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3D Printing of Gelatin Methacrylate

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Bachelor’s Thesis | Abstract

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3D Printing of Gelatin Methacrylate

The aim of this thesis was to investigate the printability of gelatin methacrylate (GelMA) with the Brinter® ONE bioprinter, whilst another goal was to synthesize our own gelatin methacrylate and compare it to the commercial gelatin methacrylate of Sigma Aldrich. The work was performed at Brinter Oy's premises and with the equipment offered by Brinter Oy.

GelMA is a biocompatible hydrogel that can be used especially in a variety of tissue engineering applications. As a hydrogel, GelMA can be printed in the shape of the desired object and, with a suitable photoinitiator, it can be cured under UV light, making its structure also physiologically resistant. GelMA is also a rheologically temperature-sensitive material, which is why it was printed by using Brinter's Pneuma Cooled printing tool.

The actual work started with the synthesis of GelMA. At the end of the synthesis, GelMA was lyophilized at Turku University of Applied Sciences and a print-ready bio-ink was prepared from it. Appropriate print settings were tested by printing a square with a 45 ^{o} grid fill. In the actual tests, corresponding pieces were printed at the height of two printing layers to determine the Pr value, and 10-layer cylinders were printed to determine the stackability of the material. These constructs were repeated with Sigma Aldrich's GelMA and the results were compared. On the basis of the tests and comparisons, it was found that the Brinter® bio printer can be used to print gelatin methacrylate. GelMA manufactured in Turku also corresponded to Sigma Aldrich's GelMA within the margins of error, but due to the high degree of methacrylation and high concentration of GelMA, the study must be continued.

Keywords:

3D Printing, Gelatin methacrylate, bioprinter, tissue-engineering

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Tekijä Joona Mölsä

Gelatiinimetakrylaatin 3D-tulostus

Tämän opinnäytetyön tavoitteena oli selvittää gelatiinimetakrylaatin (GelMA:n) tulostuvuutta Brinter® ONE -biotulostimella. Toisena tavoitteena oli syntetisoida omaa gelatiinimetakrylaattia ja verrata tätä kaupalliseen Sigma Aldrichin gelatiinimetakrylaattiin. Työ suoritettiin Brinter Oy:n tiloissa ja laitteistoilla Brinter Oy:n tilauksesta.

GelMA on bioyhteensopiva hydrogeeli, jota voidaan käyttää etenkin erilaisissa kudosteknologian käyttökohteissa. Hydrogeelinä GelMA:a voidaan tulostaa halutun objektin muotoon ja sopivalla fotoinitiaattorilla se voidaan kovettaa UV-valolla, jolloin sen rakenteesta saadaan myös fysiologisesti stabiili.GelMA on myös reologialtaan lämpötilaherkkä materiaali, minkä vuoksi sitä testattiinkin Brinterin Pneuma Cooled -tulostuspään avulla.

Varsinainen työosuus aloitettiin GelMA:n synteesillä, jonka päätteeksi GelMA kylmäkuivattiin Turun Ammattikorkeakoululla ja tästä valmistettiin tulostusvalmista biomustetta. Sopivia tulostusasetuksia testattiin tulostamalla 20 x 20 mm neliötä 45° grid-täytöllä. Varsinaisissa testeissä tulostettiin vastaavia kappaleita kahden tulostuskerroksen korkuisena Pr-arvon selvittämiseksi ja 10 kerroksisia sylintereitä, jotta materiaalin pinottavuutta voitiin tarkastella. Nämä toistettiin Sigma Aldrichin GelMA:lla ja tuloksia vertailtiin keskenään.Testien ja vertailuiden persuteella voitiin todeta, että Brinter® ONE -biotulostimella voidaan tulostaa gelatiinimetakrylaattia. Myöskin Turussa valmistettu GelMA vastasi virhemarginaalien rajoissa Sigma Aldrichin GelMA:a, mutta GelMA:n korkeasta metakrylaatioasteesta ja suuresta pitoisuudesta johtuen tutkimusta tulee jatkaa.

Asiasanat:

3D-tulostus, Gelatiinimetakrylaatti, biotulostus, kudosteknologia

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List of abbreviations

ECM	Extracellural Matrix
Fe_3O_4	Iron Oxide
GelMA	Gelatin Methacrylate
LAP	Lithium phenyl-2,4,6-trimethylbenzoylphosphinate
PBS	Phosphate-Buffered Saline
RGO	Reduced Graphene Oxide

1 Introduction

This study was made in collaboration with Brinter Oy by using their Brinter® ONE bioprinter with a Pneuma Cooled printing tool and using their facilities for both synthesizing and testing of the Gelatin Methacrylate (GelMA). Brinter is a Finnish bioprinting company which provides solutions and services for researchers in the field of 3D bioprinting. Currently, Brinter can be used in tissue engineering and drug delivery, for example, but in the future the company aims to advance bioprinting to a whole new level. This allows, even more tailored made solutions for researchers and patients. (Brinter, 2018a.)

Gelatin Methacrylate (GelMA) is known as a photocurable and physicochemically tunable hydrogel with good biocompatibility and adjustable biodegradation properties. The two-step crosslinking of GelMA is its main selling point to be used in 3D printing. This is made possible by reversible thermal gelation and permanent photocrosslinking. Researchers have gotten promising results in filament formation and printability, which is why GelMA is seen as a great candidate in complex tissue 3D printing. The results of 3D printing at different temperatures indicate that the decrease in temperature improves the formation of the filament and the accuracy of the precipitated form on the hydrogel, especially at a temperature around 15 °C. (Janmaleki, et al., 2020.)

The aim of the study was to determine the optimal printing parameters for self-made GelMA and compare it to a commercial counterpart. An application note was written for the self-made GelMA with the help of the newly found printing parameters. All the tests were carried out using the same Brinter® One 3D bioprinter and the same printing tool. Brinter's Pneuma Cooled UV print head was used to allow control over the printing temperature and to allow a UV curing of the material.

2 Theoretical background

Gelatin methacrylate (GelMA) is a semi-synthetic hydrogel consisting of gelatin derived methacrylamide and methacrylate groups. These hydrogels are used to provide cells with an optimal biological environment. GelMA can be cured quickly under UV light which allows accurate printing and stabilizes the substance to be used in a physiological temperature. (Pepelanova;Kruppa; Scheper;& Lavrentieva, 2018.) Some of GelMA hydrogel's properties closely resemble the properties of the native extracellular matrix (ECM) because they include peptide motifs that allow cells to attach to the GelMA-based scaffolds (Yue, et al., 2015). This is why GelMA can be seen as a great candidate in the eyes of tissue engineering. GelMa can be used to mimic bone-, cardiac- and epidermal tissue.

2.1 Bone tissue

As a material, GelMA is also considered suitable for printing regenerative bone tissue. Bone tissue is a very complex material, so a simple hydrogel without any additions does not meet the requirements for bone regeneration. Bone tissue's structure is anisotropic and ranges from a macroscale to a nanoscale. Various cells, such as osteocytes, osteoblasts, and osteoclasts, have been associated with the natural bone microenvironment. A particular dynamic microenvironment consisting of collagen, inorganic nanoparticles and growth factors could naturally regulate cell behavior. Taking all of this into account and in order to achieve biocompatibility, the gelatin scaffolds must have a suitable biomechanical structure and physiological properties for bone regeneration due to the complexity of natural bone tissue. To be able to mimic a natural bone tissue, various inorganic agents can be added to the hydrogel to improve the performance of the GelMA scaffolds in bone regeneration. For example, a small amount of modified magnetic iron oxide (Fe_3O_4) nanoparticles can be added to GelMA to greatly extend its mechanical stiffness and overall durability. (Dong, et al., 2019.)

2.2 Cardiac tissue

In heart tissue, GelMA can be used as a growth platform for new cells on damaged cardiac tissue. As such, GelMA can be seen as a great asset in stem cell research which could allow

GelMA to be used to cure dead cardiac tissue caused by the lack of oxygen in the heart tissue after suffering a heart attack. GelMA has the same electrical conductivity as natural heart tissue, which is extremely important as cardiac tissue contractions depend on the electrical pulses sent out from the sinus node. (Zhu, et al., 2021.) GelMA also has the needed physical properties to be able to be used as scaffolds for functional cardiac tissue. When mixed with reduced graphene oxide (rGO), the GelMA-based scaffolds could survive the stress caused by the surrounding cardiac tissue whilst contracting. They also corresponded better for externally given electricity giving them a better spontaneous beating rate, which made it better for conductivity when compared to scaffolds consisting of only GelMA. (Shin, et al., 2016.)

2.3 Epidermal tissue

GelMA can also be used to mimic epidermal tissue, such as skin. As a reference, GelMA can be used to heal wounds by adding keratinocytes to its structure. This creates a substitute scaffold for the natural epidermal tissue to grow on to. These GelMA scaffolds can support the growth, specialization and stacking of the keratinocyte layers, which in return creates a multifunctional epidermal tissue (Yue, et al., 2015). As in the case of the cardiac tissue, the epidermal tissue is also under constant stress due to the stretching and outside wear. This is why GelMA can be seen as a great candidate to speed up the natural growth of the epidermal tissue around the wound. This is due to a sufficient material stiffness for cell adhesion and keratinocyte monolayer formation, whilst collagenase degradation is kept under control and prolonged. (Zhao, et al., 2015.)

2.4 3D printing

In 3D printing, the principle of a traditional printer is used to produce physical objects instead of the traditional two-dimensional ink on a paper approach. A 3D printer can print plastics and metals as the most common materials, but sand, cement, chocolate, and living tissue can also be printed into a three-dimensional object that can be custom made to fit to its own purpose perfectly. The technique can also be called additive manufacturing, which is the opposite of reducing manufacturing, where the material is reduced from a piece. Incremental fabrication results in structures and objects that would be impossible to produce with traditional methods. This also makes it possible to manufacture the necessary components at once and

avoid unnecessary assembly. These objects are first digitally produced with the help of a computer program such as CAD software and then transferred into the 3Dprinter itself. (Niittymies, 2019.)

2.5 Printability

As an example, the printability of a certain material can be defined by measuring the filament line's uniformity or by measuring how evenly the filament layers stack on to each other. The rheology of the material itself plays a crucial part here. If the bioink has a viscosity that is too low, the material won't keep its printed shape. On the other hand, if the viscosity of the bioink is too high, it doesn't have a steady flow out of the printing head and the filament can either be cut or bent as the printing arm moves across the print bed. In a more dramatic case, the whole printing head can get jammed and the bioink either won't be printed at all, or the excess pressure caused by the stuck bioink can cause it to spit out clots on the print bed. (Wang, Backman, Nuopponen, Xu, & Wang, 2021.)

2.6 Lyophilization

Lyophilization is used to extend the shelf life of GelMA. Lyophilization is used as a stabilization process in which GelMA is frozen and the liquid is removed from the solid-state through sublimation and desorption processes. Lyophilization has three distinguished steps: freezing, primary drying and secondary drying. In the freezing stage, the liquid product is frozen below its glass transition temperature, which means that the product has reached the lowest temperature where the molecular mobility of the amorphous molecules is approaching zero. This is important because if the product warms up above this temperature during the process, it can lower its viscosity greatly and thus result in a collapse of the material as the amorphous structure changes. In the primary drying, the lyophilizer chamber pressure is lowered, which allows the ice crystals to be removed by sublimation. This is done by keeping the material in a vacuum and temperature below the glass transition temperature, which then creates a porous structure. The purpose of secondary drying is to achieve the long-term stability of the product by removing entrapped non-crystalline moisture from the amorphous phase. The remaining water is desorbed from the product at temperatures above room temperature. For pressure ranges typically used in the lyophilization of biological products, the speed is not that

dependent on pressure. Chamber pressure then often remains in the same range as used for primary drying. (Wen, Pujar, & Ellis, 2014.)

3 Materials and methods

The work began with the synthesis of GelMA. Gelatin was stirred with a magnetic stirrer in a 50 °C degree water bath for one hour, before adding 50 ml of Milli-Q water to a 50 ml of 2X PBS buffer, creating a 100 ml of 1X PBS buffer. Right after 10 g of gelatin was dissolved into the buffer and methacrylate anhydride was added by pipetting it 400 µl at a time. This was repeated 20 times, after which 70 ml of PBS was added for every ml of gelatin. The mixture was dialysed for 96 hours to get rid of the unreacted methacrylate. The product was poured into dialysis bags, which were then placed into a 10-litre glass jar full of Milli-Q water with constant stirring. The dialysis water was changed every four hours to ensure the removal of small molecule gelatins and methacrylic reaction by-products. This step was then carried out for 96 hours.

The synthesized GelMA was lyophilized to increase its self-life. An accurate amount of 1 g of GelMA was weighed into each lyophilization ampoule. The lyophilization itself was carried out at Turku University of Applied Sciences and the lyophilized GelMA was transferred back to Brinter Oy for long term storage.

3.1 Bioink preparation

The bioink was prepared by weighing the LAP initiator at 0,05 % weight per volume from the amount of wanted bioink. This was then dissolved into a PBS buffer and left stir for 15 minutes at 60 °C temperature. Lyophilized GelMA was weighed to achieve 10 % precentage in the final bioink and added to the PBS buffer containing the LAP initiator. Depending on the tests at hand, GelMA were either our own synthesis product or Sigma Aldrich's GelMA. These mixtures were gently stirred at 60 °C temperature for 60 minutes. This was done in a dark room and in a beaker that was coated with aluminium foil to prevent premature curing. The bioink was poured into the printing syringe barrel and set to cool in a fridge overnight. (Allevi, Allevi3d, 2022.)

One of the crucial parts of the bioink preparation is the addition of LAP photoinitiator, which allows the GelMA to cure under UV light. This photocrosslinking of GelMA is part of the

gelation process required to reach the wanted solid object. The main selling point of LAP is the fact that it doesn't affect the cell viability in GelMA. This is far more important if cells were added to the mixture as the free radical polymerization process of the bioink includes parts that could be potentially harmful to the cells. This approach has three main risks: exposure to harmful wavelengths, unterminated free radicals from the polymerization process released by the activated photoinitiator, or the photoinitiator itself. LAP fixes the latter of these problems as it doesn't affect the viability of the cells when mixed with the monomer as it doesn't release these free radicals that could expose the cells. (Allevi, Allevi3D, 2020.)

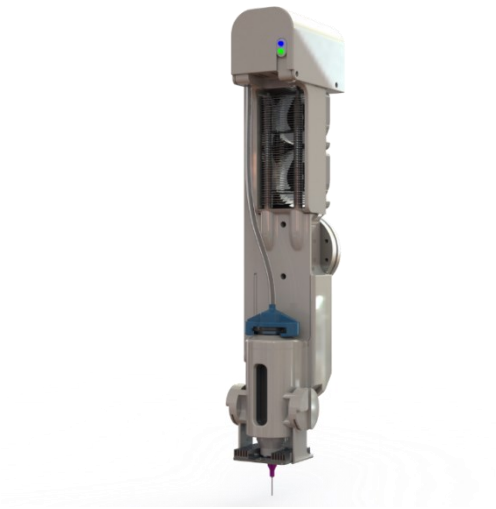
3.2 Brinter® bioprinter

The Brinter® software is completely web-based, allowing you to control printing parameters such as printer movement, height, pressure, and the slicing of the selected object. The selected and sliced object is sent from the web client to the Brinter. The printing works with the help of compressed air, and the material to be printed is placed in a printing syringe barrel, to which a printing nozzle of the desired size is attached. The syringe barrel is then inserted into the Brinter's printing tool. The Brinter retrieves the tool and calibrates itself, but the Z height is best set manually for an accurate result. Once this is set, printing will begin. During printing, it is also possible to change the height of the nozzle tip and the pressure and movement speed on the fly. The Brinter itself, as seen in the picture 1 below and the printing tool can be equipped with a UV led module to cure the printed material. This can be done while printing, between layers or after the printing has finished. (Brinter, Brinter, 2018b.)

Picture 1 Brinter® ONE 3D bioprinter (Brinter, 2018b).

3.3 Pneuma Cooled printing tool

Pneuma Cooled is a printing tool designed for temperature-sensitive materials and/or materials that have a photoinitiator added to them and thus can be cured with UV light at a wavelength of 365 nm, 405 or 450 nm. The tool itself, as seen in the picture 2 below is based on a pneumatic 3D printing method where pressure is used to push the material out of the printing head. (Brinter, Pneuma Tool Cooled, 2018.)



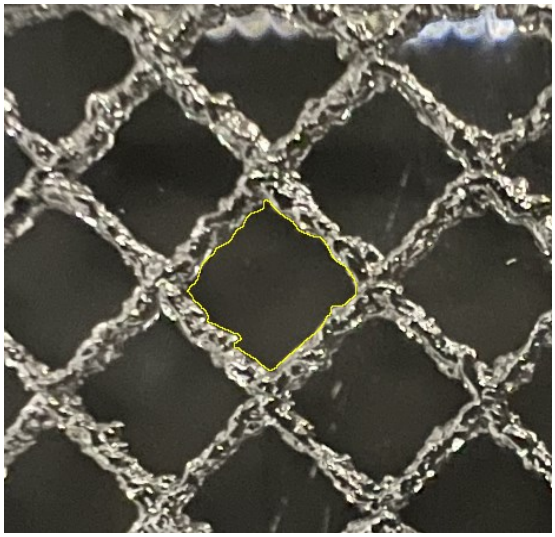
Picture 2 Pneuma Tool Cooled print head.

3.4 ImageJ program

Photos taken from printed grid filled objects were uploaded into the ImageJ program running the Fiji extension, and three pores were selected from the same spot from each printed object. Each pore's outline was selected carefully by hand and thus given for the program to calculate their circularity (C), as seen in picture 3 below. This was then used to calculate each pore's Pr-value, as seen in the equation below. Pr- value under 1 would suggest under-gelation, where as pr-value over 1 would suggest over-gelation and thus a pr-value of one would mean a prefect square pore (Ouyang, Yao, Zhao, & Sun, 2016).

$$Pr = ((\pi/4)) \cdot (1/C)$$

Equation 1 Pr-value



Picture 3 Identifying the circularity of the pore.

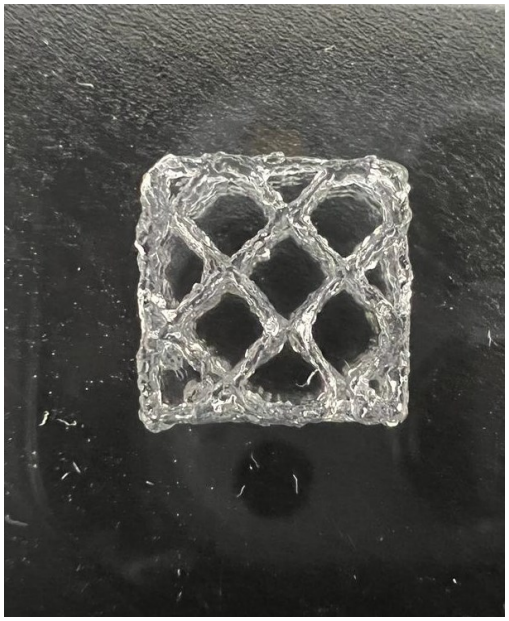
4 Printing of GelMA

Printing started by testing the GelMA's properties with a square object which was filled using a 60 % grid fill. The object was cured after the printing had finished with the printing tool's UV module, and thus, a black UV protected nozzle was used. The aim of these tests was to find suitable printing parameters for the larger scale Pr-tests. First prints were carried out using the printing parameters as seen in Table 1 below.

Table 1 Turku GelMA optimal printing parameters.

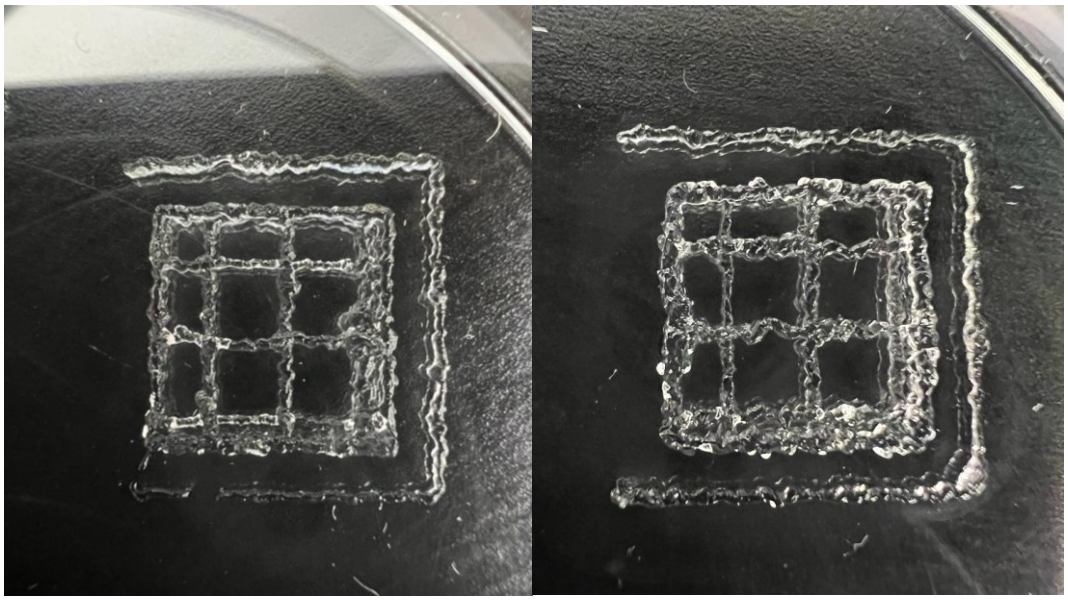
Temperature (°C)	Pressure (mbar)	Nozzle width (µm)	UV intensity (%)	Layer height (mm)	First layer height (mm)	Layers
20	945	200	0	0,2	0,2	7

The printed structure had uneven filament, which indicated that either the printing temperature was too high or that the material used had a high viscosity, as seen in picture 4, but it demonstrated that the GelMA could be printed without constant curing as this could cause the nozzle to heat up.



Picture 4 Turku GelMA 10 x 10 mm grid with 7 layers.

A few tests were carried out with a 10 mm x 10 mm slab with a 6 % solid fill and the solid fill angle set to 45 degrees. This would have been a better way of printing the objects for the Pr-value tests because all of the angles would have been 90 degrees as two of the filament layers would have overlapped each other. With these objects, the main point of interest was to study the effect of the printing tool's temperature on the printing quality. As the solid fill objects' layers overlap with each other, the filament could easily snap in half as the nozzle would constantly be one layer (0,2 mm) higher from the print bed, as seen in the picture 5. As such the printed object with solid fill was deemed to be too hard for the Brinter to be printed accurately. These tests were carried out all the way from 12 °C to RT (22 °C).



Picture 5 Turku GelMA with solid fill at 12 °C (left) and 22 °C (right).

The filament and print quality were noticed to be basically identical from 12 to 22 degrees, as seen in picture 5 above. When comparing the pictures 5 and 6 above, an assumption can be made, that it wouldn't have been wise to continue with the solid fill as printing multiple layers would have had too many variables on stackability. One mistake on the bottom layer would accumulate on the layers above.

The high level of methacrylation was, at this point, estimated to be the main problem resulting in the uneven filament. The level of methacrylation was deemed to be too high, so we decided to move on to experiment with Sigma Aldrich's GelMA. In the case of Sigma Aldrich's GelMA, it was printable within the same parameters that we had optimized for the Turku GelMA.

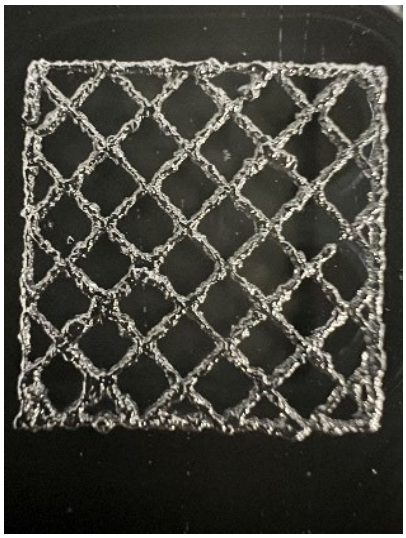
4.1 Pr-value tests

The temperature for Pr-value tests was set to RT (21 °C) because the filament and print quality were noticed to be basically identical from 12 to 22 degrees. Also, the object was changed to a 20 mm x 20 mm square slab with a 60 % grid fill, as seen in the picture 6 below. This was done because the solid fill printed object was deemed to be too difficult for Brinter to be printed accurately. Three identical objects were printed with parameters seen in the table 2 below.

Table 2 20 x 20 mm Pr-value test parameters.

Temperature (°C)	Pressure (mbar)	Nozzle width (µm)	UV intensity (%)	Layer height (mm)	First layer height (mm)	Layers
21	1200	200	0	0,2	0,2	2

Printed grids were photographed and uploaded into the ImageJ program running the Fiji extension to calculate their circularity C, which was used to calculate the Pr-value for the pores, as seen in the equation 1 on chapter 3.5.

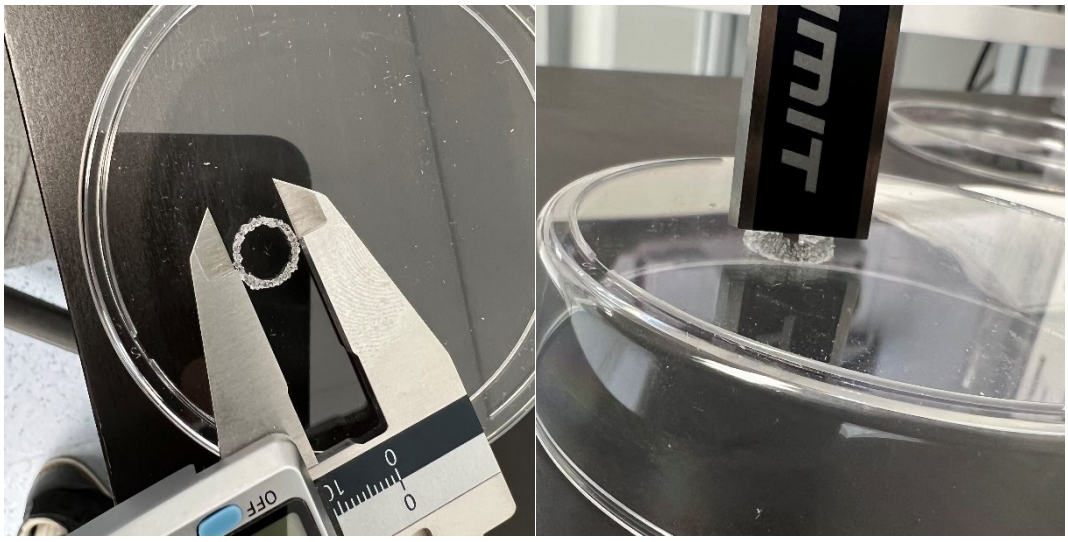


Picture 6 Turku GelMA 20 mm x 20 mm slab with grid fill.

4.2 Stackability

Stackability was tested by printing three identical cylinders with 10 layers each. Their outer diameter and height were then carefully measured by using a calliper, as seen in picture 7.

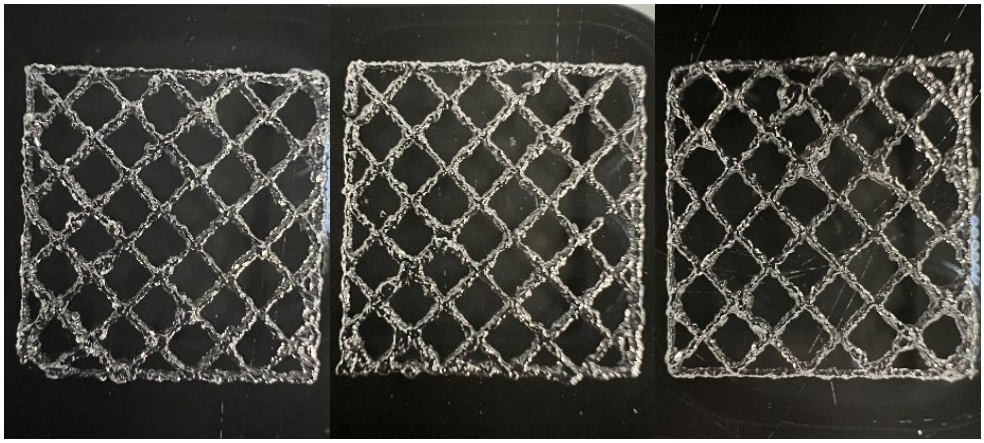
This was done for Turku GelMA and Sigma Aldrich GelMA to determine if they would considerably differ in stackability. Besides the number of layers, the parameters used were the same as in the Pr-value tests above table 2. These cylinders were printed from a 3D model that had an outer diameter of 10 mm and a height of 2 mm. A cylinder with a wider diameter could have been easier to print, but these parameters allowed the objects to be printed faster and to save GelMA that was running out at this moment. This is also the most important reason why these printing tests couldn't be continued, as a new batch of Sigma Aldrich's GelMA would have taken several months to arrive due to the worldwide material shortage.



Picture 7 Stackability measurements.

5 Results

Circularity (C) was used to calculate the Pr-value for each of the three pores in each three objects according to the equation below. Microsoft Excell was used to calculate the Pr-value, standard deviation and average. This was done for Turku GelMA and Sigma Aldrich's GelMA test results to compare their printing quality on a more accurate level. A Pr-value of 1 would indicate a perfect rectangular pore as stated in the chapter 3.5.



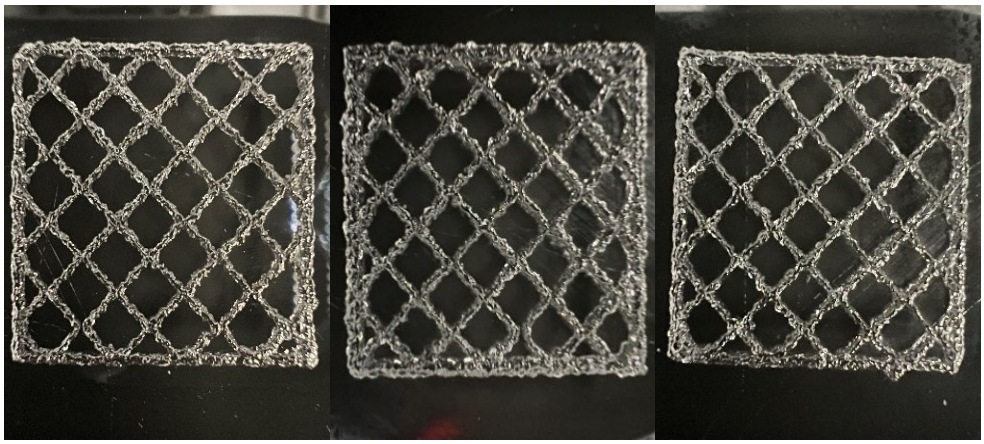
Picture 8 Turku GelMA Pr-value test prints.

Table 3 Turku GelMA Pr-values

Pore	Circularity (C)	Pr-value
1	0,681	1,153
2	0,687	1,143
3	0,613	1,281
4	0,687	1,143
5	0,691	1,136
6	0,719	1,092
7	0,657	1,195
8	0,706	1,112
9	0,669	1,173
Standard deviation (all)		0,052
Average (all)		1,159

Turku GelMA had a pretty rough filament when inspecting it by eye. The Pr-tests would indicate that the printing quality can't be considered decent. Ideal Pr-value would be between

0.9-1.1 and even a minor deviation out of this range would indicate a poor printing quality. The main problem here was the inconsistency of the mistakes in the printed object. As the printing head got sometimes blocked, the area that got either too little or too much of the filament vastly impacted the measurements. These can be seen in picture 8 above, where the excess GelMA can be seen on the outer pores of the printed object. These tests were also done for the commercially available Sigma-Aldrich's Gelatin Methacrylate for comparison.



Picture 9 Sigma GelMA Pr-value test prints.

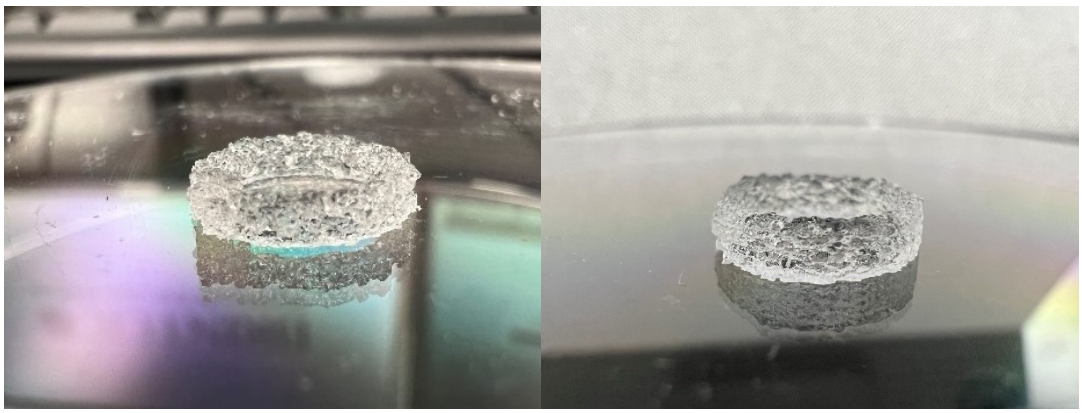
As seen in the table 3 above and table 4 below, Sigma-Aldrich's GelMA had slightly better average Pr-values when comparing it to our own, all be it with a minor difference. While Sigma-Aldrich's GelMA showed only minor improvements on the paper, we are able to see a difference when comparing the pictures above to the pictures of our own GelMA. Sigma Aldrich's GelMA would seem to suffer from the same problems as Turku GelMA, which is also seen clearly in the similarities in the pictures 8 and 9 above. However, the tests would indicate that Sigma-Aldrich's GelMA can be printed with better consistency.

Table 4 Sigma GelMA Pr-values

Cell	Circularity (C)	Pr-value
1	0,655	1,198
2	0,641	1,225
3	0,703	1,117
4	0,697	1,126
5	0,686	1,144
6	0,737	1,065
7	0,678	1,158
8	0,746	1,052
9	0,738	1,064
Standard deviation (all)		0,057
Average (all)		1,128

5.1 Comparison of printing quality through stackability

Height and outer diameter were measured from the cylinders to get a better view of how each filament layer would stack on top of each other. The parameters used to print the cylinders seen below can be found in Tables 5 and 6 below. The original 3D model that was used to print these objects had a height of 2 mm and a diameter of 10 mm.



Picture 10 Stackability with cylinder: Turku (left) and Sigma Aldrich's (right).

When looking these by eye and side by side, the Sigma Aldrich's GelMA seemed to have more even layers and better consistency overall, as seen in picture 10 above. That said the layers on

the Sigma Aldrich's GelMA also looked a bit rounded compared to our own, which could indicate that the layer height could be affected more than on our own GelMA.

Table 5 Turku GelMA cylinder measurements

Cylinder	Height (mm)	Outer diameter (mm)
1	2,11	10,93
2	2,21	10,32
3	1,91	11,05
Average	2,08	10,77
Standard deviation	0,12	0,32

Table 6 Sigma Aldrich GelMA cylinder measurements

Cylinder	Height (mm)	Outer diameter (mm)
1	2,03	10,71
2	2,19	11,19
3	1,99	11,05
Average	2,07	10,98
Standard deviation	0,09	0,20

According to the measured dimensions, an assumption can be made that Sigma Aldrich's GelMA has slightly better stackability but to the degree that can be put into a margin of error comfortably. Hence, we can say with the most certainty that the Turku and Sigma Aldrich's GelMA's can be considered identical in their printability and stackability, as seen in the tabels 5 and 6 above. The rounded layers on the Sigma Aldrichs GelMA didn't have such a negative effect on the dimensions as was predicted earlier according to the picture 10.

6 Discussion

The Turku GelMA we produced ourselves could be considered equal when comparing it to Sigma Aldrich's GelMA. The main problem with Turku GelMA was the high degree of methacrylation in addition to a too high concentration of GelMA in the ready to use bioink. Due to the uneven, crooked filament, it was extremely hard to print accurate objects because every turn of the robotic printing arm caused the filament to either break or bend gently. This made accurate 90 degree turns almost impossible. With the current way that the synthesis of GelMA is carried out in the laboratory, it would be feasible to carry out more synthesis with a more accurate way of pipetting the reagents to avoid unnesesary mistakes. Also the printnig parameters should be experimented more closely with Sigma Aldrich's GelMA. This experiment brings hope for the 3D printing of GelMA as the Brinter® platform clearly has the needed capability to process the GelMA.

As the original 3D model doesn't have a diameter for its 2D lines, the printed object can't perfectly resemble the original 3D model as the filament always has a certain width deviating from zero. As an example, most of the cylinders printed in the stackability tests, as seen in tables 5 and 6, had greater height and diameter when compared to the original 3D model due to the definite width of the filament.

A larger sample size would have been beneficial in the stackability tests, but as stated earlier, the tests had to be finished too early as we ran out of Sigma Aldrich's GelMA. As a part of a thesis, these tests had a fixed time window, and as the arrival of the new batch of Sigma Aldrich's GelMA would have pushed these tests forward for several months due to the COVID-19 pandemic-related material shortage, we had to manage with the sample size of three per GelMA. As these three were still printed right after each other, we can assume that the increase in the sample size would have had a minimum impact on the end result.
From testing, we can assume that the 10 % GelMA is too viscous and that 5 % GelMA could have been a better choice overall. This would have meant that a cooled printing bed would have been required (Ding, Illsley, & Chang, 2019.) Where 10 % GelMA was too thick, and 5 % would have probably needed the cooled print bed, a middle point at 7,5 % could have been the perfect compromise. This wasn't feasible to be tested in the restrained time period, but if the study was to be continued, this would be something to be kept in mind.

The synthesis itself would also need to be examined more closely, as we saw an extremely high amount of methacrylation. We would need a more precise way to control the synthesis and dialysis stages to combat this. This would also mean that we would need far better laboratory equipment. If the synthesized GelMA were made commercially available in the future, a quality-controlled production line would be mandatory.

There were way more variables involved in the bioink preparation phase than we had originally thought. Even the bioink preparation, which was considered to be almost bulletproof, ran into a foaming problem. This was due to a too small volume of PBS-buffer and a high amount of lyophilized GelMA in a decanter with constant stirring. To combat this, a minimum of 10 ml batch of printable GelMA was taken as a baseline. This didn't solve the whole problem, as some form of foaming was still present when large pieces of lyophilized GelMA were added to the PBS buffer. This was caused by the small openings of the lyophilization bottles, which forced us to press the pices of GelMA into smaller and thighter pieces, which caused them not to dissolve in the PBS buffer. For future experiments, a piece of advice would be to make a batch as large as possible that would still be used in a two week time period also to combat unnecessary preparation of GelMA as it could be considered a rather costly material.

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Appendix

GelMA Application note

BRINTER®
PRINTING LIFE ONE LAYER AT A TIME™

Application note

UV curable Gelatin Methacrylate (10 % GelMA, 0,05 % LAP)

Gelatin Methacrylate (component B)

Curing agent (LAP) (component A)

Suggested concentration 1: 10 % Gelatine Methacrylate + 0,05 % LAP curing agent in PBS.

Storage temperature:	+ 4 °C
Intended use:	Research use only, suitable for the printing of bone, cartilage, epidermal tissue, heart, drug delivery devices
Preparation guidelines:	First, weigh the LAP initiator in a glass decanter (0,05 % weight per volume from the amount of wanted bioink). Add the LAP to the PBS buffer and stir it for 15 minutes at 50 °C temperature. Weigh lyophilized GelMA according to the wanted percentage in the final bioink and add it to the PBS buffer containing the LAP initiator. Gently stir the solution at

50 °C temperature for 60 minutes. Pour the bioink into a UV protected printing tube and let it cool in a fridge overnight.

Suggested printing parameters with reference printer Brinter® One (www.Brinter.com):

Print head:	Pneuma Tool Cooled
Nozzle:	200 µm (UV shielded, black conical nozzle, Nordson EFD)
Syringe:	UV shielded Amber syringe barrel (Nordson EFD)
Printing pressure:	Approx. 1200 – 1400 mbar
Printing temperature:	12 °C – RT
Print bed temperature:	RT
First layer height:	0.2 mm (bottom)
Layer height:	0.2 mm (same as ID of the nozzle)
Preflow time delay:	150 msec
UV curing:	At 405 nm, layer-by-layer curing with 100% intensity and 6 second curing time/layer

Notes: Lower temperatures may require higher pressure. Needles with higher ID require lower pressure.

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