國立臺灣大學電機資訊學院生醫電子與資訊學研究所

碩士論文

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以 3D 列印方式利用投影機製造胎盤蛻膜間質幹細胞之細胞片

Projector-based 3D printing for pcMSC cell sheets

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中文摘要

此篇論文描述以 3D 列印原理，使用投影機完成幹細胞之細胞片，這個方式包含了 3D 列印機、印列墨水的製造，以及膠上幹細胞的培養。3D 列印機選擇用市售的投影機，經過改裝，利用其投影系統投射，使印列墨水曝光而凝固成膠，並且可依投影圖形，製造出不同的形狀膠體。印列墨水主要成分為：甲基丙烯酸酐化明膠(GelMa)和苯基(2,4,6-三甲基苯甲酰基)磷酸鋰鹽。細胞培養選擇用胎盤蛻膜間質幹細胞(pcMSCs)，其可以附著並培養於膠上。在往後的研究，因為 3D 列印的特性，可以完成孔洞狀結構，讓培養液可以更快流通於膠內。而以此方式所完成之胎盤蛻膜間質幹細胞之細胞片，可用於臨床研究，治療皮膚嚴重燒燙傷之傷口復原。

關鍵字：3D 列印、胎盤蛻膜間質幹細胞、甲基丙烯酸酐化明膠、苯基(2,4,6-三甲基苯甲酰基)磷酸鋰鹽、細胞片

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Abstract

In this article described a method which basing on 3D printing to producing stem cell sheets by using projector. This method included the 3D printing machine, the manufacture for printing gel and the stem cell culture. The printing gel is exposed and solidified by light projector system. The light projector system was remodeled from the commercial projector. Moreover, the outlook of the gel can be designed by the exposed pattern from projector. The printing gel is comprised by Gelatin Methacryloyl(GelMa), Lithium Phenyl(2,4,6-trimethylbenzoyl)phosphinate (LAP). In the cell culture, I choose the human placenta chorionic decidual-derived MSCs(pcMSCs). It can adhere and culture on the gel. In future work, because of the features of 3D printing, the gel can be designed with porous structure. This structure can allow the medium flowing faster in the gel. The final product, pcMSC cell sheets, completed by this method, can be used in clinical research and severe burn wound treatment.

Keywords: 3D printing, human placenta choriodecidual membrane-derived mesenchymal stem cells (pcMSCs), Gelatin Methacryloyl(GelMa), Lithium Phenyl(2,4,6-trimethylbenzoyl)phosphinate (LAP), pcMSC cell sheets

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Chapter 1 Introduction

Diabetic patients generally have an increased risk of wound infection and delayed healing. When these patients get severe burn, it will take more time to stay in hospital and suffer several operations. They require more surgical interventions than the cases of general people without diabetes. For burn patients with diabetes, the period of staying hospital is twice times than normal patients.[1] Therefore, some researchers are trying to solve this problem by using the stem cells to heal these wound faster.

In this article, it combined the 3D printing technology and the idea of cell sheets to produce the pcMSC cell sheets. There were three elements in this process: 3D printer and printing software, gelatin methacryloyl (GelMa) and human placenta choriodecidual membrane-derived mesenchymal stem cells (pcMSC).

3D printer was composed by a z-axis moving platform and a projector. The printing software controlled the projected pattern and projected time to complete the process of 3D printing.

GelMa was chose as the ECM for pcMSCs adhesion. In other words, GelMa was a cultured scaffold and pcMSCs can proliferate along the surface. After GelMa mixed with photoinitiators, it can gel by light exposure. Moreover, GelMa had high biocompatibility and can be degraded.[2]

The lab of Professor Thai-Yen Ling (Department of Pharmacology, National Taiwan University) provided the pcMSCs for this research. The pcMSCs were isolated from the choriodecidual membrane of human placentas donated by women, which were during cesarean sections.[3]

These three elements have high-quality technological process and amazing research result. So, I combined these elements to complete the pcMSC cell sheets. Moreover, I expected the pcMSC cell sheets will have a big effect on recovery and research of skin

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burns.

In the future work, the next step will be animal experiments and improve entire 3D printing process. It will be important to reducing the time and make all the process more fluent.

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Chapter 2 Literature review

Cell sheets were promising technique and an important idea in the field of tissue engineering (TE). Tissue engineering, namely regenerative medicine, officially appeared at National Science Foundation workshop in 1988[4]. Tissue engineering proposed to recover the damaged tissue by combining cells with scaffold biomaterials. For example, cardiomyocytes cell sheets can be one of the therapies for heart diseases.[5] Cell sheets were a complex material composed by cells and the scaffold biomaterials, which were as extracellular matrix (ECM). This scaffold biomaterials were templates to guide the growth of new tissue[4]. Moreover, as a role of ECM, these biomaterials provided enough stiffness for cell adherence, and had the stability of cell sheets during the surgical implantation.

At first, Gelatin was chosen as the biomaterial in this case. Gelatin was one kind of hydrogels which was designed to resemble the properties of the ECM. It was recognized a safe material by U.S. Food and Drug Administration (FDA). Gelatin denatured from collagen under high temperature.[6] It had plenty of arginine-glycine-aspartic acid sequences (RGD amino sequence), it could be adhered by cells.[7] Furthermore, Gelatin was suitable for cell remodeling and degradation because RGD sequences were also the target of matric metalloproteinase (MMP).[8]

At the end, however, gelatin methacryloyl (GelMa) was chosen and used in this research. Because the natural gelling temperature of gelatin was 35℃, it was not easy to keep solid and cultured in incubator or used in human body. On the other hand, when adding photoinitiators and exposed to light, GelMa could chemically gel no matter the degree of temperture. This kind of hydrogel was easy to tune mechanical properties and stay gelled in cultural temperature.[9] In addition, this kind of material was more suitable

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for 3D printing. It could produce cell sheets by exposed light in layer-by-layer method. In the end, it could build up 3D structure. GelMa also named as gelatin methacrylate[10], methacrylated gelatin[11], methacrylamide modified gelatin[12], or gelatin methacrylamide[7] in different research articles.

The raw material of GelMa was gelatin. The different between them was that GelMa had many methacrylamide and methacrylate groups in the side chain. Comparing with gelatin, the chemical groups modification of GelMa normally no more than 5% of the amino acid sequence in molar ratio.[12] This meant that GelMa had a good biocompatibility as gelatin. Additionally, the RGD sequences and the MA groups did not have any reaction with each other. GelMa still had well cell adhesive property.[13]

Methacrylamide and methacrylate groups were important in the gelling process. The manufacture of the GelMa, adding these chemical groups to side chain of gelatin, had already matured.[14] Thus in the recent year, researchers wanted to improve the quality of GelMa. The quality was determined by the GelMa substitution. The substitution meant the ratio of methacrylamide groups (MA group) to the NH_2 group in GelMa. The NH_2 in amino groups of gelatins need to be changed by the methacrylamide groups. The higher degree of substitution (DS) meant the higher quality of GelMa. In the gelling process, the MA groups connected to other MA groups in the different chain of GelMa molecule. Thus, the more amount of MA group (high DS) in GelMa molecule, it was easier and more stable for building connection between different GelMa molecules.[13] Moreover, the amount of this connection in GelMa molecules influenced the stiffness of the GelMa gel. According to the data, stiffness of GelMa gel can range from 3.3 to 30kPa.[15] It was suitable for many kinds of organism tissue.

The other factor influences the gelling was photoinitiators. The photoinitiators assisted MA groups connecting with others. The photoinitiators reacted with light, and

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different kinds of photoinitiators were suitable for different wavelength of light.[16] After absorbing light, the photoinitiators changed the structure and generated free radicals. These free radicals attacked the double bond of the carbon in the MA group and changed it into single bond between other MA groups. This process was called photopolymerization, and GelMa solution changed into GelMa gel.[17]

In the past, IC2959 was used as the photoinitiators in the research of the GelMa. However, the water solubility and the ability of the crosslinking were not good.[18, 19]

In the recent, Lithium Phenyl(2,4,6-trimethylbenzoyl)phosphinate (LAP) was also used in the research. It had high water solubility, not inhibited by oxygen and could be activated by visible light.[20] Furthermore, LAP could support higher cell viability.[21] It was more suitable for using in the biological research.

The biomaterial and the cell were both important for cell sheets. One kind of mesenchymal stem cells was used in this research.

Mesenchymal stem cells (MSCs) became popular in the recent year. More than two thousand patients used mesenchymal stem cell therapy to heal different kinds of diseases.[22] In the most of clinical trials, the therapy had quite efficient.[23] MSCs were isolated from donors and it would not hurt the donors during this process. So, it had less ethical issues than other kinds of stem cells, like totipotent. MSCs were multipotent stem cells which meant they had the capacity of differentiating into mesodermal lineage cells. The MSCs had the special surface markers ((CD)73, D90, CD105), and researchers also used these markers to distinguish MSCs from normal cells. In the recent findings, MSCs not only had the differentiation ability, but also had the immunogenicity, homing ability and banking. These features made it important in cell therapy, regenerative medicine and tissue reparation.[24]

After reviewing the research of the biomaterial and the cell, this article focused on

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how to produce the cell sheets. In the past, researchers developed several methods to produce cell sheets.

In normal, the method for producing cell sheets need to culture cells on dish. After cells expanded all the area, researchers used the thermo-responsive method to harvest cell sheets.[25] In this kind of method, the culture surface on dish was important. It would determine the cell sheets was easy or not to harvest. Thus, researchers put enormous attention on developing the material coating on the surface.[26] However, this article had a totally different way to produce the cell sheets. It would apply the 3D printing to produce cell sheets.

The principle of the three-dimensional (3D) printing was that rapidly accumulating the ink through layer-by-layer to build up the complex 3D structure.[27] This technique was highly precision that mean it perfectly controlled the shape, the internal structure of the product.[28] 3D printing was suitable for using in the tissue engendering area. In the recent year, there were numerous cases using 3D printing in research.[29, 30]

Focusing on 3D bioprinting (3D printing in biological area), there were 3 kinds of bioprinting techniques. They were droplet-based, extrusion-based and stereolithography-based.[31]

Droplet-based bioprinter aligned the micro-droplet to build products. The degree of precision was decided by the scale of the droplet. Extrusion-based bioprinter was based on fused deposition modeling (FDM). The ink was continuous filaments and printed out by a needle-syringe. Stereolithography-based bioprinter used photopolymerization method. Combined with laser or image projector, this kind of bioprinters could transform ink from solution to solid gel.[31]

In this research, it used the photopolymerization which meant using dynamic optical projection stereolithography (DOPsL) and exposing patterns on ink. Then ink

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solidifies and the shape depended on the light pattern. DOPsL used the digital micromirror device (DMD) to form the light pattern; this was the same principle of the projector system. The pattern need to be design in accordance with the 3D structure. The patterns were stacked layer by layer and finally build up the 3D structure.

This type of the 3D printing was different from the laser-based system. The laser-based system deposited light energy in gel[32]. But this system took too much time because of gelling dot by dot. The projector-based system printed gel layer by layer. Using this way of 3D printing, cell sheets produced faster and could be easy to shape into different patterns. The different shapes of cell sheets were suitable for different applications of regenerating new tissues. Summing up all the features, the projector-based 3D printing for cell sheets could really apply on regenerative medicine.

The research was valuable when it could solve the real problems or match the unmet need. Fortunately, pcMSC cell sheet had a real goal to achieve. It was that healing a chromic would on the patients with diabetes.

In 2017, researchers estimated 30.3 million patients with diabetes in the whole world.[33] In the same year, they estimated the total medical cost of diabetes was 327 million USD.[34] In Taiwan, it was estimated around 1.958 million patients and the prevalence in adults was 10.9% in 2017.[35] For these patients, the wound on the skin became a chromic non-healing wound.[36] The factors resulted in the healing deficiencies in these patients were more than 100. For example, the factors were abnormal production of growth factor.[37], angiogenic response, collagen accumulation, epidermal barrier function, keratinocyte and fibroblast migration and proliferation[38], number of epidermal nerves.[39], bone healing, and balance between the accumulation of ECM components and their remodeling by MMPs.[40]

84% of all diabetic lower extremity amputations came after chronic diabetic

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wounds.[41] After amputation occurring, the 5-yeat mortality of patients was 50%.[36] Thus, the better treatment of skin wounds was very important for the patient with diabetes. The pcMSC cell sheets prepared to solve this problem.

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Chapter 3 Method

3-1 The manufacture of GelMa

1. Type A gelatin (Sigma-Aldrich Corporation) was dissolved at 10% w/v at 60°C in 0.1 M CB buffer (Buph^{TM} Carbonate-bicarbonate in 1L distilled water, Thermo Scientific). The dissolving process takes about 1~2 hours at 60°C and 600 rpm stirring.
2. GelMa was prepared by reaction of free amino groups of lysine/hydoxylysine amino acids in the gelatin with methacrylic anhydride (MAA, Sigma-Aldrich Corporation) at 0.1 mL per gram of gelatin at 50 °C.

   In this case, 10g gelatin dissolved in 100ml CB buffer needed 1ml MAA. One-sixth of 1 mL MAA (167μL) was added every 30 minutes in a drop-wise format to the gelatin solutions with pH adjustment at 600 rpm stirring speed. So, MMA wasadded 6 times to the gelatin solutions and this tatoally took 3 hours. The GelMa solutions was adjusted to a pH of 9.0 by NAOH before MAA was added.

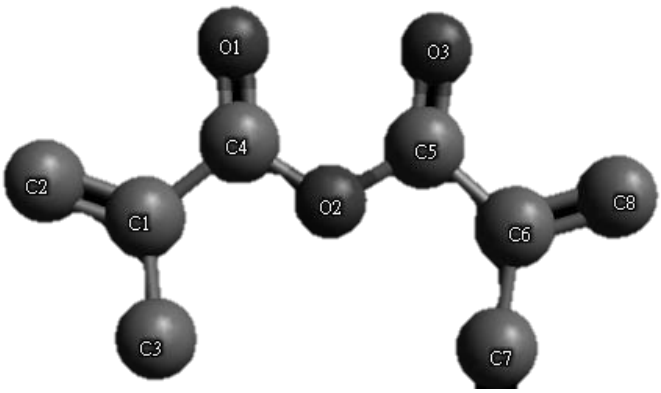

Figure 3-1: methacrylic anhydride

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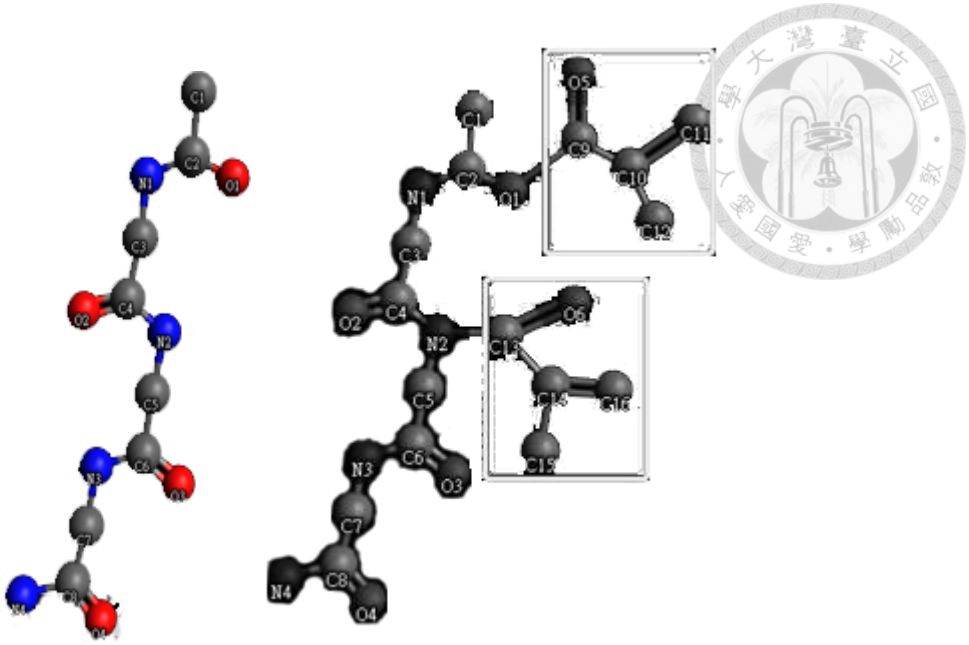

Figure: 3-2 simple pattern of gelatin(left) and GelMa(right; MA group for square-label)

3. After all the MMA adding, the GelMa solution waited 30 minutes then the solution was readjusted to a pH of 7.4 and stirring overnight at 40°C.
4. The GelMa solution was readjusted again to a pH of 7.4, then dialyzed using a dialysis membrane (12~14KDa, Spectra/Pro Dialysis Membrane Standard RC Tubing, Spectrumlabs) in ddH_2O at 40 °C for 2-3 days. We changed the ddH_2O every day.
5. After dialysis, the GelMa solution was readjusted again to a pH of 7.4, then was separated into aluminum foil dish. The upper diameter of aluminum foil dish was 30mm, the downer diameter was 22mm, and the height was 15mm. Every box was filled 3ml GelMa solutions, then stored in -20°C overnight.
6. GelMa with aluminum foil dish was lyophilized for 1 day.
7. After lyophilized, GelMa changed into white solid. Then, it was putted into plastic box with drier and stored at -20°C until further use. GelMa should stay -10°C freezer during the package process[7].

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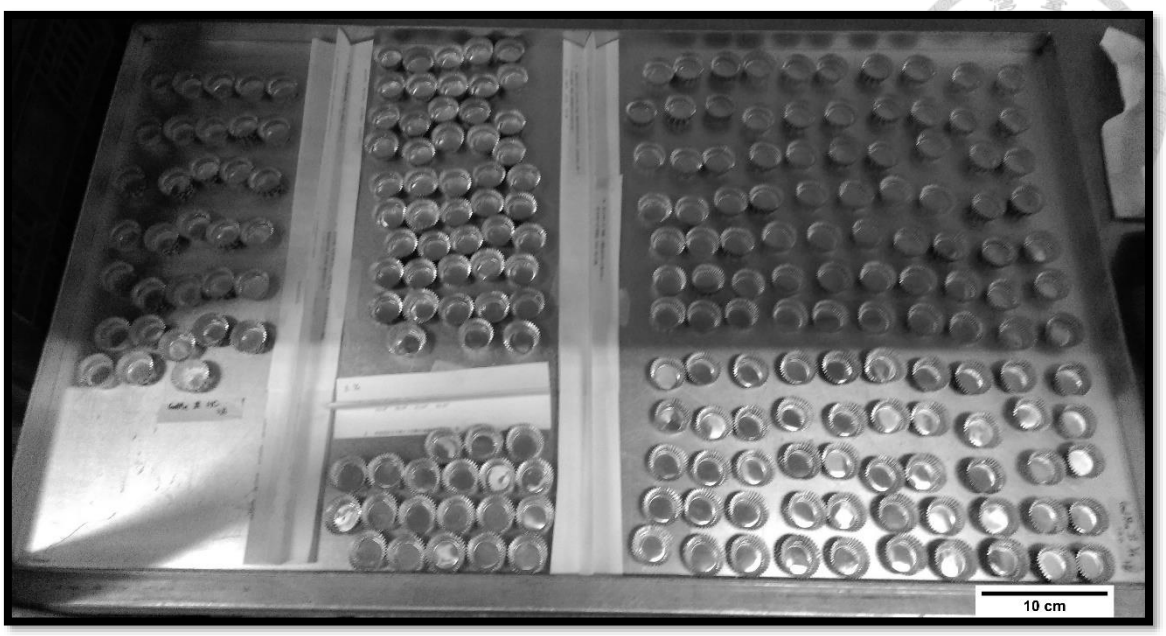

Figure 3-3: GelMa solution in aluminum foil dish before stored in -20°C

Figure 3-4: one GelMa slice after lyophilized

3-2 The manufacture of GelMa printing ink

The GelMa printing ink:

1. GelMa was dissolved at 10% w/v in PBS (Dulbecco's PBS, Simply).
2. The solution was putted into oven at 65°C for 3 minutes, then putted in room temperature for 5 minutes. This process was repeated until GelMa entirely dissolved.

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3. The lithium phenyl(2,4,6-trimethylbenzoyl) phosphinate(LAP, Tokyo Chemical Industry Co. ltd) was added at 0 .25% w/v in the solution, and it took 5 minutes until the LAP completely dissolved.
4. The solution was sterilized by using the sterile syringe filter (0.22um, PES membrane).
5. The solution was filled in the printing pool waiting for light exposure from 3D printer.

3-3 Remodeling the 3D printer

The 3D printer, Phrozen One (Phrozen, Taiwan), was remodeled to fill the need of cell culture and GelMa sterile printing. We had remodeled 3 parts of the machine, light source, printing pool and the frame of printer.

At the end, we repackaged the machine in the biological safety cabinet (Bioman scientific, Taiwan).

3-3.1 Light source

The original light source of the 3D printer was high-pressure discharge arc lamps (Osram P-VIP 210W) in projector (H6510BD, Acer, Taiwan). The photoinitiator, LAP, was reacted by the light wavelength until 400nm, and the absorbed peak was at 365nm.[42] The original light source was visible light. The light need to be changed wavelength to react LAP.

We had tried two different light source system, UV led system (405nm, 3W, EHE) and original arc lamp adding quartz filters. In the end, the original arc lamp adding quartz filter was the better choice. This filter-added light source outputted enough light power and suitable wavelength.

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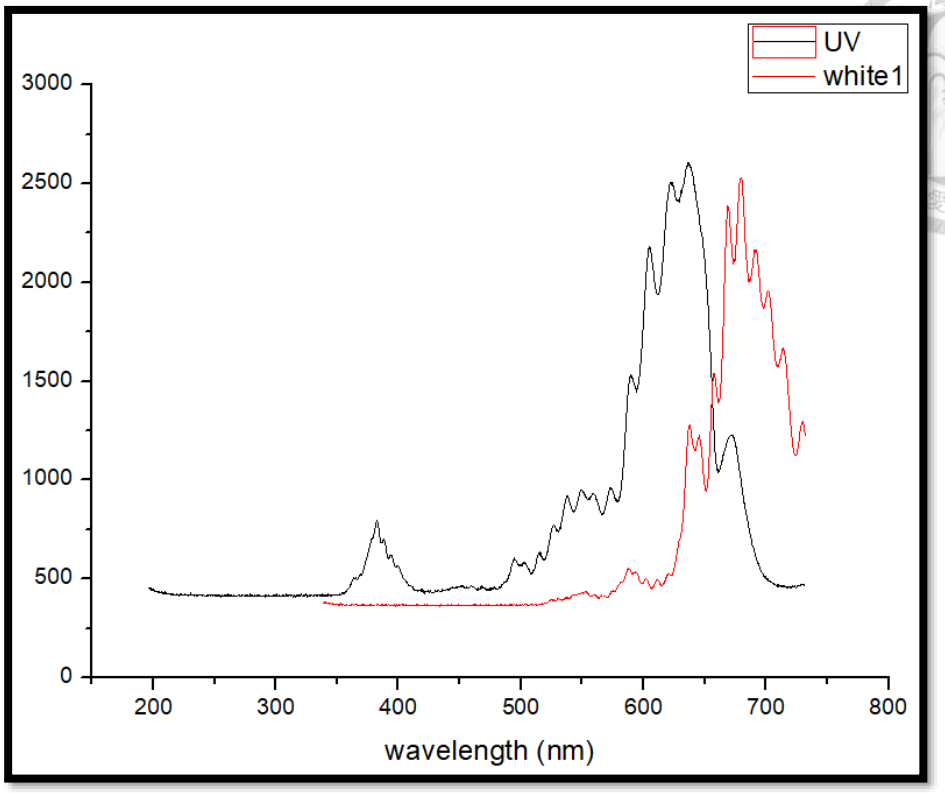

Figure 3-5: the spectrum of the original light (red) and filter-added light (black)

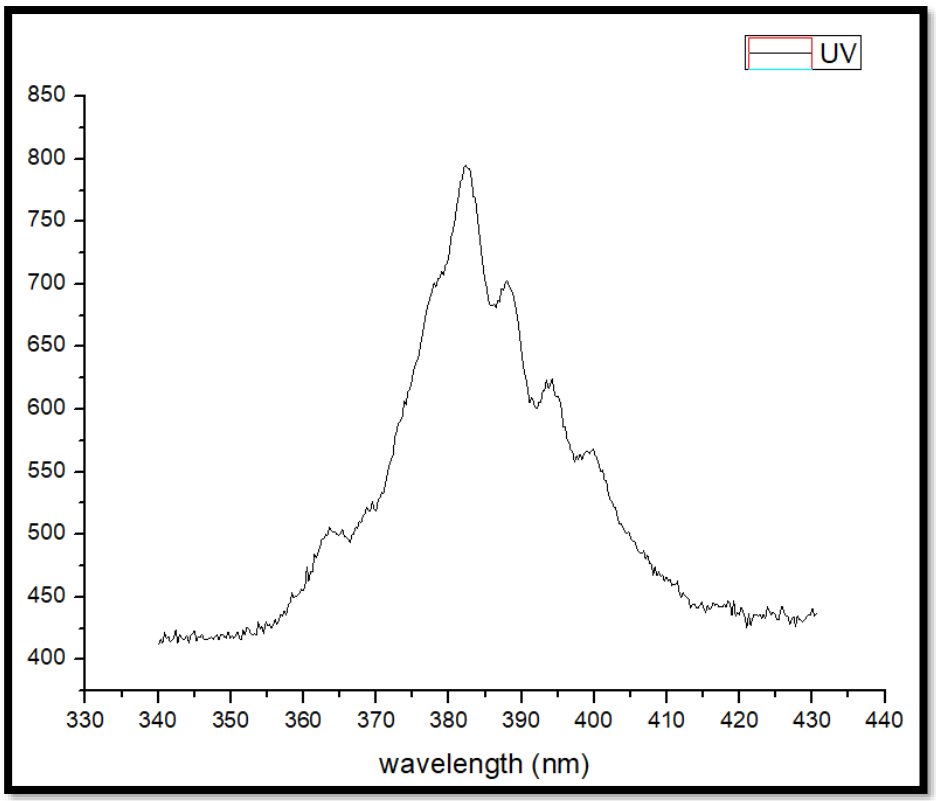

Figure 3-6: the spectrum of filter-added light source(black) in 340nm to 430nm

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In that time, it was not easy to detect the light energy of the single specific wavelength in this kind of whole spectrum. Thus, the energy value from 360nm to 420 nm was unknown.

3-3.2 Printing pool

The printing pool filled with printing ink, and the light pattern projected on the pool. The GelMa solution became GelMa gel in the pool.

The original printing pool was too big and wasted too much volume of printing ink. The original printing pool was 10cm (width) and 15.5cm (length). At the end, the scale of the 3D printing product may only in centimeter scale. Therefore, the printing pool need to be changed.

Figure 3-7: the original printing pool(10cm(width),15.5cm(length))

The new printing pool had three parts, the upper layer, Teflon film (Muybien, Taiwan,) and the lower layer. The upper and the lower layers were acrylic board.

1. The upper layer was acrylic board (thickness: 3mm) and was sculpted by CNC sculpture machine.

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2. The lower layer was acrylic board (thickness: 2mm) and was cutting by laser engraving machine.

3. The upper layer and the lower layer stacked and cut together by laser engraving machine. In this step, the pores made together for the screws on the upper and the lower layer.

4. The Teflon film (thickness: 0.05mm) was cut by the cutting machine(i-Craft™2.0, GCC company)

5. Three parts of the new printing pool, the upper layer, Teflon film and the lower layer, stacked together by the screws.

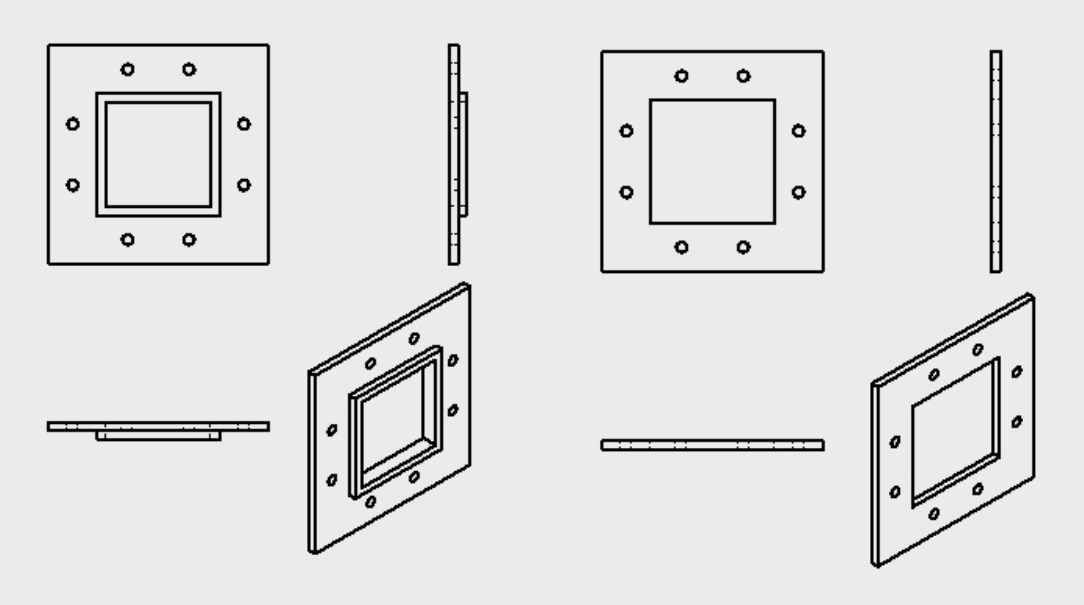

Figure 3-8: the upper layer (left) and the lower layer (right) of printing pool
The square is the place putting GelMa solution and the circles are the holes pores for the screws

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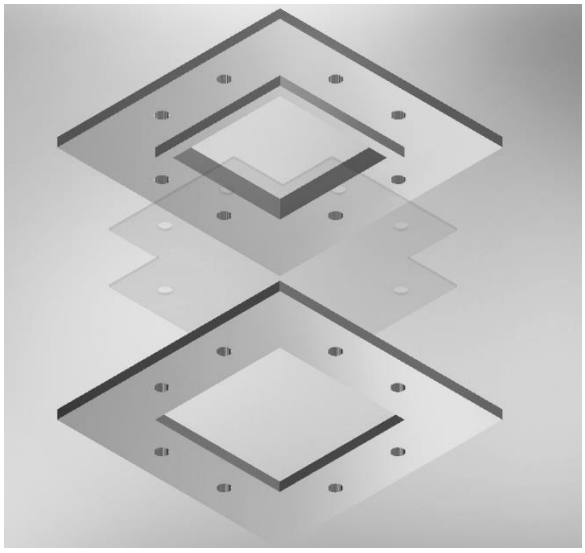

Figure 3-9: The upper layer, Teflon film and the lower layer of printing pool

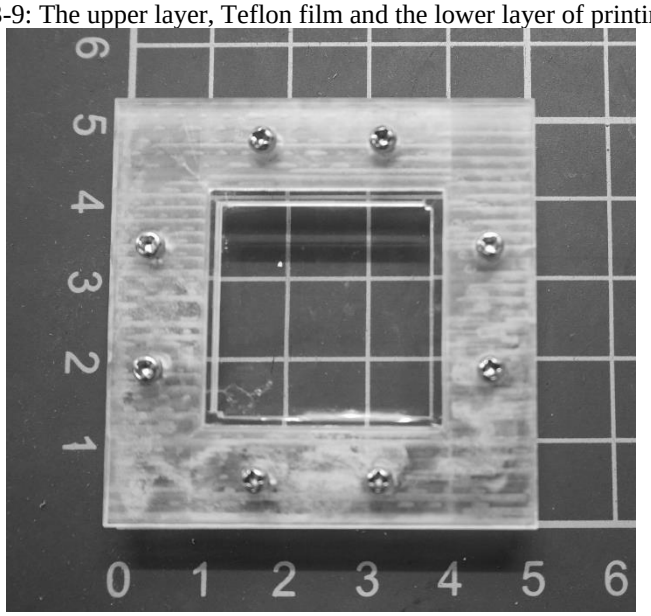

Figure 3-10: Printing pool (5cm*5cm; sink:2.4cm*2.4cm)

3-3.3 The frame of the 3D printing machine

The original scale of the 3D printing machine was 22.5cm (width), 40cm (length) and 80cm (height). It was too high to fit in the biological safety cabinet.

The new frame constructed by angle steel, and the scale was 17.5cm (width), 41cm (length) and 60cm (height). The components of 3D printing machine dismantled and conducted in the biological safety cabinet (BSC).

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Figure 3-11: 3D printer before reconstruction (left) and after reconstruction in BSC(right)

3-4 Human placenta chorionic decidual-derived mesenchymal stem cells cultures

To prepare the collagen-coated dish:

1. Human collagen typeIV(SIGMA C5533) was dissolved in 0.01N acetic acid to the concentration of 1mg/1ml.
2. The 75ul of diluted-collagen solution added on the 10cm dish (75cm^2) and then sterilized water added to cover all the surface of dish.
3. The dish took 6~8 hours to naturally dry in biological safety cabinet.

Human placenta chorionic decidual-derived mesenchymal stem cells, pcmsc, were plated onto the collagen-coated dish in MCDB201 medium (SIGMA M6770) with 1% ITS (Gibco 41400-045), EGF (PeproTech 100-15) 10ng/ml and 1% P/S (ABL02-100ml, Caisson).

The medium changed every 3-4 days. After 6~8 days, cells were washed with PBS, and detached with 0.05% of trypsin. Then cell were passaged to the new collagen-coated dish.

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3-5 3D model software and the printing control software

At first, the light pattern for projector was designed with PowerPoints (Microsoft Window). I control the exposure time through turn off the slides.

For 3D structure, it was designed with Inventor of Autodesk and MeshMixer. Then 3D model was loaded in Phrozen DLP system software to start 3D printing.

3-6 The manufacture of GelMa printing gel

After the printing ink filling into the pool, the projector was turned on. It take 90 seconds to expose one layer of GelMa gel (thickness: 0.6mm).

3-7 Quantification of GelMa substitution

To define the quality of GelMa made by ourselves, ^{1}H-NMR and TNBSA were used to determine the GelMa substitution.

3-7.1 ^{1}H-NMR(400 MHz Varian)

^{1}H-NMR (400 MHz Varian) experiments were conducted in order to verify the DS of GelMa. Around 50 mg of each lyophilized GelMa sample dissolved in 1 ml of deuterium oxide (D_2O) at 40 °C. The peak area of aromatic acids in the GelMa samples was employed as a reference in each spectrum.[43]

3-7.2 TNBSA

TNBSA solution reacts with primary amino groups, which should be replaced by methacrylic anhydride in GelMa. TNBSA solution was mixed with primary amino groups at pH 8 to form yellow adducts. Then the solution is detected the absorption at 335nm.

1. The 0.1M sodium bicarbonate buffer was prepared and the pH value was 8.5.
2. TNBSA solution (Picrylsulfonic acid solution, Sigma-Aldrich Corporation) diluted in 0.1M sodium bicarbonate buffer to 0.01% (w/v) of TNBSA solution.

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3. The sample dissolved in 0.1M sodium bicarbonate buffer at the concentration of 200ug/ml. Three different kinds of sample were type A gelatin (Sigma-Aldrich Corporation), GelMa, GelMa(Sigma-Aldrich Corporation).
4. 0.25ml of the 0.01 % (w/v) of TNBSA solution added to 0.5ml of each sample solution.
5. Then the mixed-solution incubated at 37℃ for two hours.
6. 0.25ml of 10% SDS solution and 0.125ml of 0.1N HCL added to each sample solution in order to stop the reaction of the TNBSA.

3-8 The stiffness value of GelMa gel

The Shear Wave Elasticity Imaging (SWEI) (Prospect, S-sharp, Taiwan) can measure the stiffness of GelMa printing gel. It measured the shear wave velocity of GelMa gel and then calculated the stiffness value (shear modulus G and Yong's modulus) by using Matlab.

The relation between the shear wave velocity and shear modulus G was that

$$\text{shear modulus } \mathbf{G} = \boldsymbol{\rho v^2}$$

The relation between the shear wave velocity and Yong's modulus was that

$$\text{Young's modulus } \mathbf{E} = \frac{F/A}{\Delta L/L} = \mathbf{3\rho v^2}$$

F/A: the pressure exerted on an object

ΔL/L: the change of the length/ the original length

ρ: tissue density, in this case ρ was 1000 kg/m^3

$\boldsymbol{v}$: the shear wave velocity

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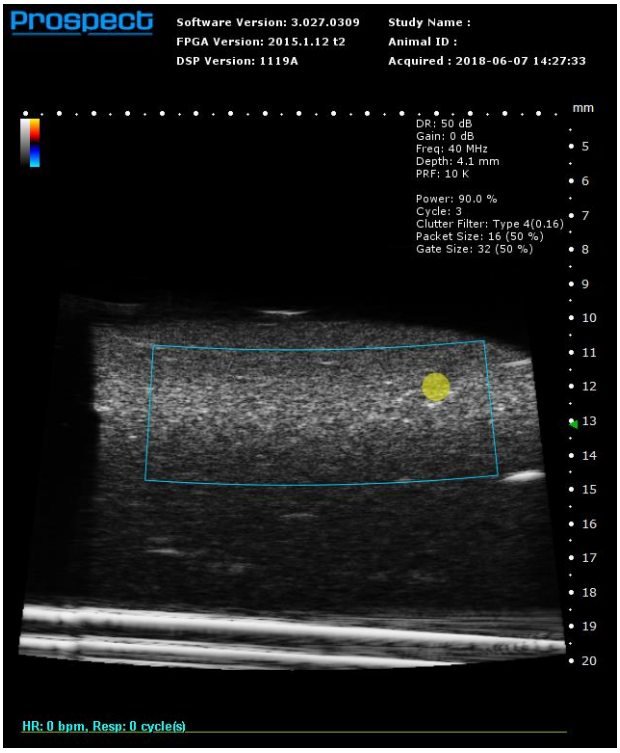

Figure 3-12: Ultrasonic B mode of GelMa gel (thickness:0.8cm)

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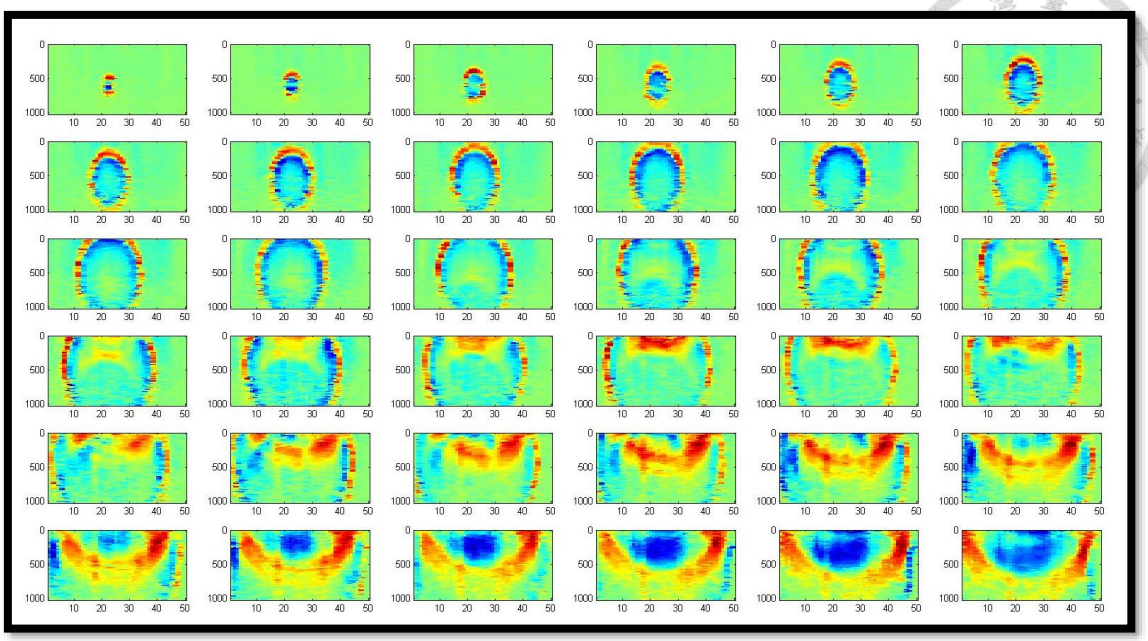

Figure 3-13: The shear wave transmited in the 5% (w/v) of GelMa gel

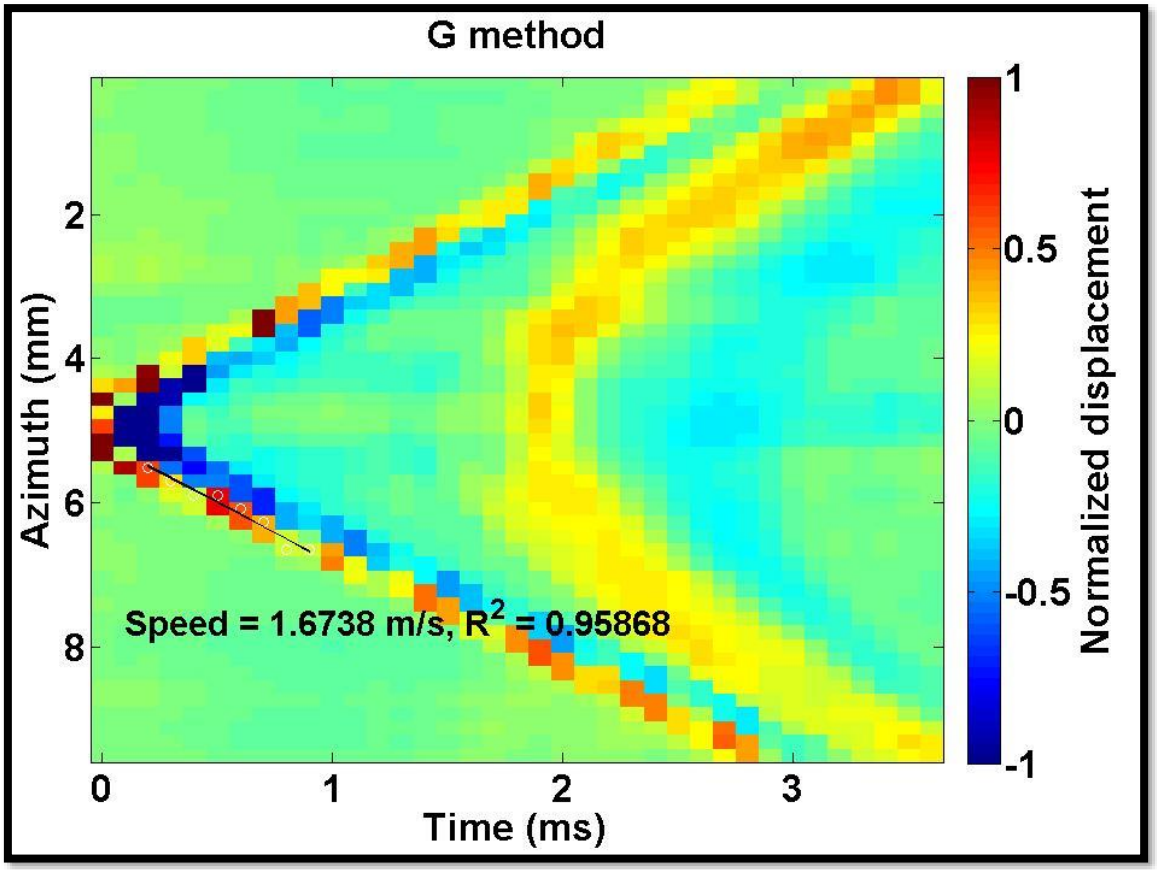

Figure 3-14: The velocity of shear wave in the 5% (w/v) of GelMa gel

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3-9 Calcein-AM and PI of Cell staining

1. 1ul of Calcein-AM and 2 ul of PI diluted in 1ml PBS.
2. The GelMa gel was washed by PBS and soaked in Calcein-AM and PI solution.
3. Then the gel cultured in incubator for 30 minutes.
4. The gel was observed by the microscope. Observing under 490nm light, the live and dead cells can be shown. Observing under 545nm light, only the dead cells can be shown.

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Chapter 4 Results

4-1 Quantification of GelMa substitution:

4-1.1 ^{1}H-NMR(400 MHz)

There were 4 samples detected by ^{1}H-NMR(400 MHz Varian).

A. Type A gelatin (Sigma-Aldrich Corporation) was the raw material of GelMa.

B. GelMa (Biobot) was claimed the degree of substitution(DS) is 50%.

C. GelMa (Sigma-Aldrich Corporation) was claimed the DS is 80%.

D. GelMa made by ourselves.

The peak of 3.1ppm responds the NH_2 groups in the gelatin. In the process of producing GelMa, the NH_2 groups were replaced by the MA groups, which responded at the peaks of 5.4 and 5.7ppm. Therefore, the higher DS of GelMa means the higher peaks of 5.4 and 5.7ppm.

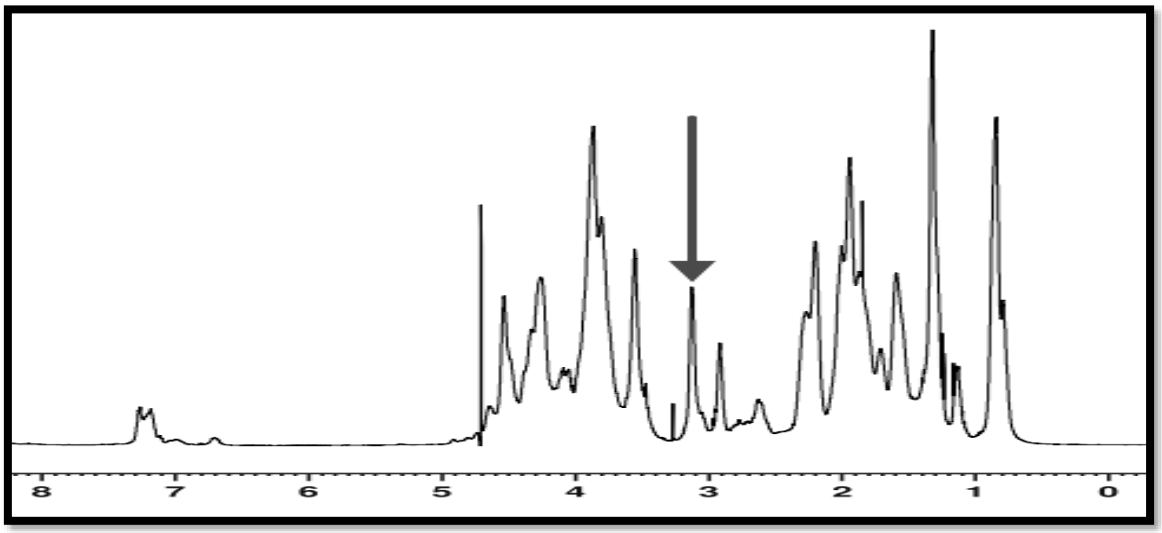

Figure 4-1: ^{1}H-NMR spectrum of Gelatin

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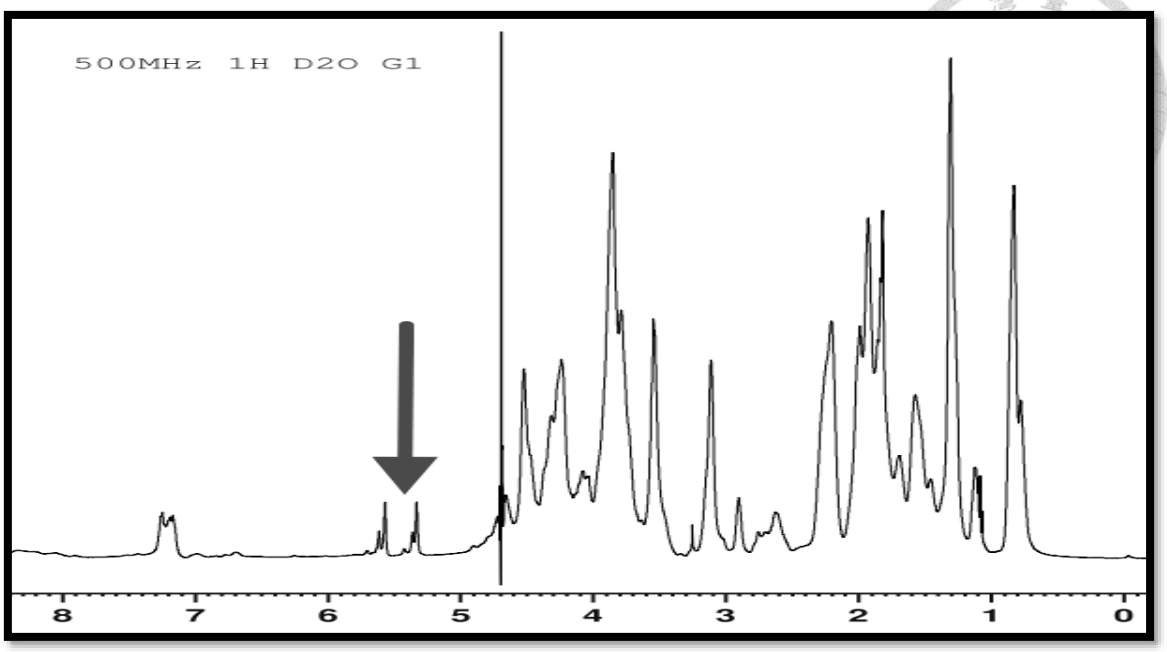

Figure 4-2: ^{1}H-NMR spectrum of GelMa (DS:50%, Biobot)

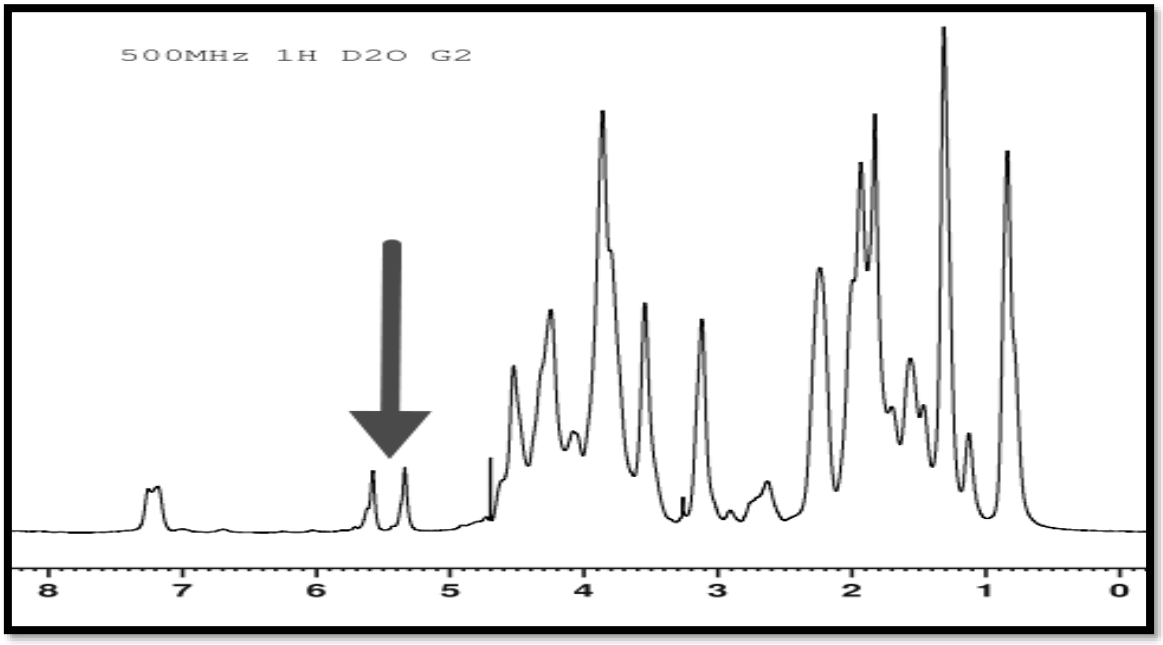

Figure 4-3: ^{1}H-NMR spectrum of GelMa (DS:80%, Sigma-Aldrich Corporation)

doi:10.6342/NTU201902957

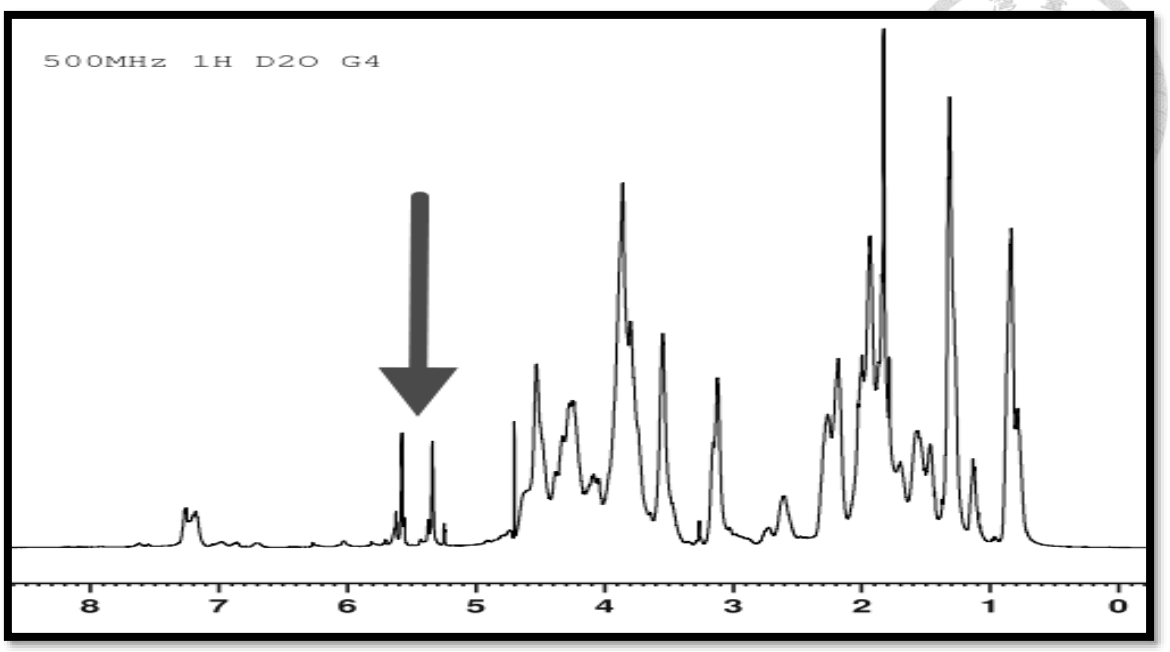

Figure 4-4: ^{1}H-NMR spectrum of GelMa made by ourselves

According the figures, the GelMa all had the peaks of 5.4 and 5.7ppm, but these data still could not quantification of DS. These figures proved that GelMa molecules were successfully produced in our lab. Then the TNBSA was used to quantify DS.

4-1.2 TNBSA

TNBSA can determine the amount of NH_2 group in the molecules. The more amount of NH_2, the absorption value at 335nm will higher. The absorption value of gelatin (type A, Sigma) was 56.65. All of NH_2 group in gelatin was not replaced by any function group. The NH_2 group in GelMa molecules had been replaced by MA groups. Therefore, the absorption value of GelMa was lower than 56.65. According to the table below, the value of GelMa (Sigma ; DS:80%) was 52.68 and the value of GelMa made by us was 51.45. However, I did not examine the background value. In other words, we did not have the value, which represented the 0% of the NH_2 groups. This experiment need to do again in the future.

Nevertheless, the conclusion was that GelMa was successfully produced and the quality was not lower than GelMa from Sigma.

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	Gelatin (Sigma)	GelMa (Sigma; DS:80%)	GelMa (made by ourselves)
Absorption value at 335nm (%)	56.65	52.68	51.45

Table 4-1: the absorption value of different GelMa

4-2 The stiffness value of GelMa gel

The velocities of 3%(w/v), 5% (w/v) and 10% (w/v) GelMa were 0.563m/s, 1.61m/s and 4.5m/s. The shear modulus of 3%(w/v), 5% (w/v) and 10% (w/v) GelMa were 0.32kPa, 2.63kPa and 20.3kPa. The Young's modulus of 3%(w/v), 5% (w/v) and 10% (w/v) GelMa were 0.96kPa, 7.89kPa and 60.9kPa. This data matched the record in the forward research.[15]

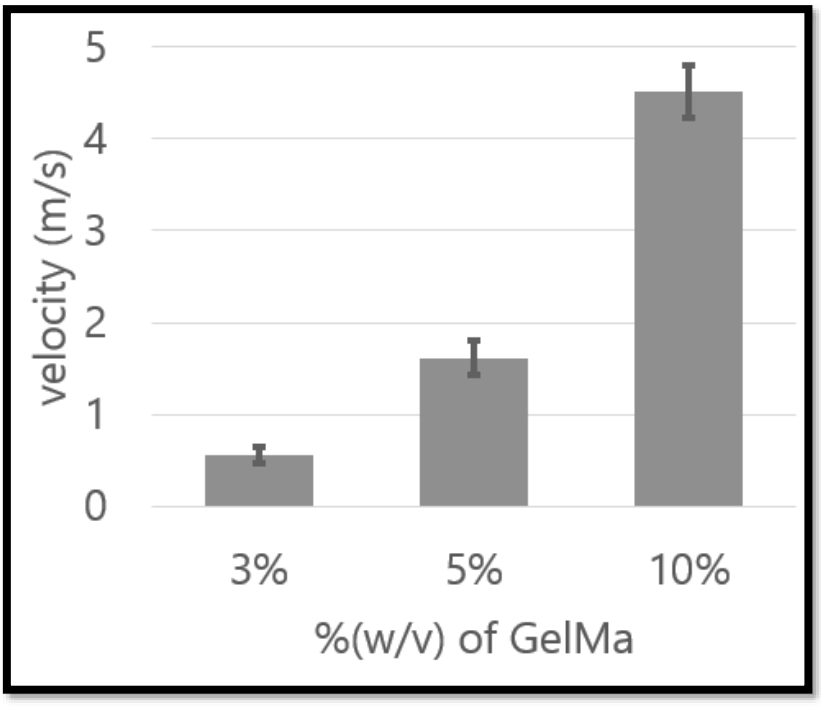

Figure 4-5: the velocities of the different concentration GelMa (n=3)

4-3 The stiffness value changing with time

The GelMa gel naturally degraded by time. The GelMa gel was cultured in incubator with PBS but without cells. The gel was recorded the velocities and stiffness values in 10 days. In this figure, D1 represented the first day, and D10 represented the 10th day.

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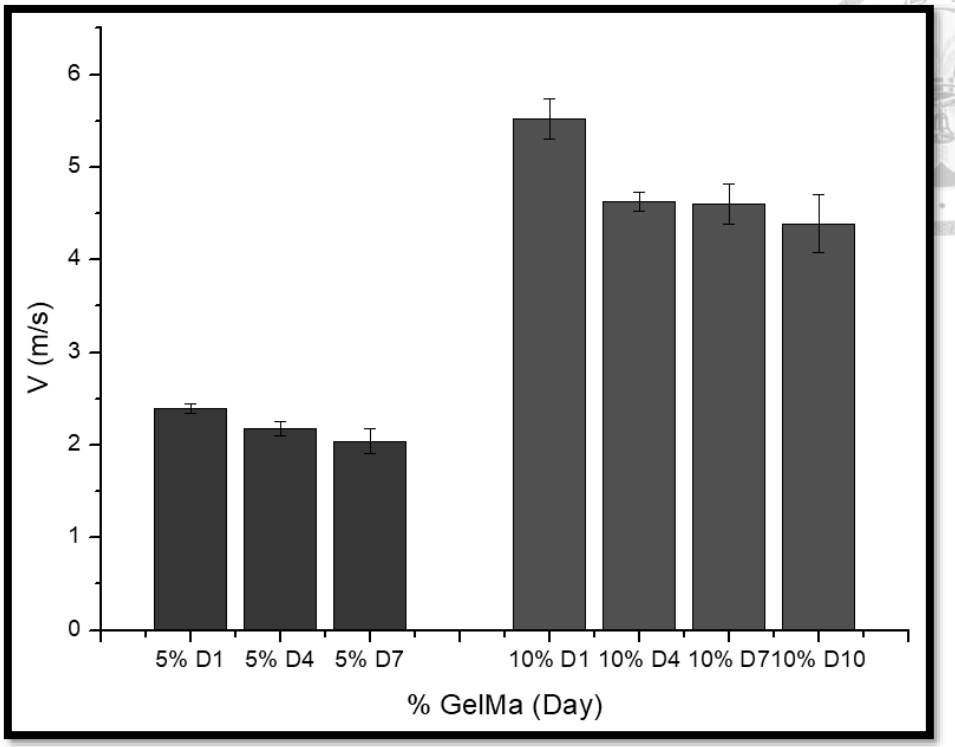

Figure 4-6: the velocities for 5% & 10% GelMa in 10 days (n=3)

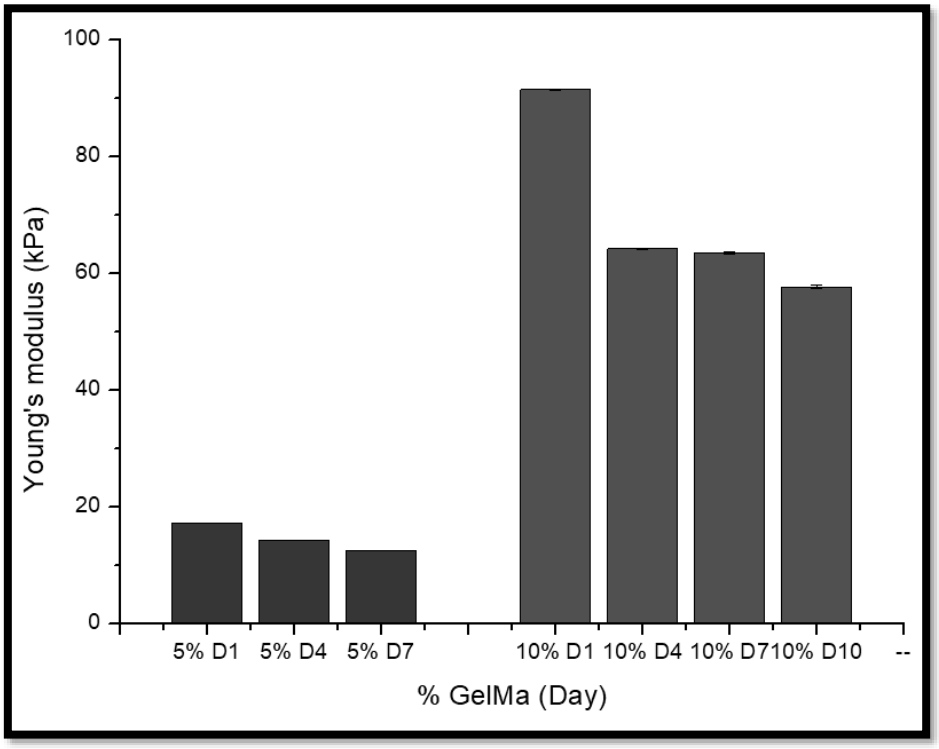

Figure 4-7: the Young's modulus for 5% & 10% GelMa in 10 days (n=3)

doi:10.6342/NTU201902957

For the 10% (w/v) GelMa gel, the velocities of the first day, 4th day and the 10th day were 5.52 m/s, 4.63 m/s and 4.6 m/s. The Young's modulus of the first day, 4th day and the 10th day were 91.5 kPa, 64.3 kPa and 63.6 kPa.

4-4 The appearance and thickness of GelMa gel changing with time

The 5% and 10% (w/v) GelMa gel were cultured in incubator with PBS but without cells. The gel was taken pictures and recorded the thickness through ultrasonic images in 10 days.

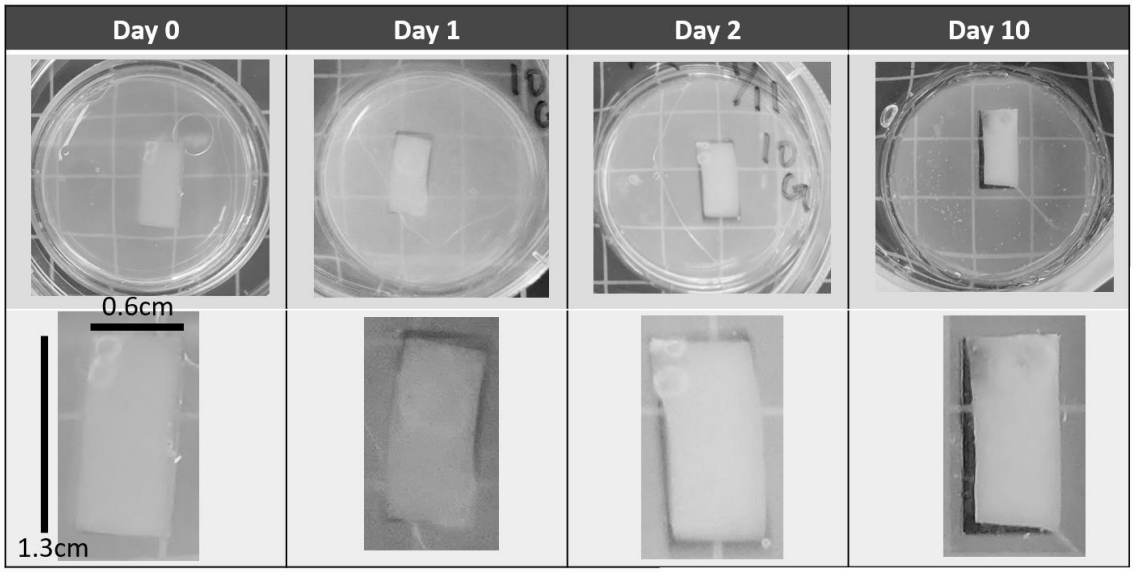

Figure 4-8: The appearance of 10% (w/v) GelMa gel changing in 10 days

The following data was record by ultrasonic images and calculated the pixel of the thickness. Then the pixels scale was transferred to millimeter(mm) scale.

Thickness(mm)	0 day	4th day	7th day	10th day
5% (w/v) GelMa	5.37	4.89	4.61	(not recorded)
10% (w/v) GelMa	6.54	5.76	5.42	5.37

Table 4-2: The thickness of 5% and 10% (w/v) GelMa gel changing in 10 days

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After normalization:

Thickness	0 day	4th day	7th day	10th day
5% (w/v) GelMa	100%	91.1%	86.0%	(not recorded)
10% (w/v) GelMa	100%	88.1%	82.8%	82.1%

Table 4-3: Normalization: the thickness of 5% and 10% (w/v) GelMa gel changing in 10 days

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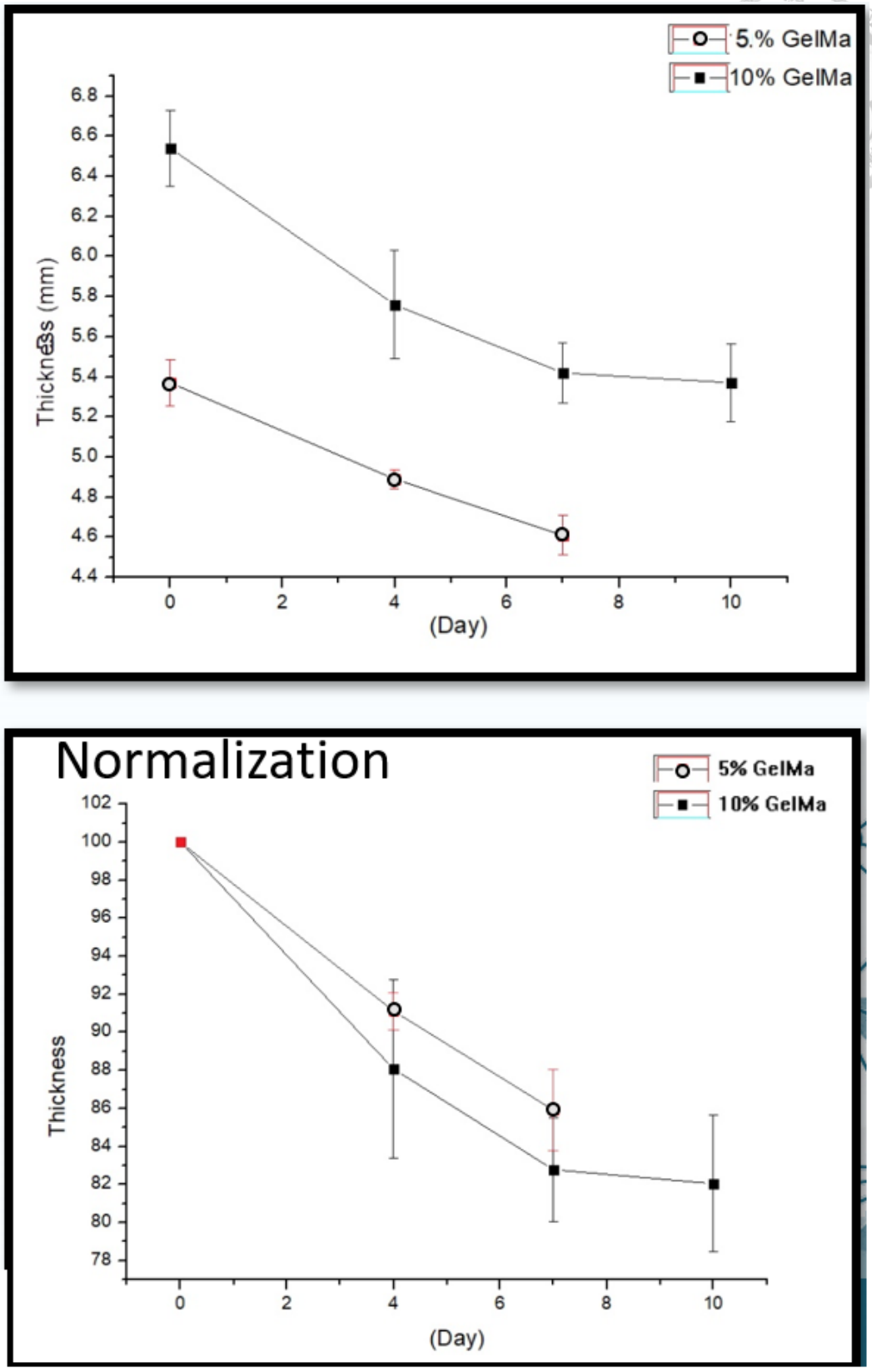


Figure 4-9:The thickness of 5% and 10% (w/v) GelMa gel changing in 10 days (Up)
The normalization(down)

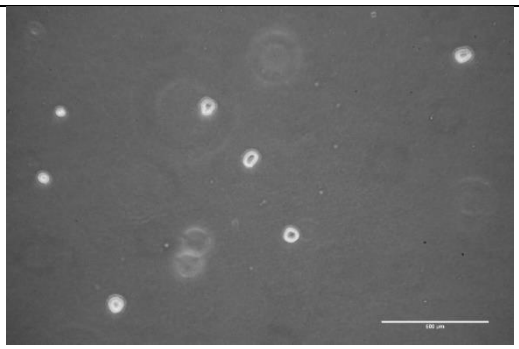
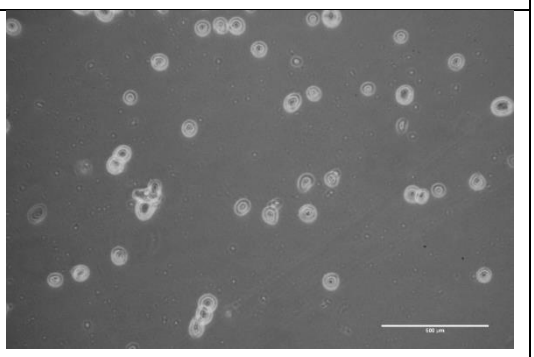
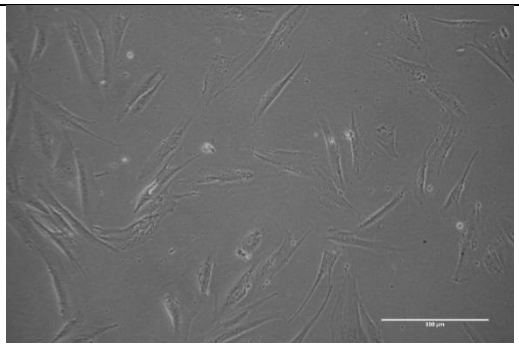
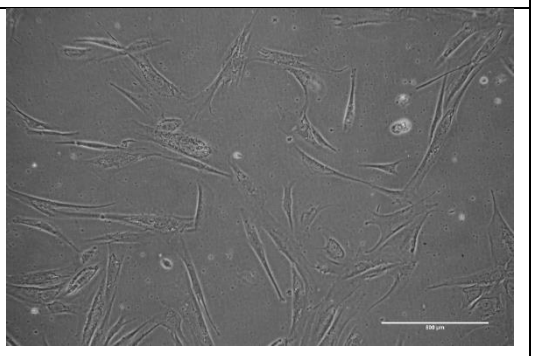
doi:10.6342/NTU201902957

According to these tables and figures, the degradation of the gel was not enough. If the gel used in the clinical case, it should degrade more and faster. Fortunately, it was possible to tune this property by improving the 3D printing process. Moreover, If the gel was cultured with cell in the incubator, it would degrade faster because of the cell had the ability to remodel and degrade GelMa.[8]

It was expected to degrade entire pcMSC cell sheets in 1-3 days. If it degraded too slow, this would block the skin cells growing on the wound. As a result, this situation would inhibit healing the wound.

4-5 Cell culture on GelMa printing gel

I cultured pcMSC on the dish coated with collagen typeⅣ and 10%(w/v) GelMa gel in 3 days. The cell was taken pictures every 24 hour. According to these figures, pcMSC were successfully cultured on the 10%(w/v) GelMa gel and the morphology not obviously differed from the situation on the dish with collagen typeIV.

	dish coated with collagen typeIV	10%(w/v) GelMa gel
0H		
24H		

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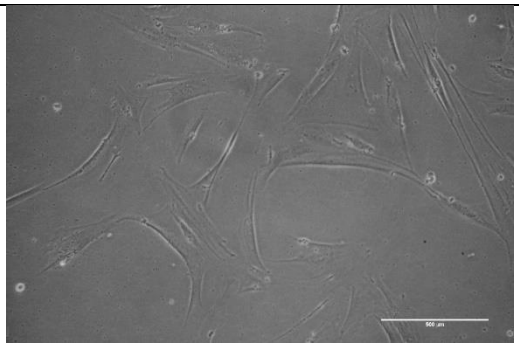
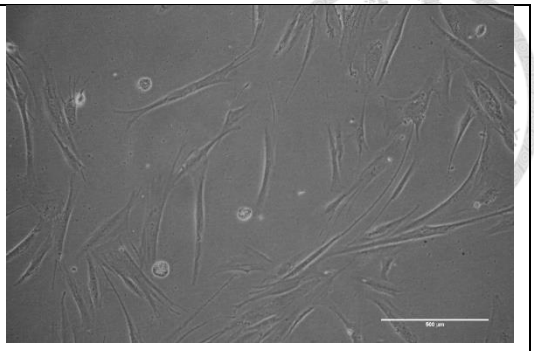
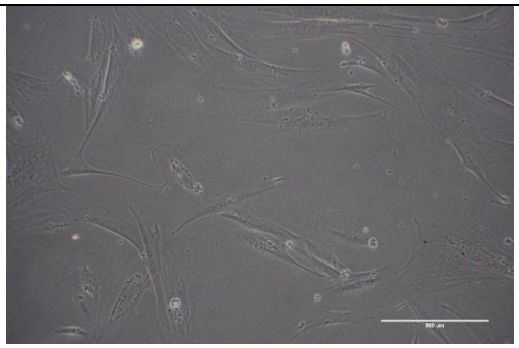
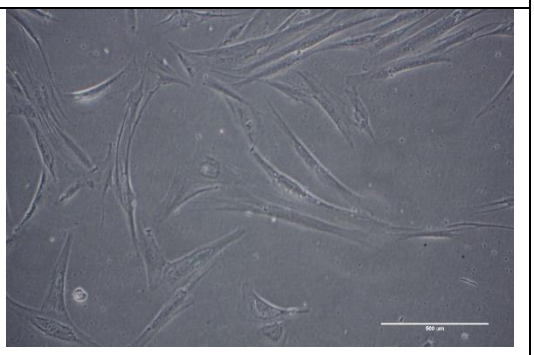
36H		
72H		

Table 4-4: figures of pcMSC cultured on the dish coated with collagen typeIV and 10%(w/v) GelMa gel in 3 days

To find the best recipe of the GelMa printing ink, I compared the cell proliferation on different percentage of GelMa and LAP.

PcMSC were cultured on 10%(w/v) and 5% (w/v) of GelMa printing gel (thickness: 0.6mm). I used Calcein-AM and PI to stain the live and dead cells. The live cell was represented by green color and the dead was represented by orange color.

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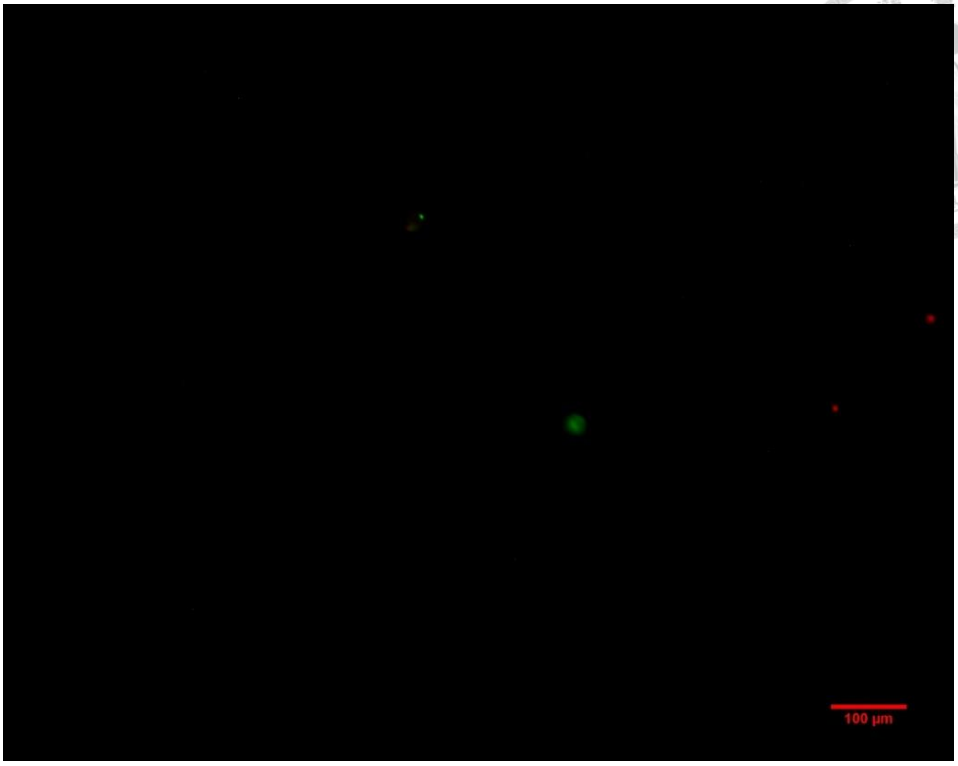

Figure 4-10: 5% (w/v) of GelMa printing gel after 4 days

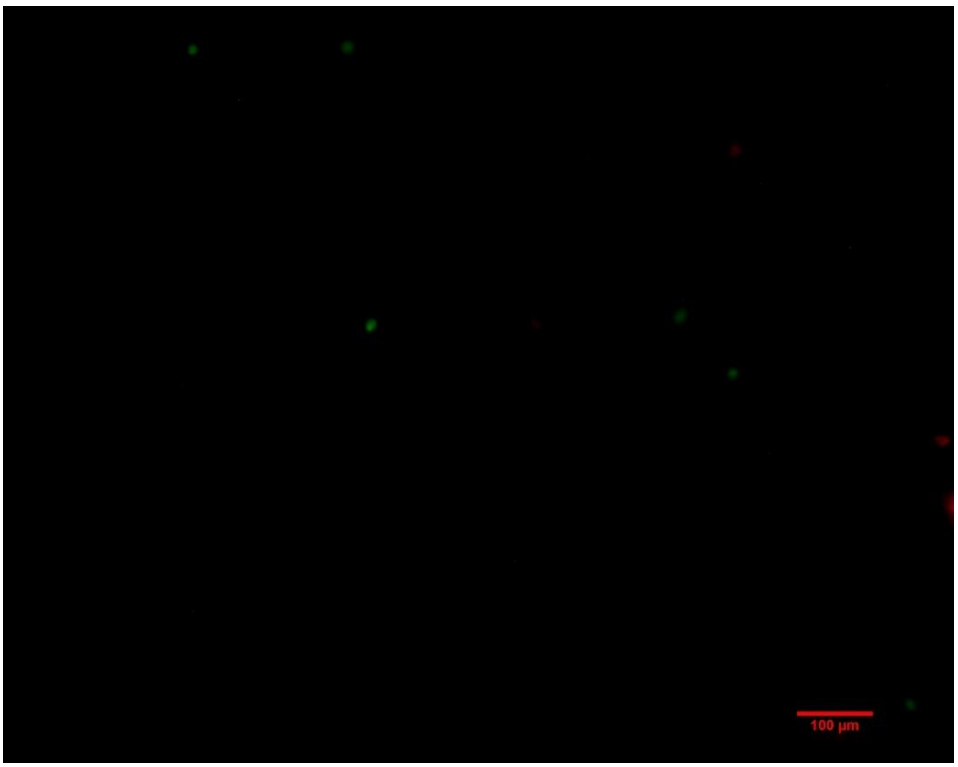

Figure 4-11: 5% (w/v) of GelMa printing gel after 7 days

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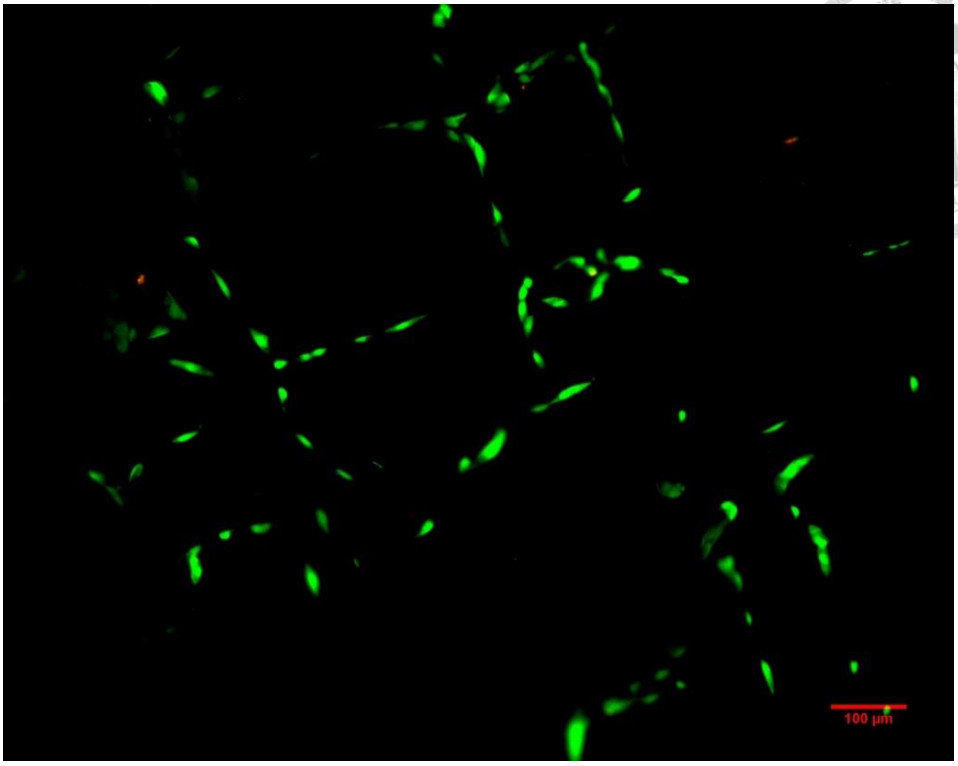

Figure 4-12: 10% (w/v) of GelMa printing gel after 4 days

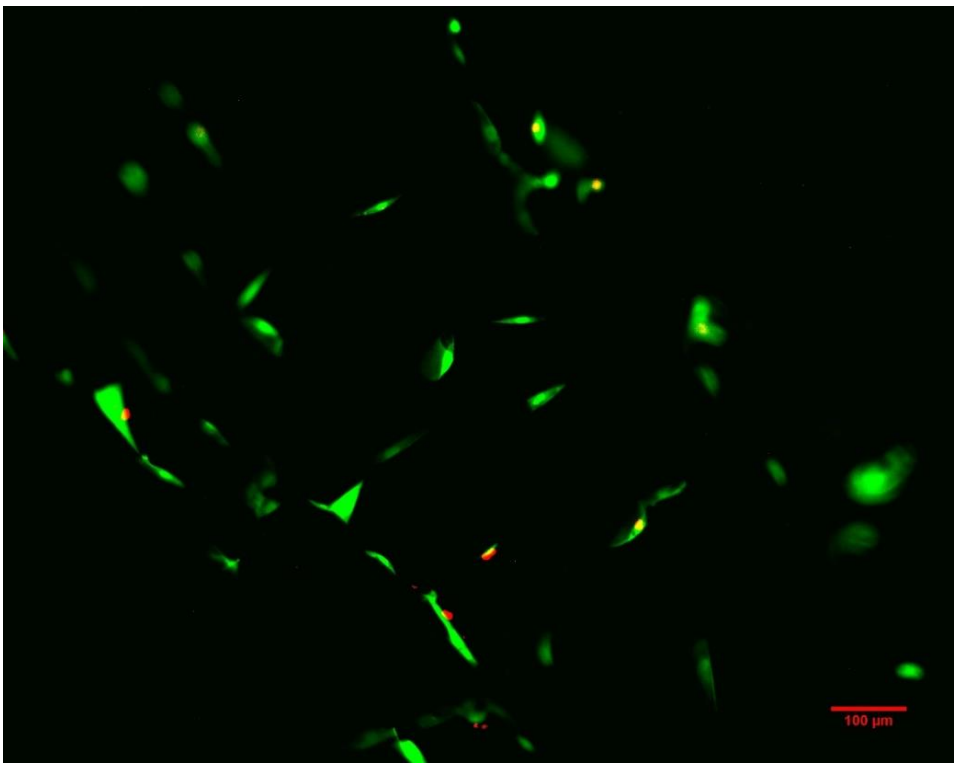

Figure 4-13: 10% (w/v) of GelMa printing gel after 7 days

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According these figures, the pcMSCs were suitable cultured on the 10%(w/v) of GelMa gel. The cells on 10%(w/v) of GelMa gel had better morphology and more live cells. Moreover, the pcMSCs can still culture on the gel for 7 days, it is important to fit the need of the cell sheets. The cell can have more change to survive on the gel in the organism after the operation

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Chapter 5 Discussion

Finally, I successfully developed GelMa printing gel for cell culture and 3D printer system. Moreover, after 3D printer remodeling, the light source of the 3D printer can be changed the filter by the need of new photoinitiators. It will have a huge benefit for the future research.

But I cannot promise the pcMSCs did not change the characteristic on the gel. I need to do other experiment to test the characteristic. One of the experiments is animal experiments, which tests the healing ability of the pcMSC cell sheets on the skin wound. During the experiment, I can also get more information about pcMSC cell sheets. According this information, I can adjust the amount of the cells on gel and the cultured days before implantation into organism. As a result, cell sheets can be improved to get the better effect on wound.

The z-axis moving platform of the projector-based 3D printer did not participate in this method, because I just printed only one layer gel for cell sheets. But z-axis moving platform will be used in the building up 3D structure. The 3D structure will have plenty of pores and pipelines, which are the place for cell adhesion. The design of 3D structure will be a big issue, because this can affect the physical properties of GelMa gel and the characteristic of pcMSC. I can observe theses data from the animal experiments.

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Chapter 6 Future work

In fact, I will start the animal experiments in the several months. For the test on rat, the GelMa gel needs to be put on the bandage. I will try to let the GelMa gel originally building on bandage in the 3D printer. Moreover, the manufacture process needs to develop more detail. The z-axis moving platform needs to be use during the process. I will learn more know-how and improve this process in the animal experiments.

It will finally become the product for the patient of severe skin burn and help these patients in the future.

doi:10.6342/NTU201902957

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