should be visibly spinning and mixing.

Add Methacrylic Anhydride dropwise in 400 μl cycles. The total amount of MA should be 8 % of the initial PBS volume for the 1 g portion of Gelatin. If the prepared portion is larger than 1 g, the amount of MA should be optimized separately. The methacrylation reaction is let to go on for 1,5 hours starting from the point when first MA drop was added into the solution. Stop the methacrylation by adding MQ water a 100 times more than the MA.

Start dialysis by warming up 5 L of MQ water to 50 °C. Place the GelMA in dialysis bags of 12−14 kDa and put to the dialysis. A slow stirring should be applied

to distribute the heat. Cover the beaker with foil. Change the MQ-water 3 times a day. After 48 hours, take a sample from the dialysis water before changing it. Run the sample on a UV spectroscope. If the peak value between 200−250 nm is close to zero, the dialysis is ready. For reference, the initial peak value of 10 %w/v MA solution is approximately 2,7 $\frac{\mathrm{I}}{\mathrm{molcm}}$. 0,05 $\frac{\mathrm{I}}{\mathrm{molcm}}$ is a good reference and when this value is reached, the dialysis has filtered out most of the unreacted Anhydrides.

Aspirate the GelMA into freeze-drying bottles. Leave the caps open or closed depending on the freeze-drier. Do not fill the bottles full because the material will expand a little while the liquid evaporates. Put the bottles at -20 °C for over night and to -80 ° for 30-60 minutes before freeze-drying.

Determine the modification degree of the ready product by comparing the absorbance spectrum of pure Gelatin and GelMA. The ratio between the peak values is subtracted from 1 and it is the modification degree. The value should be between 65−75 % to get optimal cross-linking during the printing process.

5 Discussion and conclusions

The synthesis of methacrylating Gelatin was rather vaguely described in section 2.2.1 and the experiments with it began with a training at university of Tampere with M.Sc. Jongprasitkul. The synthesis was successful at TUNI but in Turku, there were some challenges. First, the gelatin did not dissolve well in the first trial of synthesis. This was caused by a poor choice of equipment where the stirring bar did not fit the beaker. Gelatin requires vigorous stirring and it can only be achieved with a stirring bar that is the same length as the beaker is in diameter.

In the next step of the synthesis, Methacrylic Anhydride was added dropwise to methacrylate the free amino groups of Gelatin. In the TUNI training, pH level adjustment was mentioned and conducted prior to the initialization of methacrylation. The pH level was in the optimal level already at TUNI, and it did not require any

modifications. In the synthesis at Turku, the pH level adjustments were discarded because the ordered pH paper never arrived, and it was not expected to impact the results greatly after the TUNI experiment was successful without the pH adjustments. After researching about the pH levels, it became clear that pH should be modified and adjusted during the addition of MA for the reaction to be the most effective [1, 5, 24]. A study was even made to determine the effects of pH adjustment on the DS, as shown in Figure 29 [24]. The study found out that by not adjusting the pH, lowest DS is achieved compared to adjusting the pH only in the beginning and adjusting the pH constantly [24]. The result of adjusting pH in the beginning was almost as high as the constant adjustment and it could be concluded that the pH adjustment in the beginning of the MA addition could lead to the most effective synthesis of GelMA.

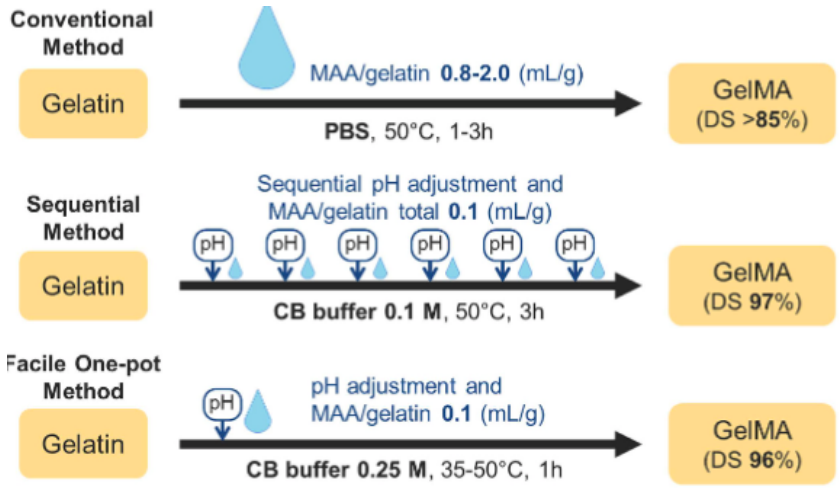

Figure 29. The methods of MA addition were studied by Shirahama et al. [24]. The synthesis without pH adjustment (conventional method) had the lowest DS value, constant pH adjustments (sequential method) had the highest DS, and only adjusting the pH level in the beginning (facile one-pot method) had almost as high DS as the constant adjustment method [24].

In the addition of MA, another concern arose. The addition is done by one drop at a time and there should be a small wait in between for the reaction to proceed steadily. If a much larger volume of GelMA was prepared, the pipetting

time might take longer than the methacrylation itself. Also, the relationship between the amount of MA proportional to the amount of Gelatin should have further studies made on. The amount of MA might not be directly proportional to the amount of Gelatin to achieve the same DS.

Next in the preparation of GelMA, was the filtering of unreacted MA and the reaction byproducts. Dialysis is a widely used method of filtering GelMA and it was tested first. The method is simple but one note from dialysis procedure is that a foil should be placed on top of the beaker for the dialysis bags to stay wet from both sides. Another concern was also present from the beginning of learning about the dialysis. The procedure does not have a specific end point where the dialysis should be stopped. A method for testing the dialysis water was then invented and developed based on the measurement of the degree of substitution with UV spectroscopy. A sample was taken from the dialysis water before most water changes and tested with the UV spectroscope for the peak values indicating the amount of MA in it.

The peak values decreased rapidly during the first 24 hours and then the values stabilized close to zero. Hence, it could be concluded that the dialysis had worked well and it worked even faster than expected. After 48 hours the process could even be stopped and the result would be 97,6 % down in the peak value. Overall, the greatest intake from this experiment is the knowledge of the peak placement of Methacrylic Anhydride and its reference value. In the future, the dialysis water purity could be tested after 48 hours to see if the peak value has settled close to zero and the dialysis could even be stopped then. The original assumption was that dialysis could take up to 7 days [6] and the information of the dialysis to be effective even after only 48 h is inspiring. The method of determining the end point of dialysis developed in this thesis could be more widely used because it could cut down the dialysis time by a lot.

Another aspect of this method is the fact that the reference graph is only from a

solution of MA and MQ water. There are no byproducts of Methacrylic acid in the reference graph and it cannot be concluded that Methacrylic acid would be filtered out faster, or in the same rate as the unreacted MA. On the other hand, MA is larger molecule than Methacrylic acid and it could be assumed that the acid is therefore filtered out faster, but this would require further testing.

The other tested method of filtering GelMA was preparative chromatography. There the buffer solution was replaced with MQ water while filtering out everything smaller than 10 kDa. The molar mass of GelMA is 901,982 g/mol. The largest reagent in PBS is Na_2HPO_4 which has a molar mass of 141.99 g/mol and the MA molecule has a molar mass of 154.165 g/mol. The molar masses were calculated based on the table of elements. The MA molecule is slightly larger than any PBS molecules but far away from the GelMA molecule. Therefore, it could be concluded that the MA molecules were effectively filtered out in the preparative chromatograph along with the salts in PBS. The GelMA was dialysed with 12-14 kDa membrane dialysis bags and the filter used in the preparative chromatograph was 10 kDa, which means that the chromatograph filtered out even smaller particles than the dialysis. The product was never lyophilized and tested in printing so it cannot be known if the chromatography changed any properties of the GelMA.

The filtering time of preparative chromatography is significantly shorter than the dialysis time but the process requires constant human supervision. During the 3 hours of chromatography, the percentage of impurities decreased much less than with overall decrease of impurities in dialysis. With the new method of testing when the dialysis process has filtered out the unwanted molecules, the dialysis time can even be decreased down to total of two days and the active work in dialysis would decrease to approximately 75 minutes. The dialysis is also a low-cost option that is easy to implement in any laboratory.

After filtering GelMA it was lyophilized. The freezing was optimized to be

done in two sections. First, by freezing GelMA overnight at -20 °C and then before transferring the material to lyophilizator, 30 minutes at -80 °C. The goal temperature of the material is -30 °C when it is placed in the lyophilizator but the machine was at the University of Applied Sciences and it requires a short walk of 500 meters. Therefore, the material was cooled lower than the required temperature because the walk would warm up the product. If there is no transportation between the freezer and the lyophilizator, the cooling could be done at only -30 °C.

From the lyophilized product, the degree of substitution was measured. As a result, 81 % degree of substitution was achieved. The 81 % degree of substitution was higher than expected. High DS could be a consequence of too long methacrylation or too large volume of the MA added. In this first synthesis, the beginning of the 1,5 h wait for the methacrylation begun later than from the beginning of the addition of MA. The reaction was then continued for too long and the prolonged duration of the methacrylation might be the cause for too high DS. Another reason could be in the scalability of the amount of MA. There might not be a linear dependency between the final volume of GelMA and the added MA. In this case, the amount of MA was multiplied by 3 because the amount of Gelatin was increased by 3. The relationship between MA and Gelatin might not be this simple and the relationship would require further studies. Also, the addition of PBS to stop the methacrylation might not work as well as adding pure MQ water.

In conclusion, the study of the process of preparing Gelatin Methacyloyl produced good discussion and more information and questions about the procedure. The issue of the scalability of Gelatin and MA should be studied further and pH adjustment should be added to the beginning of the MA addition. Preparative cromatography could be used as a filtering method of removing unwanted molecules from GelMA as a faster alternative to dialysis. The method of preparative cromatography should be examined for the impact on the molecular, rheological, and

mechanical properties of the ready bioink. Freeze-drying program represented in Table I in Chapter 3.3 seems to be an effective drying method that preserves the mechanical properties of GelMA. The degree of substitution can be effectively measured with UV and H NMR spectroscopies. The new method of determining the end point of dialysis could be more widely used and introduced to the scientific community. The experiments and analysis done in this thesis provide a good platform for anyone to develop their own GelMA for bioprinting or to search information about a specific step in the synthesis.

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