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Supercritical Processed Decellularized Extracellualr Matrix As Regeneration Therapeutics Applying 3d Printing

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SUPERCRITICAL PROCESSED DECELLULARIZED EXTRACELLUALR MATRIX AS REGENERATION THERAPEUTICS APPLYING 3D PRINTING

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Abstract

Extracellular matrix has been broadly applied and show great promise in medical applications and tissue engineering. Product of extracellular matrix (ECM) should be treated with decellularization and purification process. Supercritical carbon dioxide treatment is of particular interest for purifying ECM because of its medically available and rapid speed of process. However, it is not fully researched in treatment of biomaterials for tissue engineering. Therefore, we investigated optimal conditions of supercritical carbon dioxide processing in different extracting parameters from porcine adipose tissue. The 3-day, dual treatment including enzymatic decellularization and supercritical fluid extraction of pork adipose tissue were performed. Two types of protocols using different extracting parameters were applied in order to evaluate the influence of extracting pressure, and temperature on the extraction yield, DNA concentration and remaining collagen amount of product. Yield rate was increased when higher temperature or pressure were applied, and pre-enzyme treatment had higher percent of yield rate than the pre-supercritical processing. DNA removed nearly 90% when extracted at highest pressure (3.04×10^{7}Pa) and low temperature (30±5℃) in the pre-enzyme sample. As for collagen concentration, pre-enzyme process showed efficient extracting ability at each temperature and pressure. Remaining collagen steadily decreased with increasing of extracting pressure and temperature. At the lowest temperature

(20±5℃) and lowest pressure (1.01×10^7Pa), remaining collagen was 75.74±1.83%. Supercritical extraction technology can produce DE-ECM eliminating DNA content efficiently and remaining proper collagen amount successfully. This study evaluated the feasibility of utilizing supercritical extraction technology with bio-materials and it is proven that it is successful. Through this technology of controlling extracting pressure and temperature, it has a potential for the DE-ECM mass production can be useful as tissue regeneration therapeutics as well new drug delivery paradigm.

To improve bio-compatibility, 3D printing technologies (piston type of extrusion method) are applied to fit for bio-environment and this thesis purpose, and material processing for producing printable bio-ink was conducted. PTE method was successfully applied to print 2D pattern for medical patch application and 3D structure for implantable application.

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Chapter 1: Tissue Engineering

Generally, tissue engineering is the multidisciplinary application of cells, engineering and bio-materials development, and applicable biochemical and physicochemical factors to develop or replace damaged human tissues. (Figure 1.1) These days, tissue and organ failures are serious and commonly happen in organ transplantation, surgical repair, artificial part transplantation and drug therapy.[1] For example, researches are going on about bioartificial organ such as liver, pancreas, bladders, artificial skin, artificial bone, tissue engineered vessels and cartilage. Thus, tissue engineering extends the area from controlling cellular responses to material implants, controlling the curing environment of regenerated tissue, producing replaceable tissues and cells for human body and studying bio-compatibility and rate process between material and human body. Tissue engineering uses tissue scaffold for the construction of new viable tissue for a regenerative medicine for tissue repair or therapeutic purpose. To construct a basic tissue, cells are generally planted on scaffolds which has proper microenvironment such as extracellular matrix (ECM) in order to develop tissue development artificially. Various of research has been reported on the nanocomposite scaffolds for tissue engineering. Scientific advances in biomaterials, stem cells, biomimetic environments like scaffolds have provided unique chances to produce tissues in laboratory from engineered ECM.

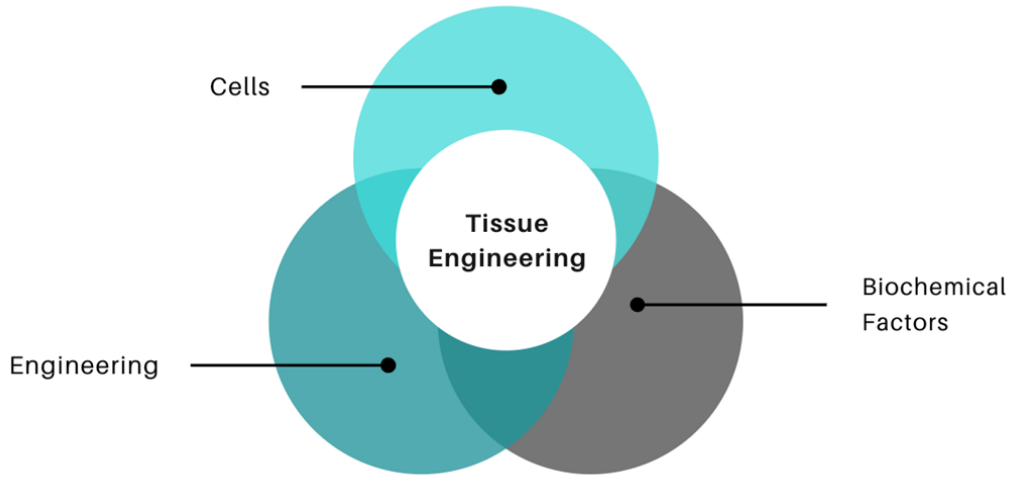


Figure.1.1: Multidisciplinary area of tissue engineering with cell science, engineering and biochemical factors. [1]

1.1 STEM cells

STEM cells are cells that have ability to divide in self renewal to produce same type of STEM cells. Self-renewal process of STEM cells occurs with asymmetric cell division which means dividing into one mother cell that is similar of the original STEM cell and another new cell that is differentiated. It has the capacity to differentiate into specialized cell types which means potency. Since it is rare to obtain, the research with STEM cells is conducted prospectively and sterilized carefully.[2] The research of STEM cells is critical part in tissue engineering because it replaces destroyed or damaged organs or cells that our body cannot replace naturally. There are four types of STEM cells; embryonic STEM cells which are obtained from the inner cell mass of the blastocyst; Tissue-specific STEM

cells which generate different cell types for the specific tissue or organ; Induced pluripotent STEM cells which are cell that have been engineered in the laboratory by changing tissue-specific cell; Mescenchymal STEM cells which are isolated from stroma.

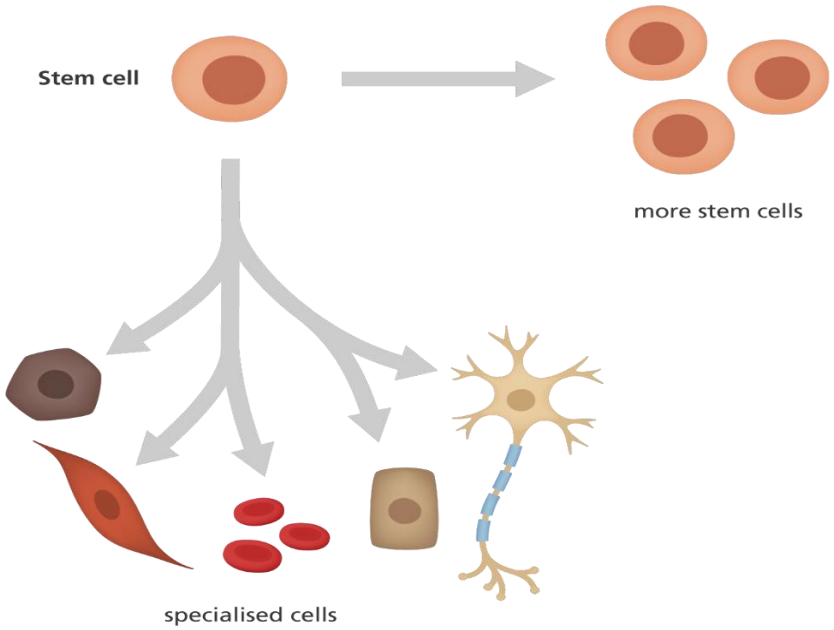


Illustration 1.1: An illustration describing a STEM cell growing cells by self-renewal process or specialized cells. [3]

1.1.1 Tooth tissue engineering

Dental STEM cells are investigated for tooth regeneration and repair. Further research was done with bone marrow mesenchymal STEM cells and embryonic STEM cells to prove the possibility of creating teeth by imitating embryonic development mechanisms. Tooth regeneration technology has studied into two main methods; implantation of artificial cells obtained by reiterating tooth embryonic

developmental processes in vitro and in vitro cell culture and polymers for in vivo implantation.[4]

1.1.2 Wound healing

Regenerative medicine for wound healing is collaborative area in nonmammalian and human development, STEM cell biology, genetics, materials science, bioengineering and tissue engineering. It is new therapies to replace or restore lost and damaged cells. The specific research has continued about limb regeneration, fetal wound healing, STEM cell biology and tissue engineering as foundation for developing new clinical therapies.

Traditional therapy has been ongoing for years and focused on understanding the natural regeneration in lower species. However, STEM cell biology has developed that manipulation and provide bio-compatible cells and tissues could recapitulate the regenerative process and more functional actions. Illustration 1.2 shows that a common approach to engineered replacement tissues adds with biocompatible and bio-degradable scaffolds seeded with appropriate STEM. [5]

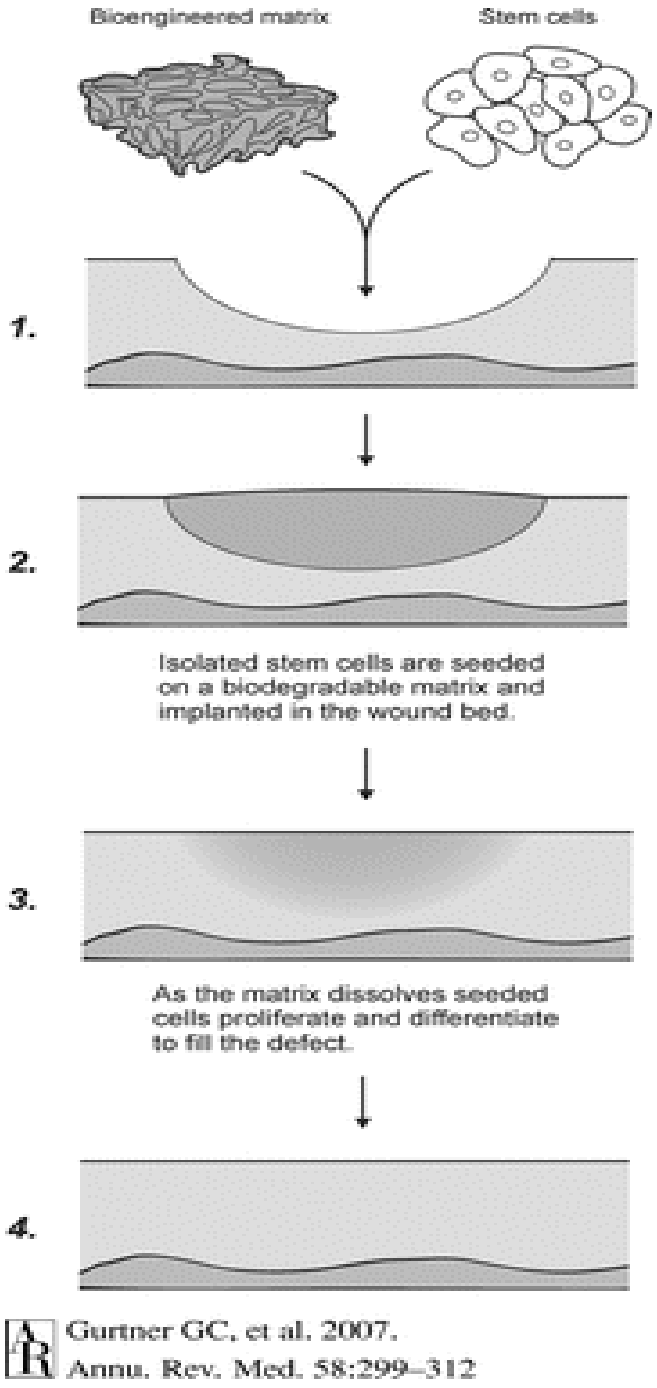


Illustration 1.2 Common approach to engineered replacement tissues adds with biocompatible and bio-degradable scaffolds seeded with appropriate STEM. [5]

1.1.3 Brain neurons replacement

STEM cell technology can apply in brain part to replace neurons that have died as a result of degenerative disease or stroke. Researchers generate dopaminergic and serotonergic functional neurons in vitro from mouse, and induce functional recovery when transplanted into parkinsonian rats. [6-7]

1.2 Scaffolds

Scaffolds are materials that have been produced to make desirable cellular interactions to form the new functional tissue for regenerative purpose. Scaffolds almost mimic the ECM of native tissue. Cells are seeded into this biostructure which is able to support 3D tissue formation; allowing cell attachment and migration between them; delivering and keeping cells and biochemical factors, diffusion of vital cell nutrition and products, exerting specific mechanical to modify the cell phase.

Well-made scaffolds have high porosity and adequate pore size to make convenient of cell seeding and diffusion over the whole structure of cells and nutrients. Scaffolds should have property of biodegradability which means absorbing by the surrounding tissues without physical removing action. Scaffold can provide structural integrity to the body and it will remain the newly formed tissue with dissolve itself.[8-9] However, the possibility of toxicity with non-biodegradable nano substances is not fully researched and still studying about it. To

overcome the limitation of current scaffold production, 3D printing technology has been applied to solve difficulty of maintenance for bio-structural features and develop bio-compatibility. (Showed in Figure 1.2 and Figure 1.3)

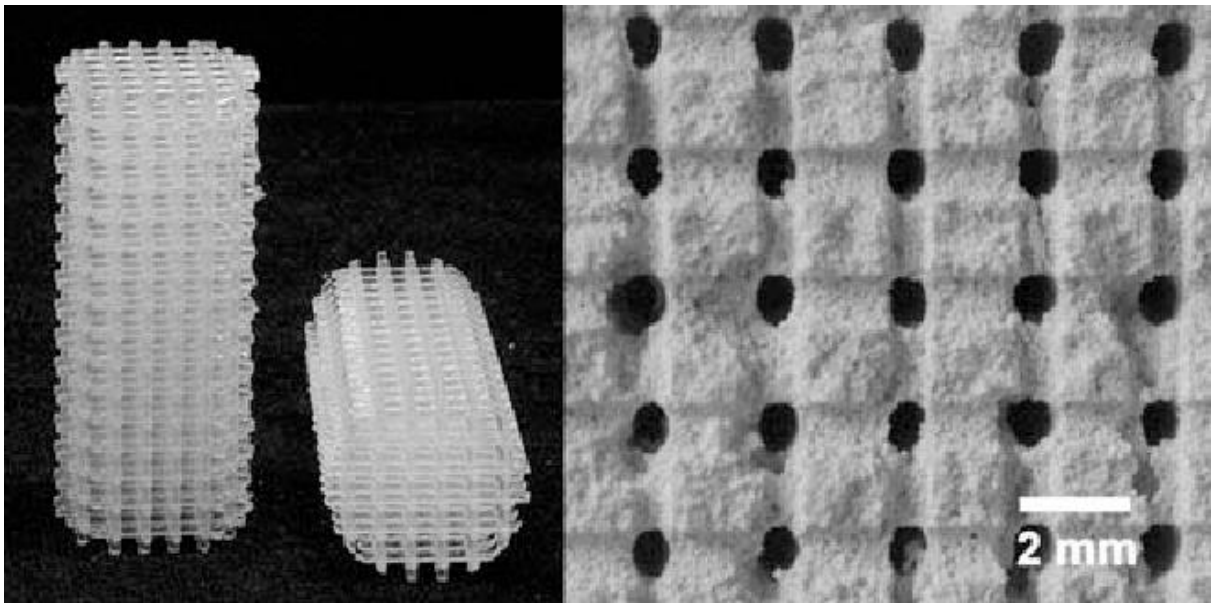


Figure 1.2 3D printed bone tissue scaffold using polymer material. [8]

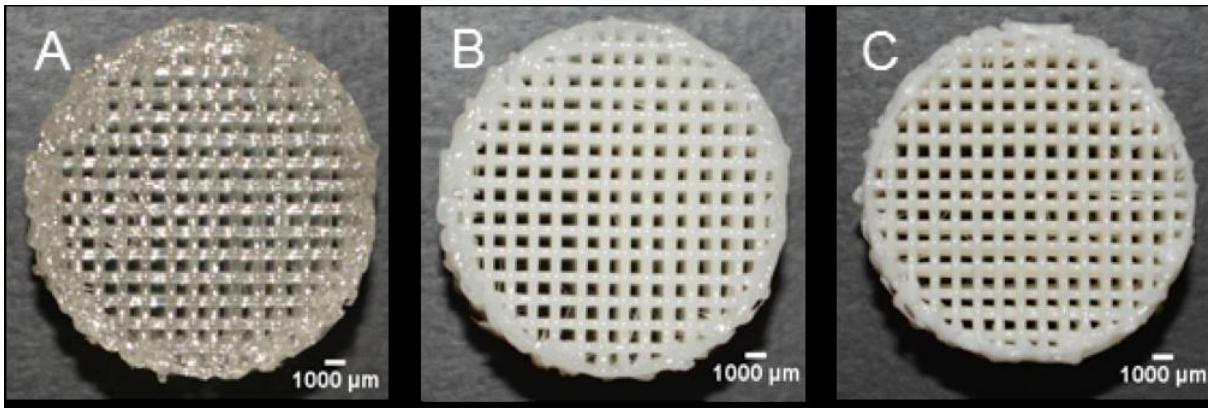


Figure 1.3 Printed filament scaffolds for researching bone regeneration technology, A) 100% PLLA and composite matrix B) 5% of TCP C) 20% of TCP.

[9]

1.3 Extracellular Matrix (ECM)

Natural bio-materials which have high degree of bio-compatibility as well as ability to direct cellular proliferation and constructive tissue remodeling get attention from researchers and showed great promise in medical applications. [10-11] Especially, biological scaffold including extracellular matrix (ECM) has been broadly applied in medical field. [12-16] The ECM is a complex matrix surrounded by cells and take structural and biochemical supporting functions from bioactive ingredients such as collagen, elastin, glycosaminoglycans (GAGs), growth factors and cytokine [17-18]. The main function of ECM is supporting cell adhesion, cell to cell communication and differentiation. ECM consist of microfilaments, phospholipid bilayer, integrin, proteoglycan, fibronectin, collagen and elastin. (described in Figure 1.4) Collagens are the most abundant protein in the ECM. It exists in ECM as fibrillar proteins and provide structural support to resident cells. According to the concentration of collagen and elastin, it has various of stiffness and elasticity. And those properties have shown to take influential role in regulating numerous cell functions such as cell migration, cell proliferation, differentiation and death.

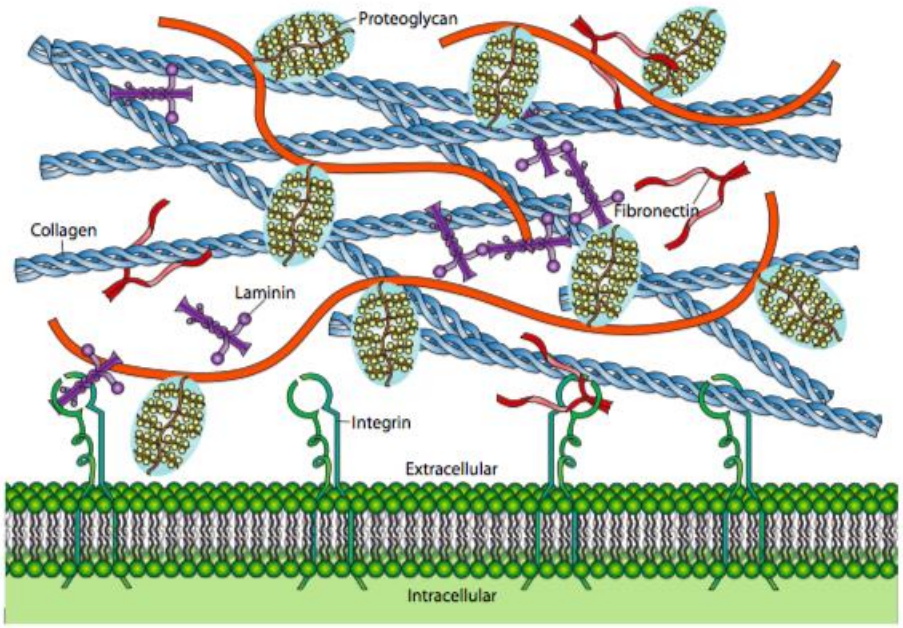


Figure 1.4 Biostructure of ECM which contains proteoglycan, collagen, laminin, integrin and fibronectin. [19]

Most bio used metals or polymer composite materials have difficulties when they are implanted in human body. They occur lowering the pH degree in the transplanted site and remaining toxic chemicals in implanted area which occurs terrible immune problems. However, the most attractive feature of ECM is causing regrowth and healing of tissue. The ECM works with STEM cells to grow and extend all parts of the human body. Coming to the injury repairment, the ECM prevents the immune system triggering from the injury and reaction with inflammation and polluted tissue.

1.3.1 Synthesis of ECM (Decellularization)

Decellularization is the main process used in ECM synthesis. It isolates the ECM of a tissue from its inhabiting cells, and makes it available to use in artificial organs and tissue regeneration area.[20] Decellularized ECM has already been applied in many areas of research including tissue engineering, drug delivery system and implantation. Various decellularization processes are used to make ECM because different structure and composition of ECM are used in different area.

1.3.2 Limitation of traditional decellularization process for ECM production

There is limitation of traditional decellularization method because it generally uses chemical detergents which are cytotoxic and can damage ECM structure and has disadvantages such as high costs and time-consuming process. [21-23]. According to Table 1, it shows that most of the decellularization methods can transform the 3D bio structure of tissue which relate with mechanical properties, and also the chemical detergents and acids can remain inside the tissue after being processes and cause substantial adverse immune effects. [24-25]

Table.1.1 Summary of decellularization agents and technique for producing DE-ECM tissue

Agent/ Technique	Mechanism	Effect	References
Acids and base	Solubilize cytoplasmic components of cells and tend to denature proteins.	Decrease collagen amount and GAG.	[26-27]

Hypotonic and hypertonic solutions	Occur Cell lysis by osmotic shock and disrupt DNA-protein interactions.	Non-effective in removing cellular residue.	[28]
Tributyl Phosphate	Form stable complexes with metals and disrupt protein-protein interactions.	Mixed results with efficacy dependent on tissue, dense tissues lost collagen.	[29]
Nucleases	Catalyze the hydrolysis of ribonucleotide.	Difficult to remove from the tissue and invoke an immune response.	[30]
Freezing	Intracellular ice crystals disrupt cell membrane.	Ice crystal formation disrupts or fracture in product.	[31]
Electroporation	Pulsed electrical fields disrupt cell membranes.	Electrical field oscillation disrupts ECM structure.	[32]
Agitation	Lyse cells and facilitate chemical exposure.	Aggressive agitation or sonication damages ECM.	[32]
Perfusion	Facilitate chemical exposure and removal of cellular material.	Pressure associated with perfusion disrupt ECM.	[33]

1.4 Biomaterials used in tissue engineering

Biomaterials take role of important portion in tissue engineering to control the structure of regenerated tissue and its environment, producing cells and tissues for transplantation into the human body, and controlling rate process of material to body

tissue. [1] They are designed with architectural of bio-structure (like ECM…) to facilitate cell growth and tissue regeneration in laboratory. Generally, many different materials are utilized for tissue engineering such as natural and synthetic, biodegradable and permanent sources. These days, researches have continued about its properties and customized function such injectability, bio-compatibility, non-toxic synthetic process, non-immunogenicity, transparency, resorption rate, nano scale materials and biomimetic structure.

PLA is a polyester which degrades in human body system to form lactic acid which is naturally occurring chemical and easily removed from the body. Polyglycolic acid (PGA) and polycaprolactone (PCL) are similar with PLA. But they show different rate of degradation compared to PLA. [1] And also, they have well maintained mechanical strength, hydrophobic nature and structural integrity. The hydrophobicity inhibits biocompatibility which means disadvantage for tissue scaffolding. Overcoming the disadvantages of polymer kind of materials, hydrogel was suggested because of its superior biocompatibility and hydrophilic nature. Hydrogels are watery polymer networks forming as crosslinked polymer chains in nature. The 3D network of hydrogels can encapsulate cells to react with their bio environment in all directions. It can also contain therapeutic cells at specific cite of cells. However, they lack the structural integrity than polymers. Subsequently, natural based materials come to replace. Proteic materials such as collagen or fibrin,

and chitosan or glycosaminoglycans fit in terms of bio compatibility and structural features. But some issues occur about potential immunogenicity and problem during the material processing. As a result, another form of scaffold has researched. It is decellularized tissue extracts whereby the remaining cellular contents and extracellular matrix act as the scaffold.

Chapter 2. Supercritical Extraction

Supercritical extraction is the novel process of separating one component from base solid or liquid matrix utilizing supercritical fluids for the extracting solvent. It has advantage of selectivity and speed. Supercritical fluid's soluble ability can be altered by manipulating pressure and temperature, doing selective extraction. Moreover, supercritical extraction is a diffusion-based process. Because of the lack of surface tension and intermediate level of viscosities, the solvent can penetrate into the base matrix quickly.[34] The disadvantage of supercritical extraction is that it occurs deformation of materials after extraction. For example, materials become slurry and gel-type of material after extraction.

As shown in Figure 2.1, the system of supercritical extractor mostly consists of pump for the gas where provide gas to extracting chamber, a cell to put the sample, controller where maintain pressure and temperature system and collecting tube for waste after extraction. The gas can be recycled or discharged to atmosphere.

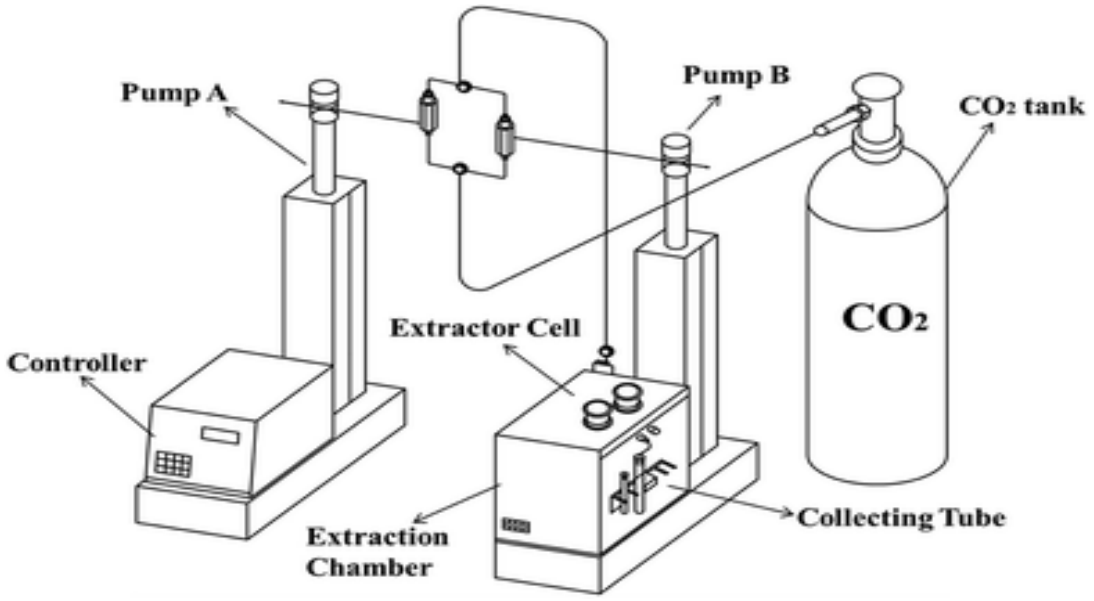


Figure 2.1 Supercritical extractor consist of pumps for the gas, extracting chamber, gas tank and controlling system. [34]

2.1 Supercritical fluid

Supercritical fluid is the unspecified substances at temperature and pressure above critical points which means the end point of phase equilibrium curve. (described in Fig2.2) It has strong selectivity, expeditiousness and high degree of dissolution ability.

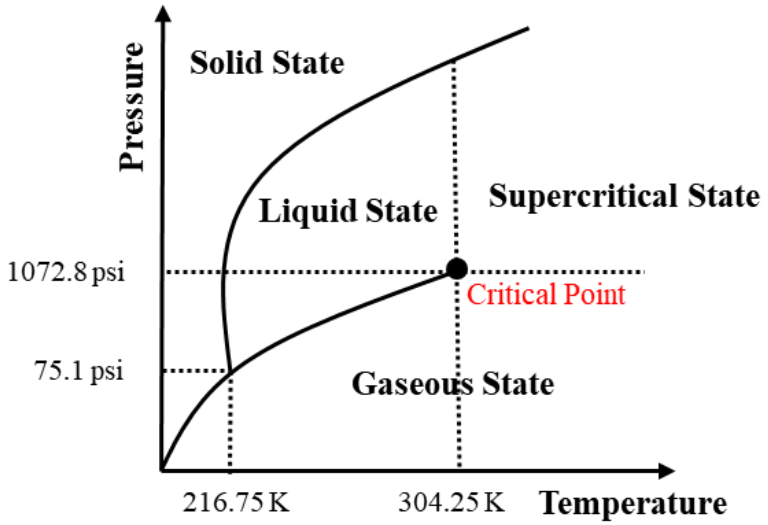


Figure 2.2 Phase diagram of carbon dioxide which states solid state, liquid state, gaseous state and supercritical state above critical point.

This state cannot be specified as liquid or gas. It can penetrate solid like a gas and dissolve materials like a liquid. As shown in Table 2.1, supercritical state has intermediate level of density and viscosity between gas and liquid state.

Table 2.1 Value of density, viscosity and diffusivity at the state of gas, liquid and supercritical.

	Density (kg/m^3)	*Viscosity ($\mu Pa \cdot s$)*	*Diffusivity (mm^2/s)*
Gases	1	10	1-10
Supercritical fluids	100-1,000	50-100	0.01-0.1
Liquids	1,000	500-1,000	0.001

Moreover, there is no surface tension in a supercritical fluid between liquid and gas. By controlling the pressure and temperature of the material, the properties can be change like liquid like or gas like properties. It is called as manipulating of solubility of material in the fluid. To increase the solubility of the solvent, density of the solvent should be increased by controlling extracting pressure and temperature. Additionally, using the nature of polar or non-polar, co-solvents are used to increase solubility with solvent.

2.2 Solvent for supercritical extraction

Various kinds of gases are used for supercritical extraction. Each of them has different value of critical point. (Describe in Table 2.2) Carbon dioxide gas is the most popular solvent for supercritical fluid. Because it has mild condition of critical temperature of 31 ℃ and critical pressure of 74 bar. [35-38]

Table 2.2 Various kinds of solvent for supercritical fluid and their molecular mass, critical temperature and pressure.

Solvent	*Molecular Mass (g/mol)*	*Critical Temperature (K)*	*Critical pressure (Mpa)*
Carbon Dioxide	44.01	304.1	7.38
Water	18.015	647.096	22.064
Methane	16.04	190.4	4.60

Ethane	30.07	305.3	4.87
Propane	44.09	369.8	4.25
Ethanol	46.07	513.9	6.14
Methanol	32.04	512.6	8.09

2.3 Application of supercritical extraction

Supercritical extraction technology is already used in various of daily life. Since it is cost-effective, rapid process, strong extracting ability and non-toxic process, it is used in food and drink industry, medical area and material area. [39-40] The extraction is possible to be selective to specific extent by manipulating the solvent density. Carbon dioxide is the most popular supercritical solvent because it is non-toxic substance and has mild condition of critical point value.

Chapter 3. 3D Bioprinting Technology

Upon the development of 3D printing technology, it has moved from the fused deposition modelling (FDM 3D printing technology) to metal printing and ceramic printing, and recently bio 3D printing area has been studied. A lot of new challenges are coming up on the surface in the area of tissue engineering for certain special cases like complicated implanting structures, mechanical property and biocompatibility of tissue for implantation. 3D printing technology is important to be employed in sophisticated system which needs enough to build the 3D structure with complex inside features. [41]

Reformation in materials processing technology becomes critical part for high-performance and diverse materials for diverse applications. Comparing with conventional methods such as milling, grinding and cutting, additive manufacturing method gets spotlight because of its precision, speed, high product performance and flexibility. Additive manufacturing has developed in its applications like aerospace and automotive field. It is suitable for strict criterion of tissue engineering for processing highly customized medical products or structures. Advance of 3D printing technology promotes the bio-researchers and doctors to get more functional scaffolds or suitable bio parts which has similar environment with actual human tissue. This technology also solves the problem of current biological research

process, testing on animal process. It has critical effect on reducing the need for animal trials.

The materials for bioprinting are known as bio-ink to create bio structures in a layer-by-layer shape. Bio-ink usually consist of collagen, gelatin, alginate or cellulose which are used as isotropic structure of hydrogel. Recently, bio-ink is produced with combination of living cells and hydrogel material for base. It forms scaffold to grow cells and nutrition to survive on.

3.1 Conventional bio-printing technologies.

Described in Figure 3.1, inkjet printing technology is the first to be applied. The advantage of this technology has high printing speed and cost-effective. But thermal and mechanical pressures can damage cell structure. And there is limitation of low cell densities because it has poor ability for printing high viscosity material. [42-43] The research was done about regeneration skin and cartilage.

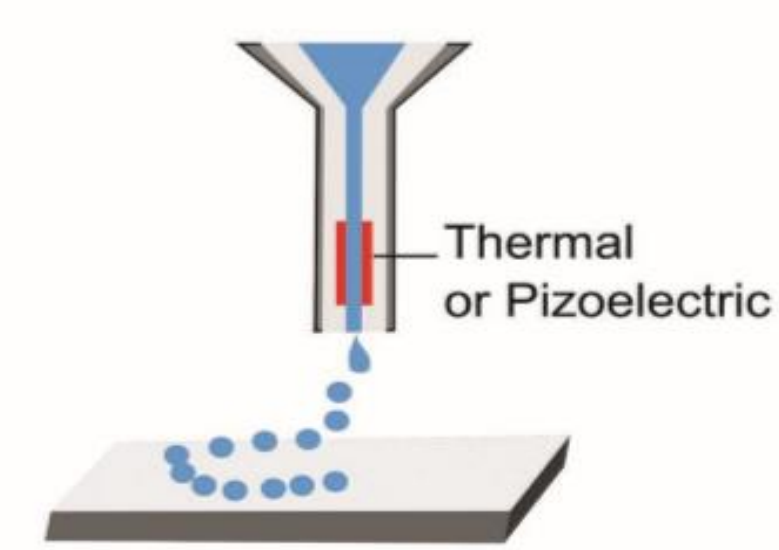


Figure 3.1 Diagram of inkjet bioprinter by small droplet of deposition using hydrogel. [42]

As shown in Figure 3.2, micro-extrusion printing technology operates similar as inkjet method, but it can print with high viscosity material and deposit to make structures. It applies on vascular graft and cartilage constructs. [44-45]

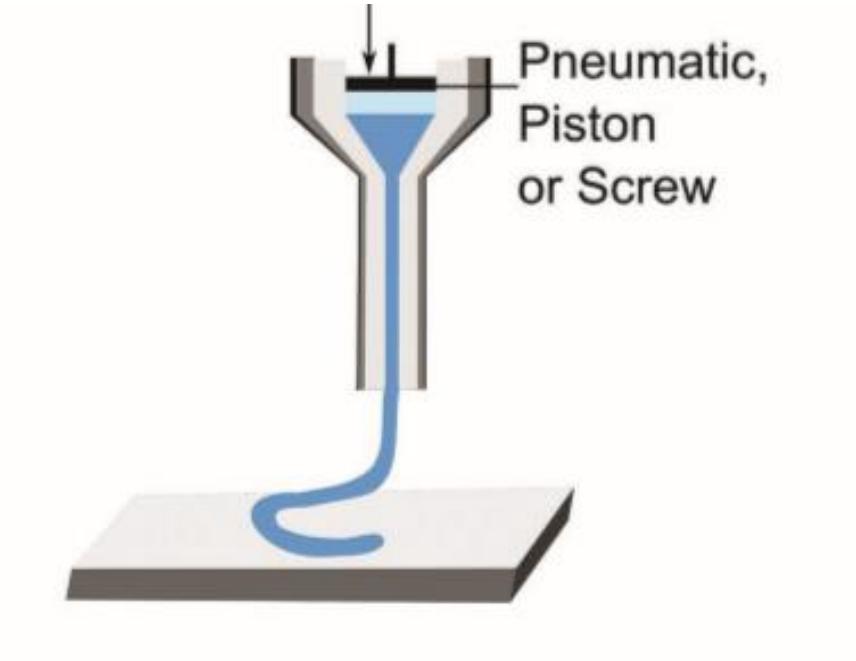


Figure 3.2 Diagram of micro-extrusion bioprinter using a cell laden liquid solution. [42]

As described in Figure 3.3, laser assisted bioprinting (LAB) and stereolithography can produce structure as micro-scale which is critical for mimicking micro environment of tissues. Moreover, these methods are nozzle free process which is problem of the other bioprinting technology. It utilizes light to print and operates by selectively solidifying bio-inks as layer-by-layer method.

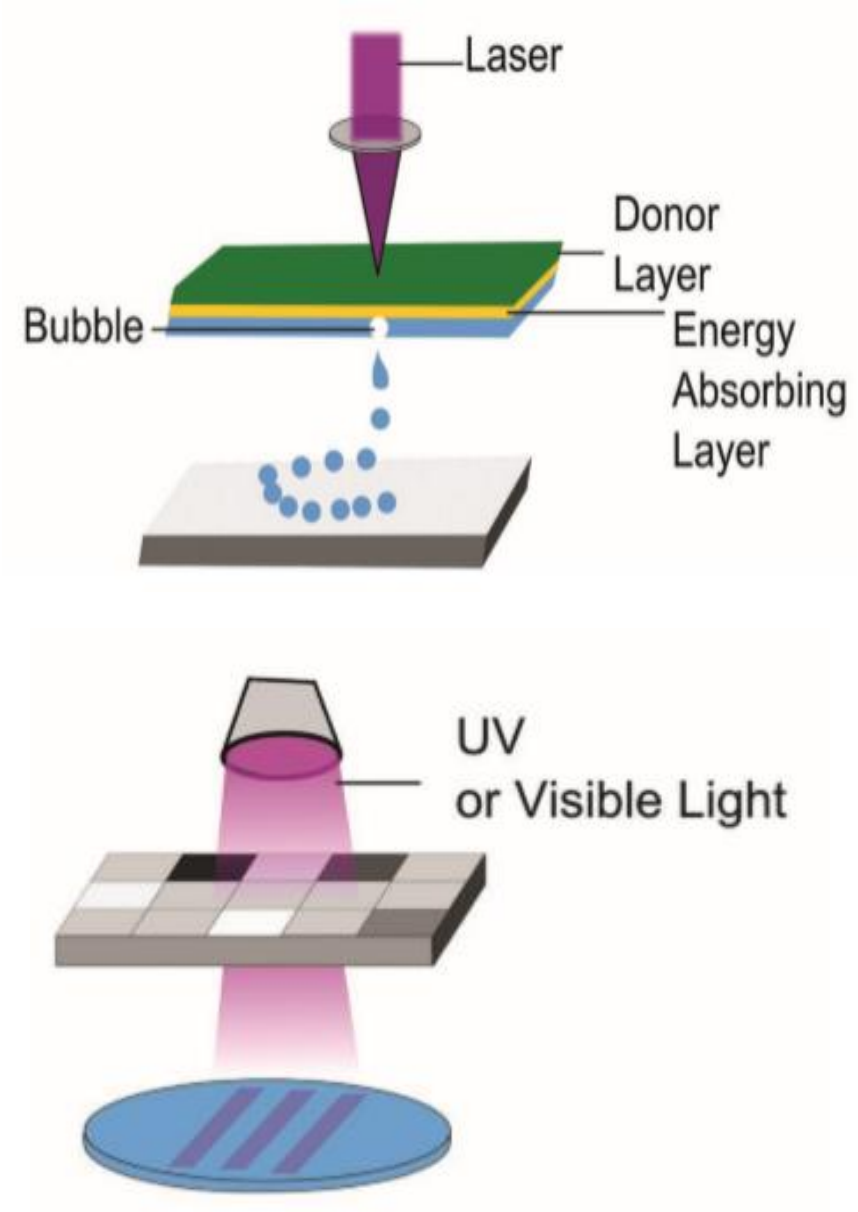


Figure 3.3 Diagram of LAB bio printer and stereolithography printer. [42]

3.2 Piston Type Extrusion (PTE) 3D printing

Applications to the area of artificial implantable bone, artificial skin or organs have been researched, there is a negative result about biomaterials with 3D printed metal because of surface oxidation, stability of structures and biological immune problems. To solve this problem, direct printing method using high viscosity

materials is researched, and PTE technology was suggested for bio 3D printing. PTE printing realizes hygienic process, non-toxic method, rapid and flexibility of material customization. [41]

As shown in Illustration 3.1, PTE adaptor was installed in the 3D printer to print high viscosity material as 3D structures. This printer maximizes the availability of printing in various shapes with different kinds of materials with high pressure. Fluidic dynamics behavior, with a constant viscosity consistent with changes in the shear rate on the Arduino and the Raspberry Pi was monitored and controlled in the range of a force of 5~50 N for high viscosity of material. No additional vacuum packaging or processing was applied to save time to maintain the freshness of the material. Tests are carried out to mount the piston filled with material in the 3D printer and to prevent any substances or contaminated air other than the piston, tip and material from entering the space occupied by the isolation system. The power booster system controlled the force on the piston in the range of 5 to 50 N. As a result of printing, Figure 3.4 shows that the precision and controllable printing using PTE printer with various high viscosity materials.

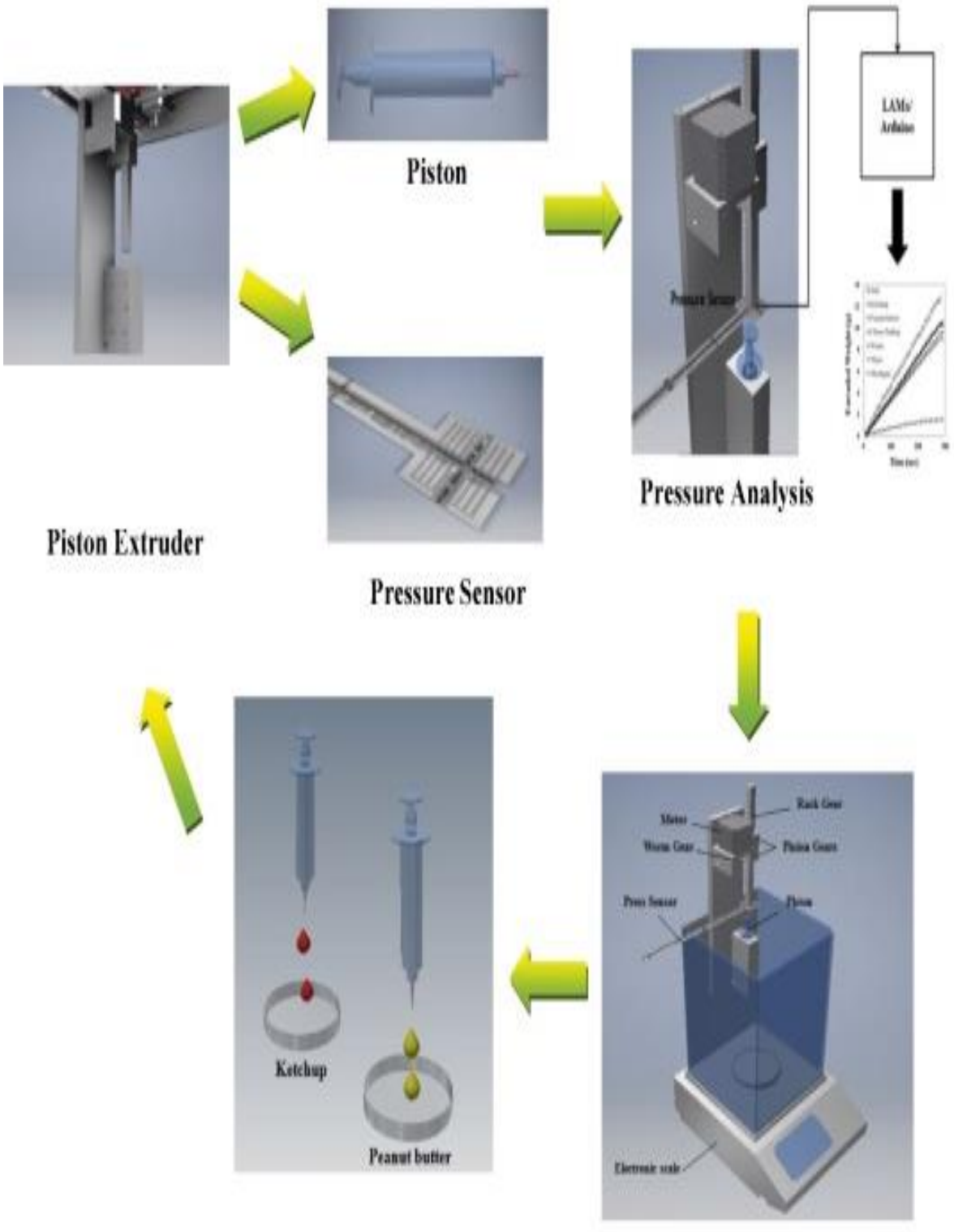


Illustration 3.1 Progress of PTE printing method using high viscosity material. [41]

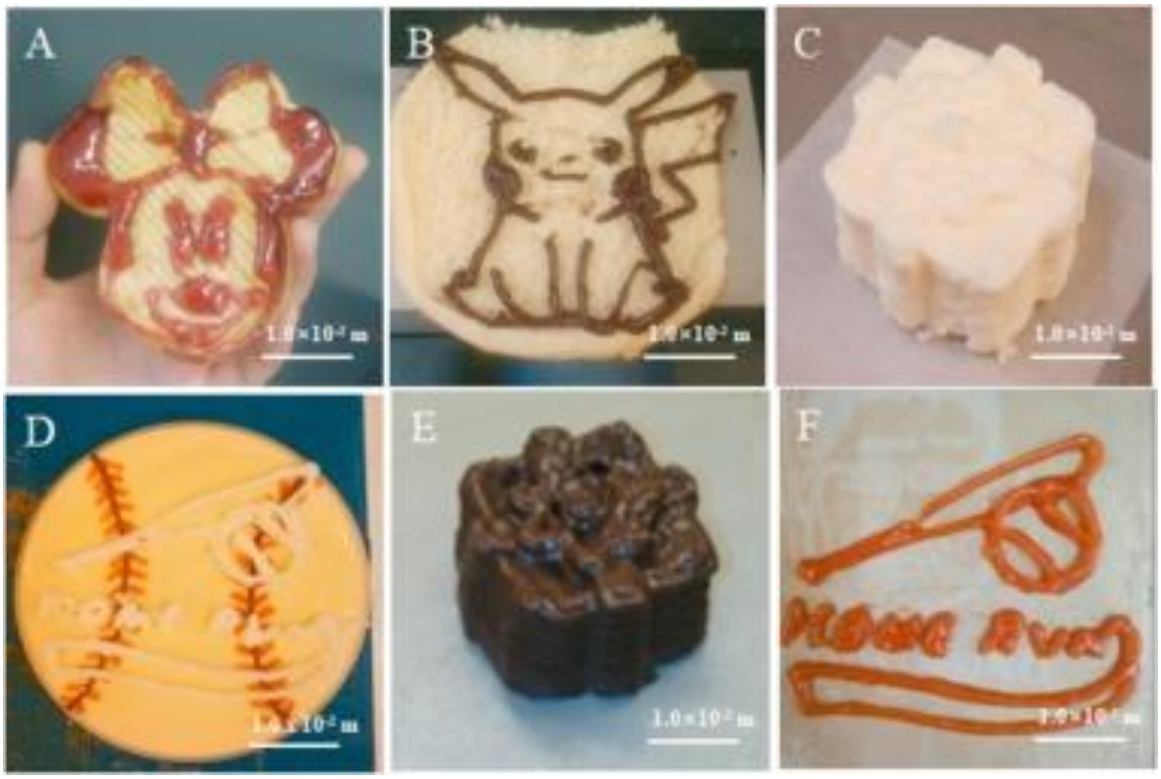


Figure 3.4 3D printed result by various of materials using PTE printing method. A) Jam; B) Chocolate Pudding Paste; C) Mayonaise; D) Mayonaise; E) Cholate Pudding Paste and F) Ketchup. [41]

3.3 Screw Type Extrusion 3D printing

Screw type extrusion 3D printing technology was researched to print high viscosity type of material as delicate 3D structures. It has been studied to use 3D printing method and high viscosity slurries for application like construction field, manufacture process, electrical area (conductive lines or solar cells) and other area for ceramic tools production. Fused deposition modelling (FDM) applies thermoplastic filled with ceramic material, and case of slurry micro extrusion utilizes moderate degree of viscosity of porcelain slurry. However, they have limitations of

cost and precise performance using high viscosity materials such as bio-materials. Bio 3D printing technology is different promising area where 3D printing method could be applicable to produce artificial scaffold tissue, bone or tooth and organs using bio-materials. Screw type extrusion 3D printing method is designed for using high viscosity material like clay or paste type ink which is a non-Newtonian material; shear stress varies with time ad pressure depending on the difference of shear rate for the material. As shown in Figure 3.5, it consists of screw adjusted to an extruder and monkey bar style head which operates x,y,z axis separately on the table. High viscosity materials could be inserted in the screw, and the head moves along the monkey bar style head which is installed on the ceiling. The printing tip which is connected with screw through hose extrudes linear velocity of discharge of materials within 2% error. [46] The result of using STE printing is described in Figure 3.6.

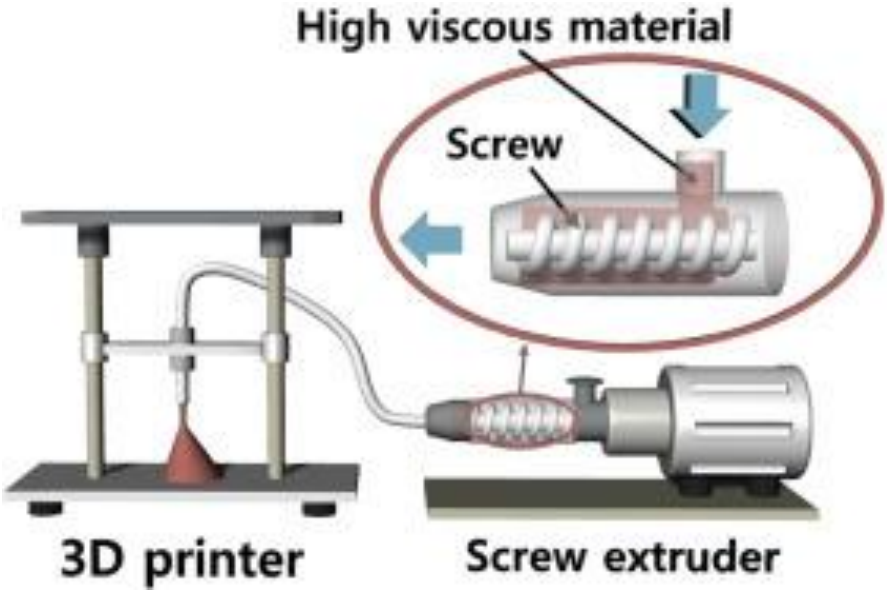


Figure 3.5 Image of Screw type extrusion type of 3D printer which consist of monkey bar style of printing part and screw extruder. [46]

Fig 3.6 Various of 3D printed ceramic structures using STE printing method. A) Korean traditional astronomical building; B) Porcelain; C) Porcelain; D) Vase; E) Vase; F) Cup; G) Fox character; H) Animation Character; I) Taj Mahal [46]

There are advantages of both PTE and STE for application in bio 3D printing. Both methods are available of printing high viscosity material within a few minutes and extracting precise amount of material with sophisticated 3D design. And also, actual extracting amount is similar as mathematical model result which is Hagen Poisoulle (HP) equation; $q = \frac{\pi(P1-P2)r\hat{}4}{8\eta L}$ (q: Flow rate/ P_1-P_2: Pressure difference/ r: Tip radius/ η: Dynamic viscosity/ L: Tube length). HP equation is useful to find printing condition such as extracting amount, printing speed, tube length and tip size. Both printing method make available of finding exact printing condition.

Chapter 4. Materials and Experiment

In this paper, two types of decellularizing and purifying methods are used to produce medically available DE-ECM. The pork adipose part was used for current research, because it contains abundant and useful source of cells such as collagen, peptides and cytokines. [47-48]

Decellularization was performed with enzyme solutions which separate proteins and eliminate lipid or cellular contents. [49-51] To overcome time-consuming limitations of chemical decellularization, the supercritical extraction by carbon dioxide (SC-CO_2) is applied as additional purification process. [52] All of the produced DE-ECM was treated by decellularization with enzymatic solution and supercritical extraction with CO_2 (SC-CO_2). The experimental processes are summarized in Illustration 4.1. In brief, the 3-day, cost-effective and non-toxic procedure involved enzymatic decellularization and supercritical fluid extraction treatment.

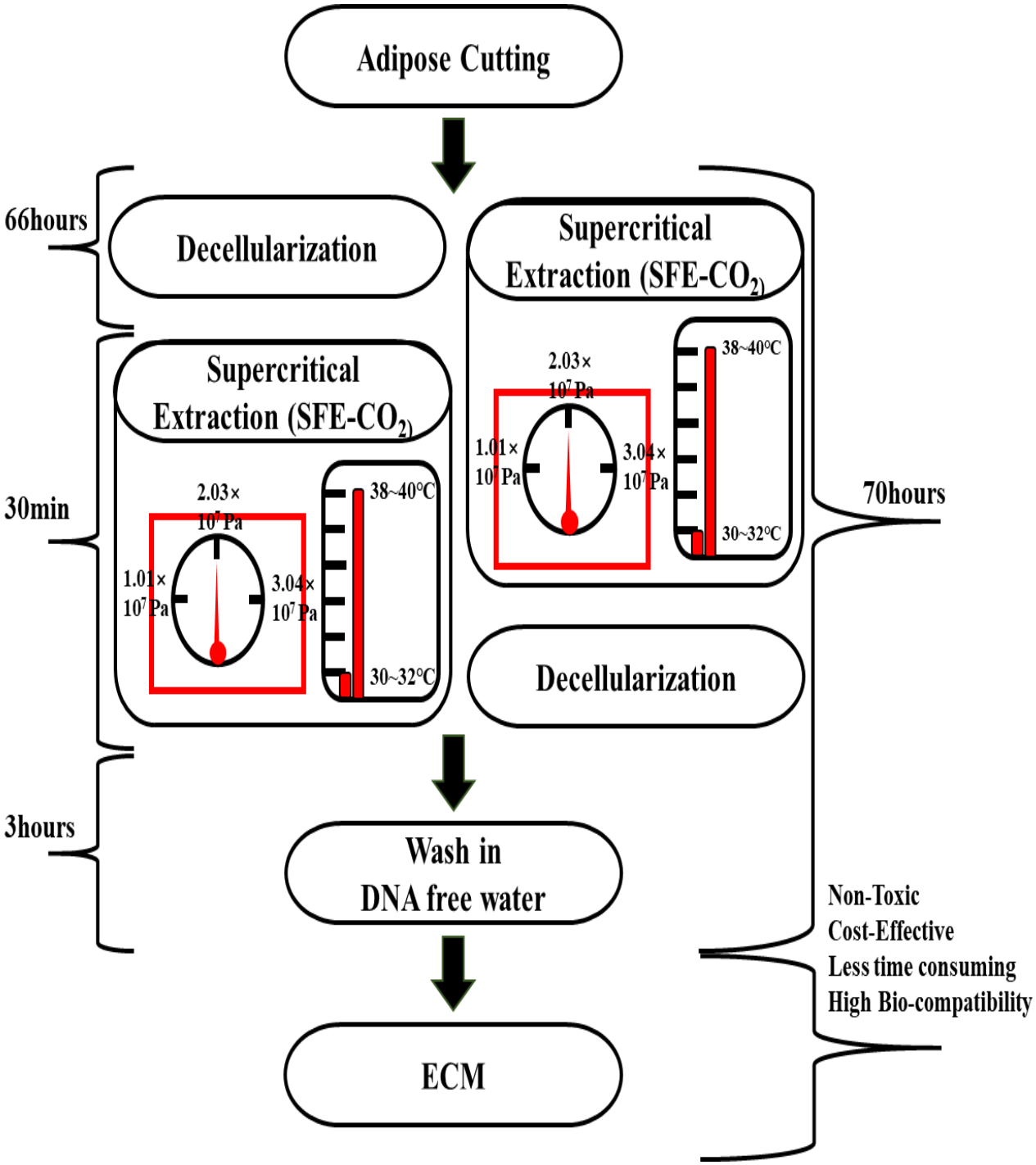


Illustration 4.1: Scheme of the decellularization process and supercritical fluid treatment procedure

Describe in Illustration 4.2, two types of treatment protocols were followed. According to the first treatments method, the supercritical extraction was carried out after enzyme decellularization process (pre-enzyme treatment). According to the second treatments, the enzyme process was carried out after supercritical extraction (pre-supercritical treatment). Different extracting parameters were applied for these experiments, extractions were carried out with different extracting pressure and extracting temperature in order to evaluate the influence of extracting pressure and extracting temperature on the extraction yield, DNA concentration and remaining collagen amount. All the decreased weight data were recorded.

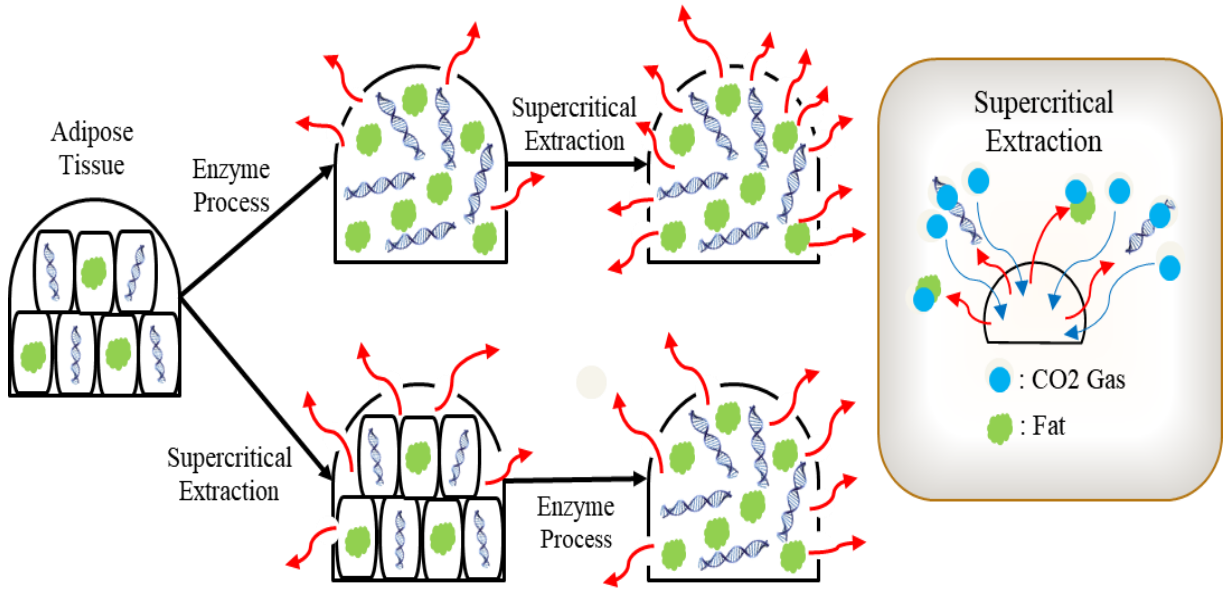


Illustration 4.2 Two types of process which are used in ECM treatment; Pre-Enzyme process and pre-supercritical process.

4.1 Raw adipose tissue Preparation

Adipose section of the pork was used to produce DE-ECM. The raw pork was obtained from local grocery store and kept in the freezer to avoid degradation. The comminution of the raw adipose tissue was harvested by using electric grinder and each tissue was cut into small pieces of different thickness (T1: 1.9×10^{-3}m, T2: 5.7×10^{-3}m and T3: 9.5×10^{-3}m). The raw adipose tissues were prepared in same volume which is 3.8×10^{-7} m^3. It was possible to control volume of all the tissues.

4.2 SC-CO_2 Treatment

The supercritical extractions were carried out to remove DNA components and purify by non-toxic and non-destructive way by the apparatus described in Figure. 4.1 (OCO-LABS, California, CA, USA). The extractor includes an extraction vessel with a maximum operating pressure of 5.07×10^{7}Pa. Pressure within the extraction vessel (described in Figure 4.1 (c).) kept constant by monitoring gas gauge, and the temperature is set by internal sensors Extracted ingredients were collected in flask (Figure 4.1 (d).) by depressurizing after 30 minutes. The extractions were conducted in 30 minutes cycles to give enough time for the supercritical fluid to dissolve the target components. Carbon dioxide gas was utilized as solvent material, which has medically available feature. To eliminate residual extracted ingredient on the surface, tissues were purified by deionized water during 180 minutes. Different extracting parameters were applied in order to compare

extraction efficiency. (temperature: 20±5℃ 30±5℃ and 40±5℃, pressure: 1.01×10^7Pa, 2.03×10^7Pa and 3.04×10^7Pa). Mass of produced tissue was measured before and after the extraction as well. Three times of experiments for each condition were conducted to investigate the effect of extracting variables.

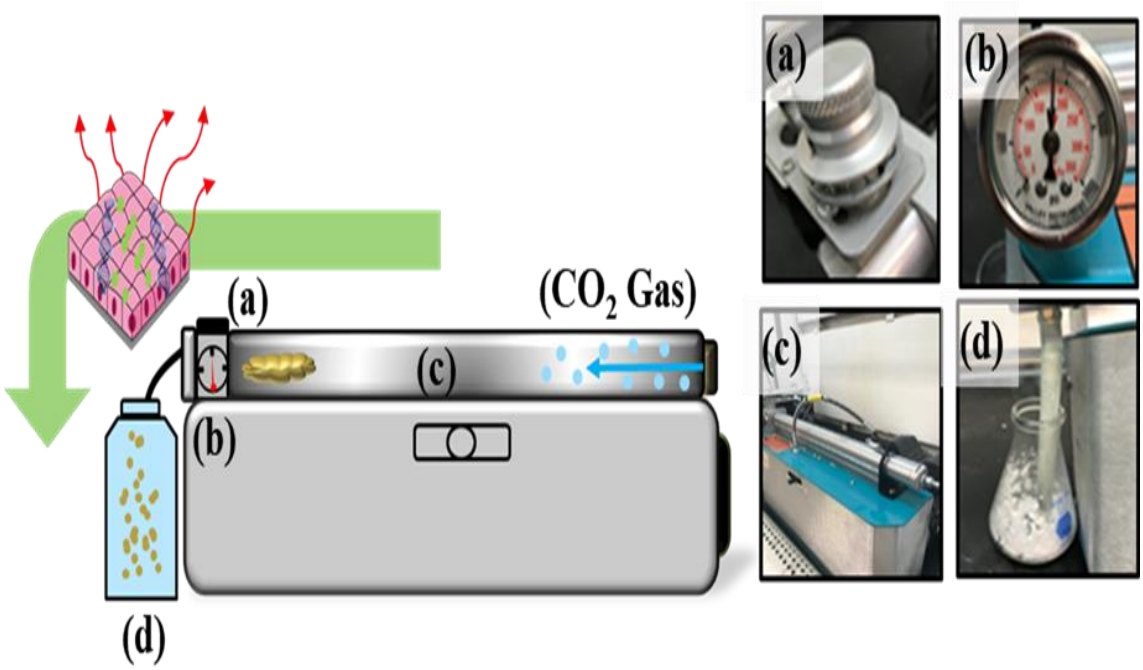


Figure 4.1 Supercritical CO_2 extraction system (OCO-LABS, California, CA, USA): (a) Gas Control Valve; (b) Gas Gauge; (c) Extracting Chamber; (d) Waste collecting flask.

4.3 Decellularization Process (Enzyme Treatment)

Decellularization was processed with 3-day protocol. The adipose tissues were subjected to chemical decellularization with enzymatic solutions and deionized water. The tissues were submerged into the solution which involves 0.05% Trypsin-EDTA and 0.032M deoxycholic acid (Sigma-Aldrich, Missouri, MO, USA) was

included in process. The adipose tissue was thoroughly submerged in solutions mixed with 0.02% Trypsin and deoxycholic acid at 20℃, and incubated overnight for 48hours. This enzymatic digestive solution dissociates protein and adherent cells, and its amount was 5 times of tissue mass and each solution was dissolved with 1:1 ratio. Subsequently, tissues were rinsed through 30mL deionized water. Temperature and neutral state of pH degree (approximately pH 7) were measured to determine whether other accidental chemical reaction is occurred. Following a washing course in deionized water, the tissues were transferred into the solutions containing Triton X-100 (Sigma-Aldrich, Missouri, MO, USA) and deionized water for 6 hours. After this course, the tissue was put in deionized water to rinse during 6 hours again. At the end of the processing, the adipose segments were mechanically compressed for dehydration. All produced tissues were stored under 4℃ after treatment.

4.4 DNA Assay

To investigate the DNA concentration after treatment, Pico-green dsDNA assay (Invitrogen, California, CA, USA) was performed.[53] Since it is a colorimetric procedure, the test materials for analysis must be free from particular materials such as cell debris and insoluble ECM fragments. To expose DNA contents, acid-pepsin digestion was applied to tissues. They were submerged in the pepsin concentration of 0.1mg/ml 0.5M acetic acid, which works as an enzyme activity for eliminating the terminal non-helical telopeptides to release the

ingredients into solution. After pre-experiment to prepare samples for evaluation, DNA concentration were measured by VERSA max Tunable Microplate Reader (Molecular Devices Corporation, California, CA, USA).

4.5 Sircol Collagen Assay (SCA)

The amount of remaining collagen after treatment was evaluated by assaying soluble collagen with a SCA kit (Biocolor Ltd, County Antrim, CA, UK). Briefly, SCA is a colorimetric procedure therefore tissue should be decomposed from cell debris and insoluble ECM fragments. The test samples were homogenized in acetic acid including pepsin per 10mg sample. Each sample was incubated at 4℃ and separated into pellet and supernatant by centrifugation. Sircol dye reagent that binds to collagen was applied to each sample. After centrifugation, alkali reagent was added to pellet. The spectrophotometric inspection was taken at 540nm wavelength on a microplate reader.

4.6 2D/ 3D Printing applying piston type extrusion (PTE)

2D and 3D printing experiment was conducted to research about application of 3D printing technology using DE-ECM material. PTE printing method was selected to increase possibility of extruding high viscosity and hygienic printing environment is essential for bio-usage. 2D and 3D printing was performed for different usage of each structure; 2D design can be applied for medical patch which is used for wound healing, burnt wound and drug patch.; 3D design can be used for

implantable organs or parts and printing biostructure which has high degree of bio-compatibility. For the printing material, DE-ECM after treating with supercritical extraction and enzymatic process was inserted and extruded through piston.

Following the purpose of this experiment, different bio-inks are applied. As shown in Figure 4.2, 100% of DE-ECM bio-ink was prepared through dry-freezing process for medical applicable direction. Moreover, 50% of DE-ECM and 50% of collagen bio-ink was prepared for cosmetic applicable prospection. Both of samples were slurry type of material, and they kept in hygienic environment for experiment.

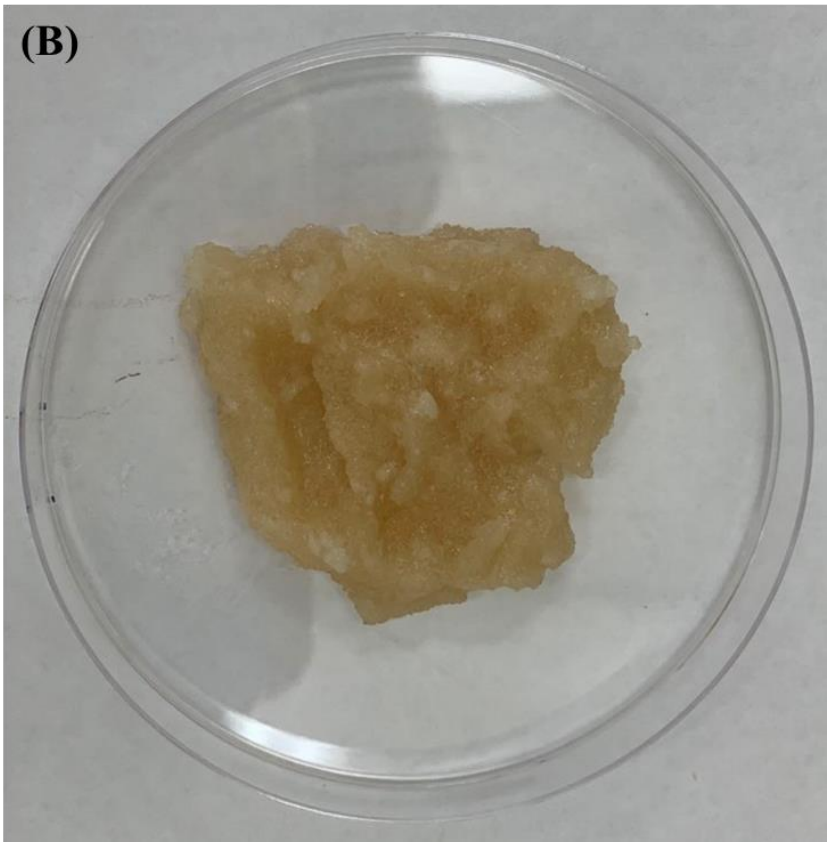


Figure 4.2 Prepared printable materials for PTE printing. A) 100% DE-ECM Bio-ink; B) 50% DE-ECM and 50% of Collagen Bio-ink

Based on information in Section 3.2 Piston type of Extrusion, PTE 3D printers were supported from CPMC laboratory (Center for Printable Material Certification/ University of Texas at El Paso/ TX/ USA). 1mm diameter of tip was used for printing process.

Chapter 5. Result

Non-toxic, cost-effective and short term of DE-ECM processing methodology including supercritical extraction and enzymatic decellularization was established. Adipose tissues were treated with different process protocols (pre-enzyme process or pre-supercritical process). After whole processes, the white color of matrix described in Figure 5.1 was collected as significantly decreased mass. Macroscopically, the size of raw adipose tissue was different following with its thickness because of mechanical compression, but produced DE-ECM had a similar appearance with getting a whiter colouration after entire processes.

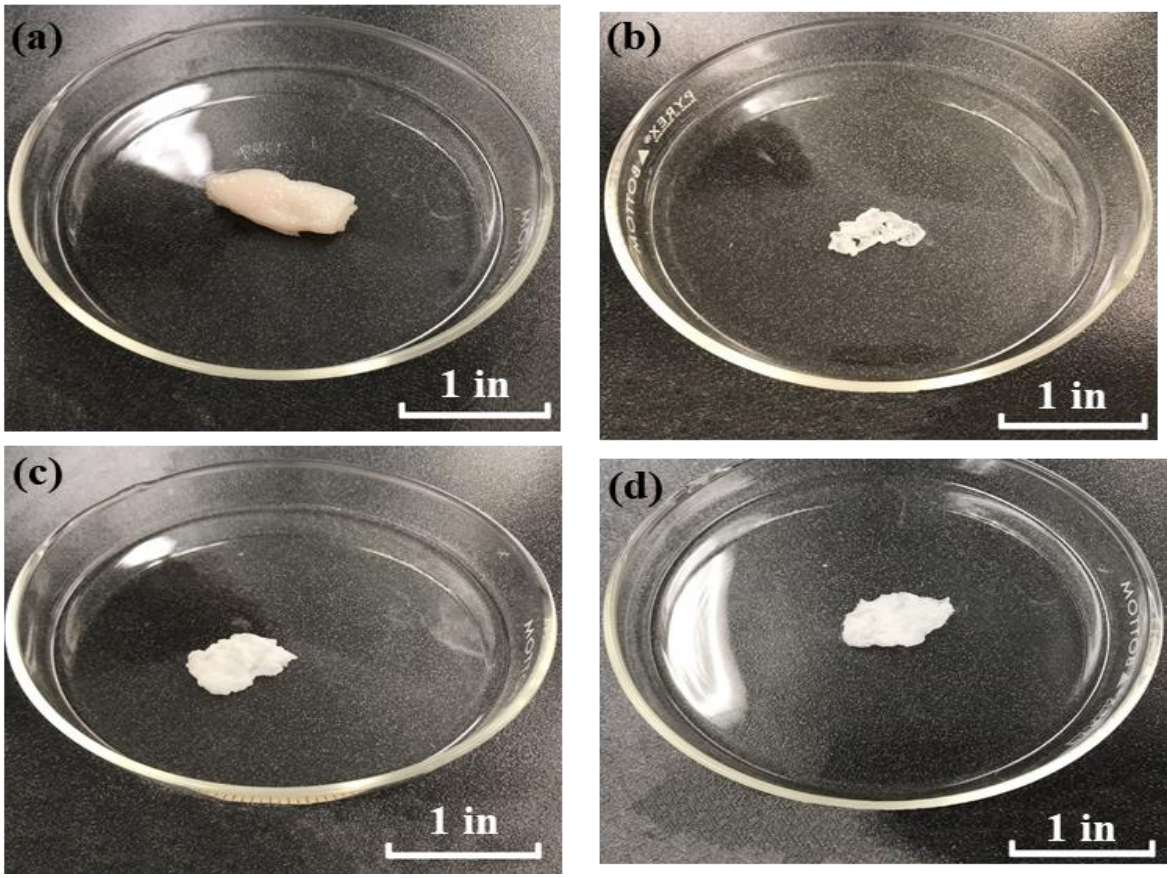
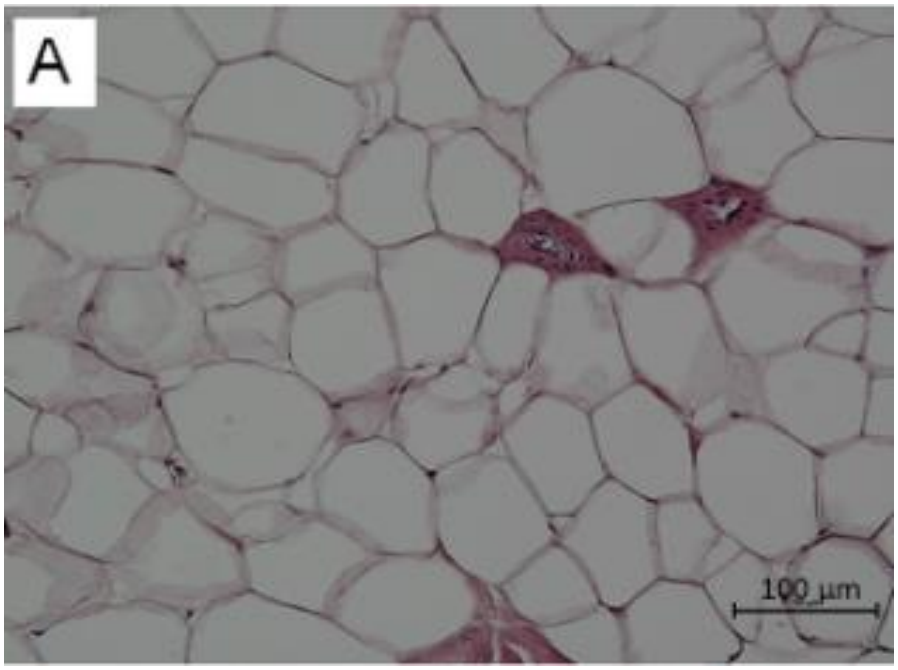


Figure 5.1 Images of tissues before and after treatments. (a) Raw adipose tissue; (b) 1.90×10^{-3}m produced DE-ECM tissue; (c) 5.70×10^{-3}m produced DE-ECM tissue; (d) 9.50×10^{-3}m produced DE-ECM tissue.

5.1 Hematoxylin & Eosin staining (H&E staining)

De-ECM was soaked in 4% paraformaldehyde solution under 4 ºC for an hour, dehydrated state with ethanol, and washed with a xylene/ethanol solution. To investigate DNA content, nucleus and collagen fiber, de-ECM was fixed in paraffin and sectioned as 5 um thickness. Figure 5.2 displays the images of the H&E staining of de-ECM tissue under the microscope. Comparing with native tissue which has well-shaped cell boundary, De-ECM processing with supercritical extraction and enzymatic decellularization process shows destroyed boundary and proving DNA contents or bio-burden are extracted efficiently.



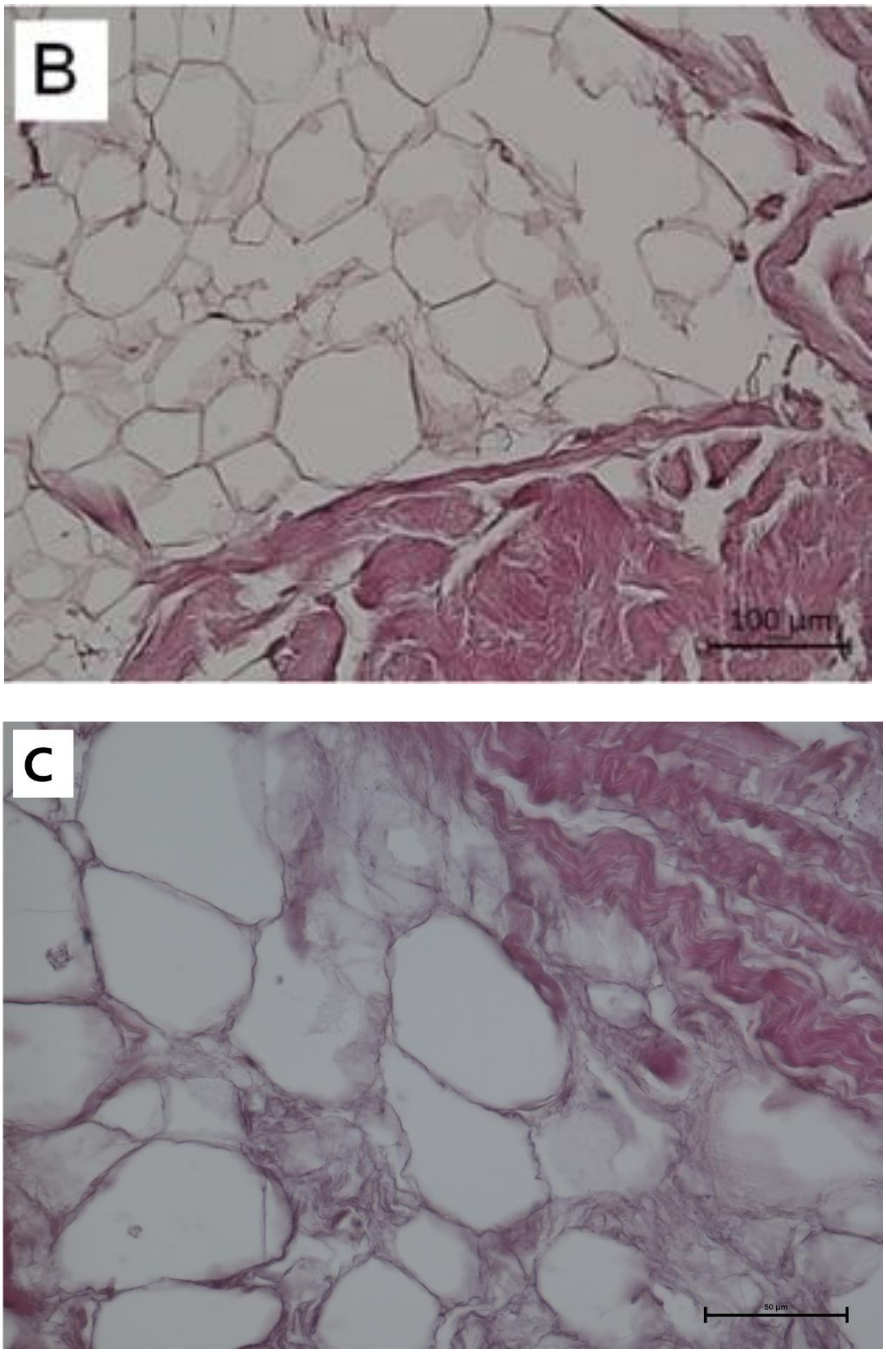


Figure 5.2 Image of hematoxylin and eosin staining test result using microscope. A): Native tissue, B) Decellularized ECM and C) ECM product processed with enzymatic decellularization and supercritical extraction.

5.2 Yield Rate

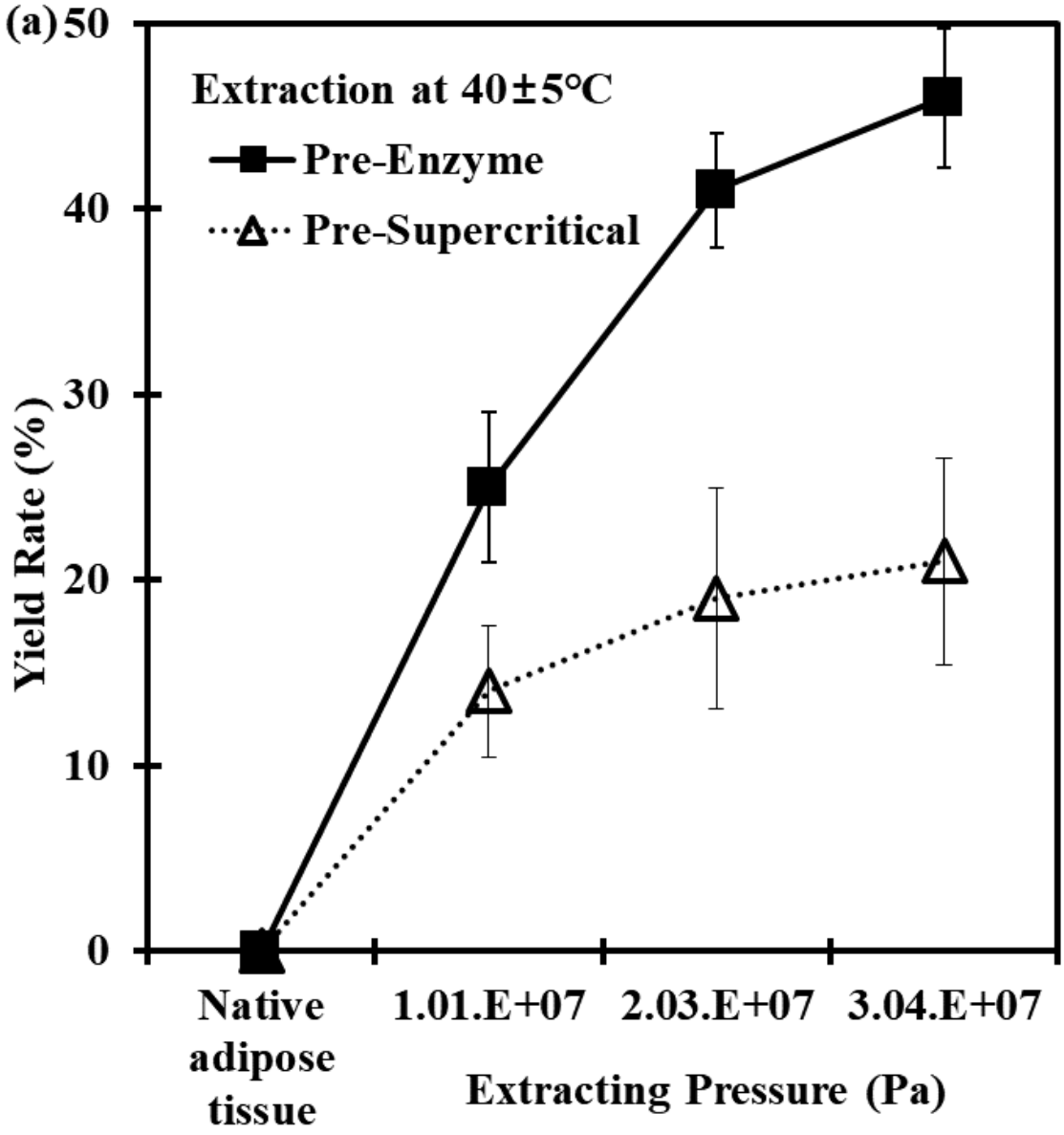
The yield rate of DE-ECM tissue with different extracting parameters compared. (Described in Table 2) Three kinds of adipose tissue are sectioned as different thickness and processed with pre-enzyme process or pre-supercritical process at 3 different pressure and each of them processed at 2 kinds of temperature condition. The weight of before processing and final product was measured. As a result, yield rate depended on the extracting temperature and pressure. Following Figure.5.3, it shows the influence of extracting pressure and temperature on yield rate after treatment. When the experiment temperature was 40±5℃, different yield rate value was figured out according to protocol and extracting pressure (Pre-enzyme process: at 1.01×10^7Pa- 25.13±4.02%, at 2.03×10^7Pa-40.87±3.09 and at 3.04×10^7Pa-45.62±3.75%: Pre-supercritical process: at 1.01×10^7Pa- 14.40±3.54%, at 2.03×10^7Pa-19.31±5.95 and at 3.04×10^7Pa-21.47±5.58%). When the experiment temperature was 30±5℃, the result also showed different yield rate following protocol and pressure (Pre-enzyme process: at 1.01×10^7Pa- 18.34±7.46%, at 2.03×10^7Pa-26.58±4.35 and at 3.04×10^7Pa-34.04±4.04%; Pre-supercritical process: at 1.01×10^7Pa- 4.85±3.04%, at 2.03×10^7Pa-15.49±2.47% and at 3.04×10^7Pa-21.74±8.12%). Yield rate was increased when higher temperature or pressure were applied. Furthermore, pre-enzyme treatment had higher percent of yield rate than the pre-supercritical processed DE-ECM. The maximum yield rate was nearly 46% and

the condition for the extraction is as follow: the extraction pressure of 3.04×10^{7}Pa, the temperature of 40±5℃ and treated through pre-enzyme process.

Table.5.1 Extraction yield of DE-ECM product with different extraction condition. (Pre-enzyme process: PE; Pre-supercritical process: PS) (Adipose tissue 1: Thickness 1.9×10^{-3}m; Adipose tissue 2: Thickness 5.7×10^{-3}m; Adipose tissue 3: Thickness 9.5×10^{-3}m)

Adipose tissue	***Process***	***Pressure (×10⁷ Pa)***	***Temperature (±5 ℃)***	***Weight of original mass (g)***	***Weight of product (g)***
1	PE	1.01	30	0.21	0.17
			40	0.20	0.16
		2.03	30	0.22	0.15
			40	0.25	0.15
		3.04	30	0.23	0.15
			40	0.20	0.09
	PS	1.01	30	0.15	0.15
			40	0.24	0.20
		2.03	30	0.24	0.19
			40	0.19	0.14
		3.04	30	0.20	0.14
			40	0.17	0.12
2	PE	1.01	30	0.47	0.37
			40	0.45	0.34
		2.03	30	0.61	0.47
			40	0.49	0.29
		3.04	30	0.52	0.33
			40	0.64	0.38
	PS	1.01	30	0.44	0.35
			40	0.43	0.38
		2.03	30	0.46	0.39
			40	0.53	0.46
		3.04	30	0.45	0.36
			40	0.42	0.35
3	PE	1.01	30	0.62	0.56
			40	0.62	0.44
		2.03	30	0.60	0.42
			40	0.62	0.35
		3.04	30	0.70	0.48

		40	0.60	0.35
PS	1.01	30	0.67	0.64
		40	0.70	0.60
	2.03	30	0.89	0.80
		40	0.62	0.51
	3.04	30	0.78	0.66
		40	0.67	0.55



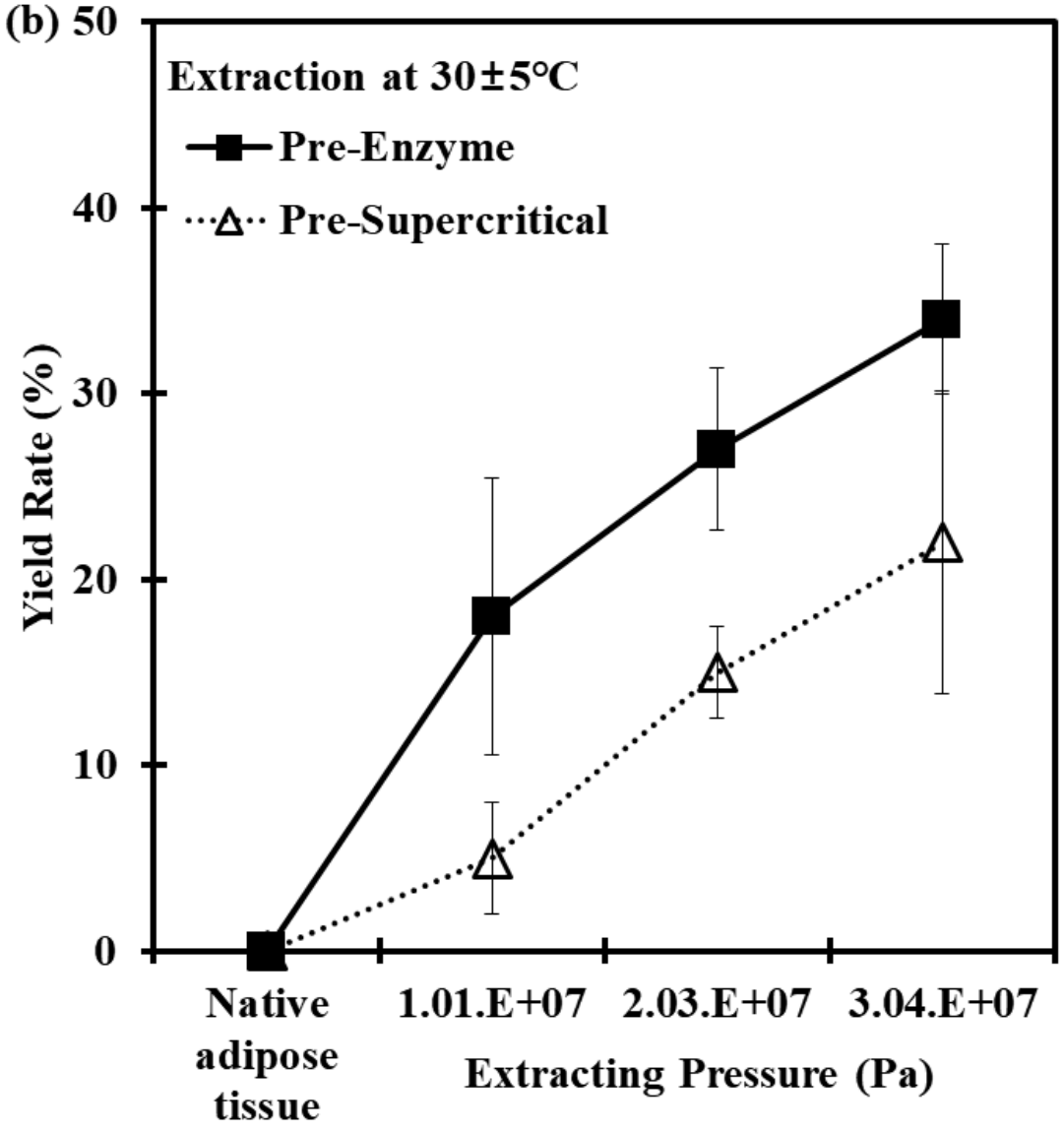
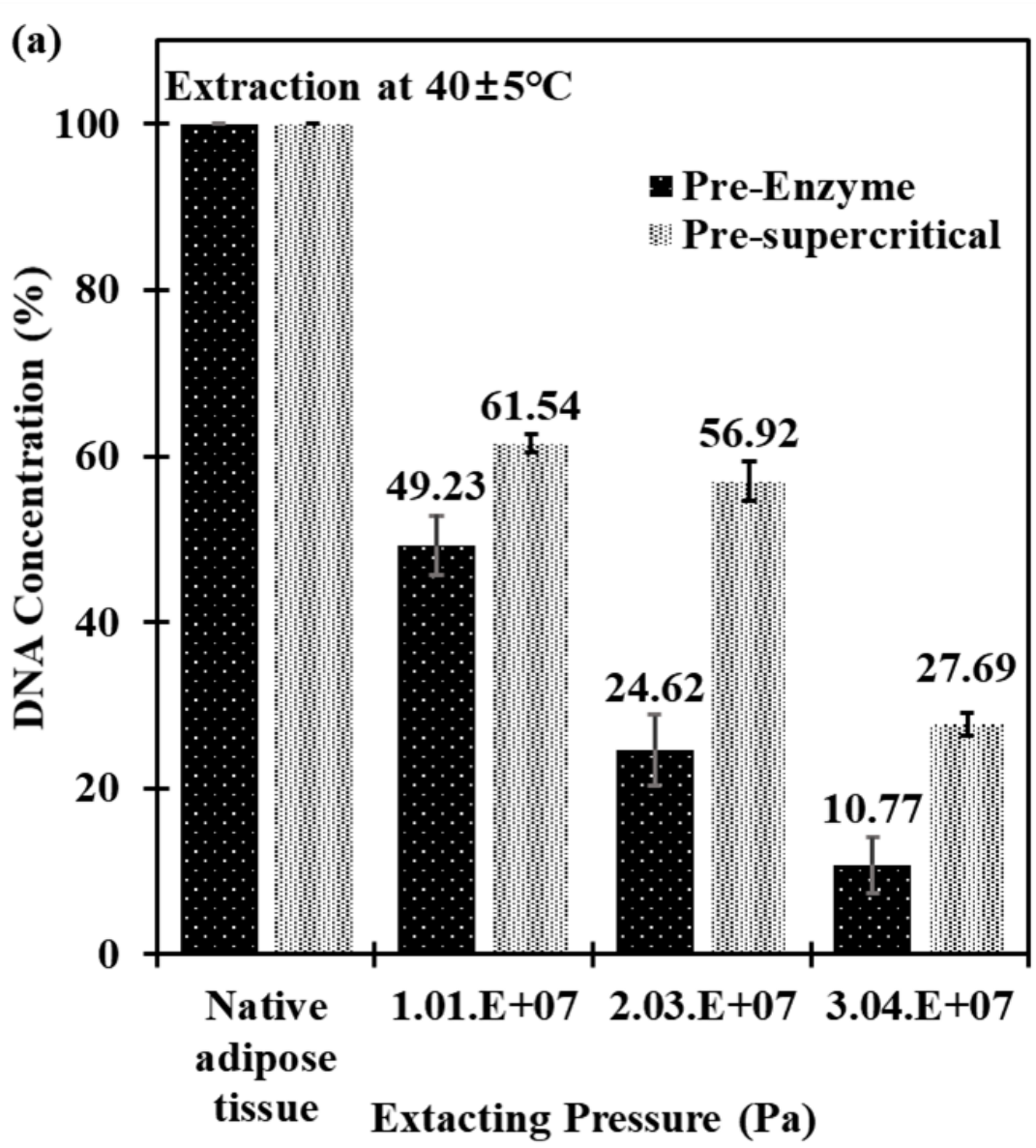


Figure 5.3 Average yield rate (%) following different extracting pressure (1.01×10^7Pa, 2.03×10^7Pa and 3.04×10^7Pa) for different treatment process (Pre-enzyme process and pre-supercritical process). (a) Extraction temperature at 40±5℃.; (b) Extraction temperature at 30±5℃.

5.3 DNA Assay

The DNA contents were compared with native adipose tissue (measured average DNA concentration: 0.065㎍/㎖). Figure 5.4 shows the remaining DNA concentration after decellularization and supercritical extraction for different extracting temperature and pressure. DNA contents were extracted more efficiently when the higher pressure, processing at lower temperature and pre-enzyme process (DNA concentration of produced DE-ECM tissues using pre-enzyme process at 40±5℃: 1.01×10^7Pa- 49.23±3.48%, 2.03×10^7Pa- 24.62±4.35% and 3.04×10^7Pa- 10.77±3.43%; produced DE-ECM tissues using pre-supercritical process at 40±5℃: 1.01×10^7Pa- 61.54±1.10%, 2.03×10^7Pa- 56.92±2.39% and 3.04×10^7Pa- 27.69±1.31%; produced DE-ECM tissues using pre-enzyme process at 30±5℃: 1.01×10^7Pa- 24.62±5.52%, 2.03×10^7Pa- 18.46±3.36% and 3.04×10^7Pa- 9.23±0.38%; produced DE-ECM tissues using pre-supercritical process at 30±5℃: 1.01×10^7Pa- 38.46±6.16%, 2.03×10^7Pa- 30.77±10.22% and 3.04×10^7Pa- 21.54±3.18%). DNA concentration of pre-enzyme tissues extracted at 3.04×10^7Pa and 30±5℃, its DNA removed nearly 90% of DNA. This result indicates that the different kinds of ingredient which contains different value of DNA contents are extracted because of solubility difference.


(a)
Extraction at 40±5°C
Pre-Enzyme
Pre-supercritical
DNA Concentration (%)
100
80
60
40
20
0
49.23
61.54
24.62
56.92
10.77
27.69
Native adipose tissue
1.01.E+07
2.03.E+07
3.04.E+07
Extacting Pressure (Pa)

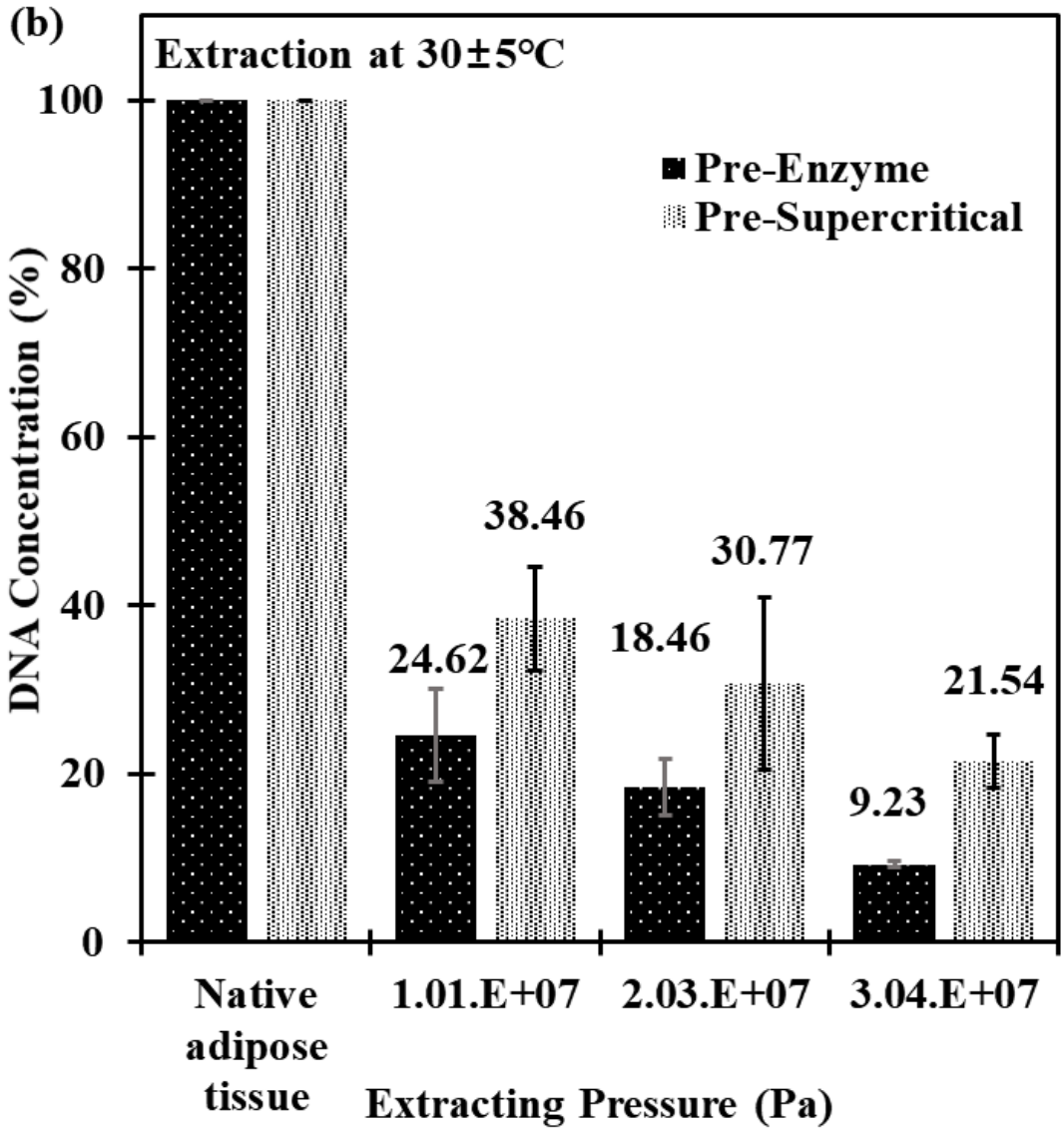


Figure 5.4 Effect of extracting pressure and temperature on remaining DNA concentration after treatment. Amount of remaining DNA concentration was compared between raw adipose tissue prior process with produced ECM tissue. The adipose samples were subjected to various extracting pressure (1.01×10^7Pa, 2.03×10^7Pa and 3.04×10^7Pa), extracting temperature and different processing

methods. (a): DE-ECM samples extracted at 40±5℃.; (b): DE-ECM samples extracted at 30±5℃.

5.4 Collagen Assay

The influence of the extracting temperature and pressure on the collagen concentration is presented in Figure 5.5. Produced DE-ECM tissues were compared by amount of collagen with original native adipose tissue (contain about 0.90±0.03 μg/㎖). Figure 5.5 indicates that collagen value steadily decreased with increasing of extracting pressure from 1.01×10^7Pa to 3.04×10^7Pa. Moreover, produced DE-ECM tissues subjected to extraction at the lowest temperature range (20±5℃) remained more collagen concentration than the other tissues processed under higher temperature range (30±5℃ and 40±5℃). At the highest temperature range, produced DE-ECM tissues contain collagen of 24.88±2.44% (extracted at 1.01×10^7Pa), 14.08±2.10% (extracted at 2.03×10^7Pa) and 8.11±6.87% (extracted at 3.04×10^7Pa). At medium temperature range (30±5℃), produced DE-ECM tissues contain collagen of 33.97±0.67% (extracted at 1.01×10^7Pa), 22.59±0.79% (extracted at 2.03×10^7Pa) and 17.72±4.86% (extracted at 3.04×10^7Pa). At the lowest temperature range (20±5℃), produced DE-ECM tissues contain collagen of 75.74±1.83% (extracted at 1.01×10^7Pa), 43.17± 3.57% (extracted at 2.03×10^7Pa) and 32.11±4.78% (extracted at 3.04×10^7Pa). The products produced at 20±5℃ were

compared with products produced at other temperature to show effect of extracting temperature to the remain collagen amount.

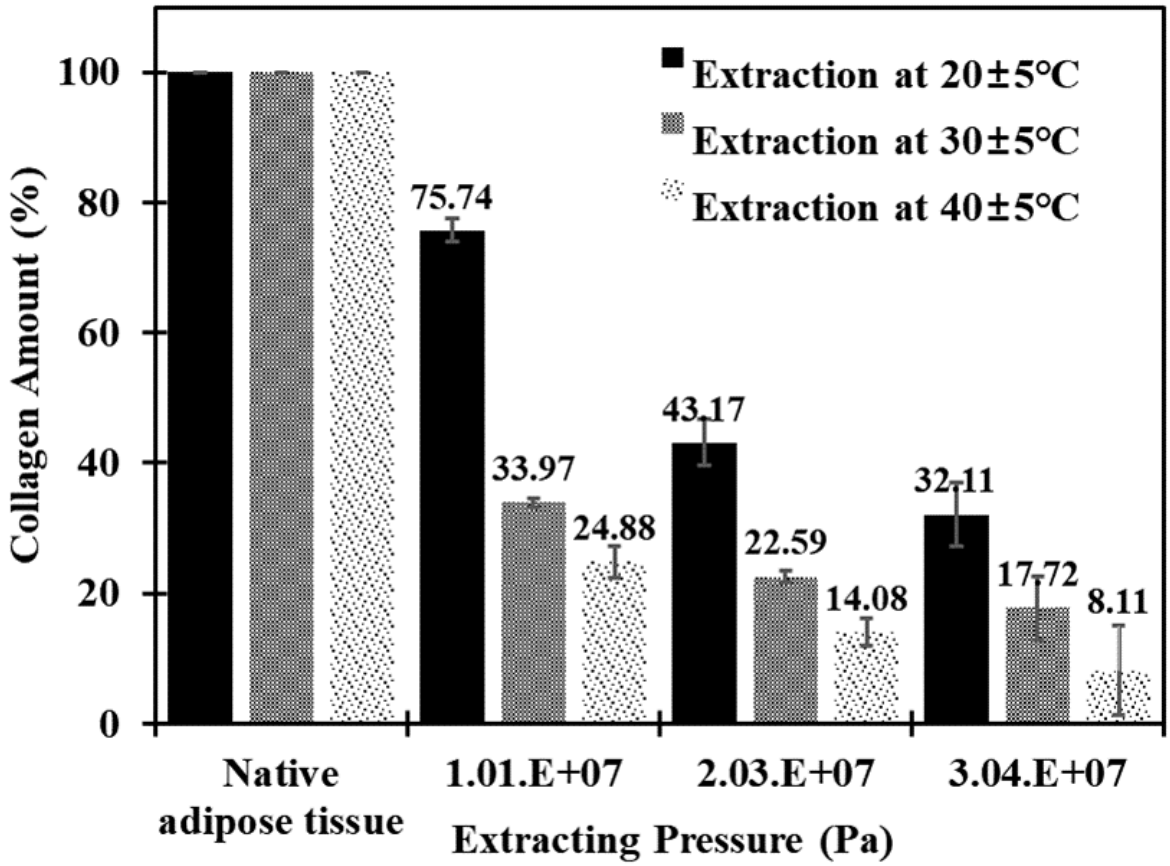


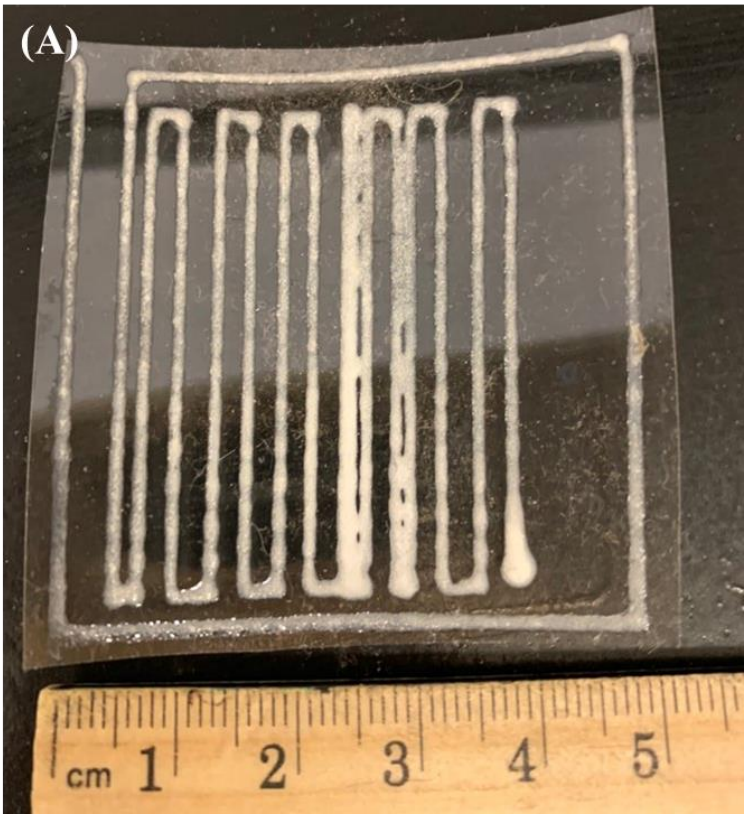
Figure 5.5 Remaining collagen concentration in produced DE-ECM tissues. DE-ECM tissues were processed with pre-enzyme process, but different extracting temperature (20±5℃, 30±5℃ and 40±5℃) and different pressure (1.01×10^7Pa, 2.03×10^7Pa and 3.04×10^7Pa) was applied.

5.5 ECM 3D Printing

To open up new application of DE-ECM in 3D printing area, PTE type of 3D printing method was utilized in further experiment. Advance treatment process

which is supercritical extraction and enzymatic chemical process assist to make printable state material. The material became enough slurry, and kept it in the hygienic environment. Specifically, tooth 3D structural design and 2D patch pattern design were used as draft.

As a result, Figure 5.6 shows that DE-ECM was printed as 2D pattern which can be applied in medical patch application. In printing process, 1mm diameter of tip was used to extrude material. (Figure 5.6 (A)) Moreover, DE-ECM went through material processing steps, it was convenient to print as preferable shapes. To evaluate the various usage of 3D printing technology with DE-ECM, collagen added DE-ECM bio-ink was printed in Figure 5.6 (B). It proves that DE-ECM can be applied in medical area as well as cosmetic area. Whole 2D printing was processed under controllable parameters such as printing speed, extruding pressure and substrate material. DE-ECM 2D printing was successfully conducted as expectation.


(A)
cm 1 2 3 4 5

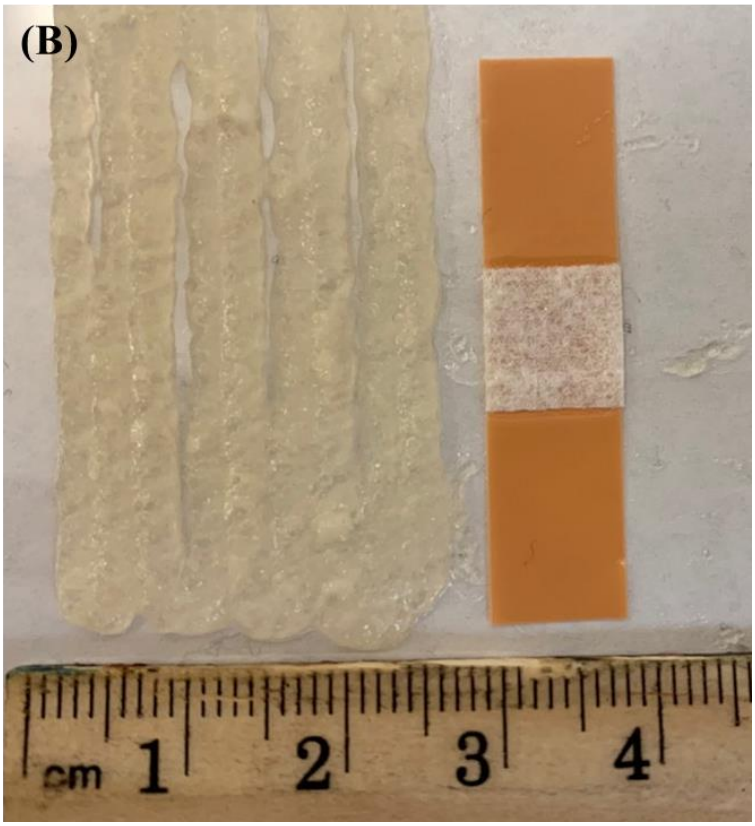

(B)
cm 1 2 3 4

Figure 5.6 Images of 2D printed DE-ECM pattern for medical patch or cosmetic application. A) 100% DE-ECM used medical patch type sample; B) 50% DE-ECM and 50% Collagen used cosmetic applicable sample.

Furthermore, printing 3D structures using DE-ECM was attempted, but it was impossible due to the lack of the material. Thus, protein paste was replaced of DE-ECM to print tooth 3D structure, since it has similar printable state with DE-ECM. Same printing condition was applied in this trial. As shown in Figure 5.7, tooth 3D printing using protein paste was successful applying PTE type of 3D printing. 1mm of tip was utilized to print small size of structure. Through this trial, 3D printing using DE-ECM was assumed that it would be printed successfully, and it contributes to development of bio 3D printing and printing super bio-compatible 3D structure for human body system.

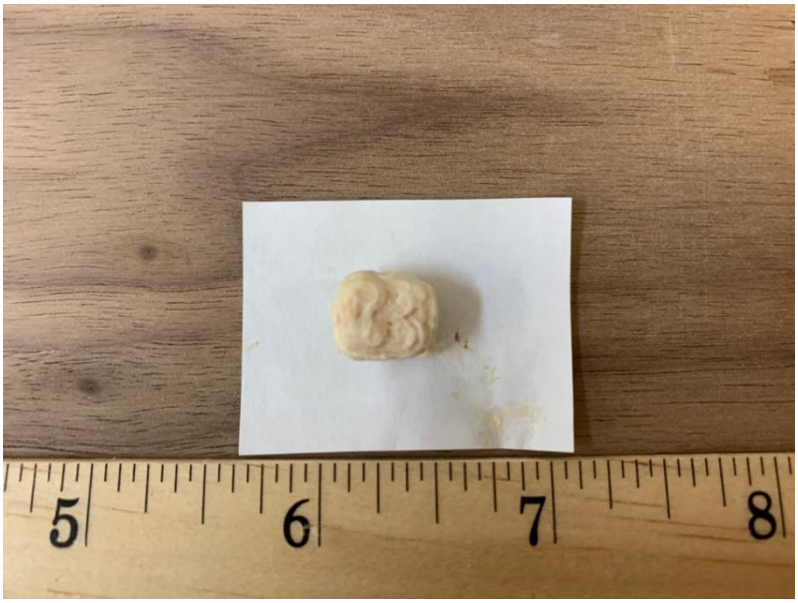


Figure 5.7 Image of 3D printed tooth structure using protein paste applying PTE printing method. (1mm tip was used in this trial)

Chapter 6. Discussion

The purpose of this thesis was to produce bio-compatible DE-ECM by cost-effective, short term and non-toxic process applying on adipose tissue. The supercritical fluid extraction with carbon dioxide (SC-CO2) and decellularization with enzymatic solutions were applied to treat DE-ECM. Produced DE-ECM matrix contains biological growth factor (collagen, elastin, glycosaminoglycans and cytokine) without past DNA information. The combination of SC-CO2 and enzyme treatment produced DE-ECM samples in 70 hours cost-effectively, and also demonstrated the satisfactory elimination of DNA contents. Supercritical extraction eliminates DNA contents applying the concept of polarity transformation from liquid and gas state to supercritical state. At supercritical state, carbon dioxide becomes polar substance from non-polar at normal state. Thus, supercritical state of carbon dioxide dissolves with non-polar ingredient in adipose tissue. To increase the solubility of solvent, different experiment parameters are investigated on extracting pressure and temperature by increasing the solvent density. [54-55] But in different aspect, the temperature gives severe effect to the collagen composition of DE-ECM since it is vulnerable from high temperature. [56-57] DNA analysis showed that extraction using pre-enzyme process at 30±5℃ and 3.04×10^{7}Pa was the optimal condition to decrease DNA concentration, that is, DNA concentration was decreased from 0.065 ㎍/㎖ of original adipose to 0.006㎍/㎖ of produced DE-ECM. The

experiment result proves that previous research which has done in CPMC about DE-ECM kinetics. [58] The diffusion of the reactant is heterogeneous chemical reaction kinetics between solid and liquid interface and mass transfer contributions. As shown in Figure 6.1, the diffusion of reactant penetrates adipose tissue surface through channels, and make chemical reaction inside the cell. And diffusion-out of products through surface. Thus, pre-enzyme process makes more critical purification efficiency by creating channels in advance of supercritical extraction.

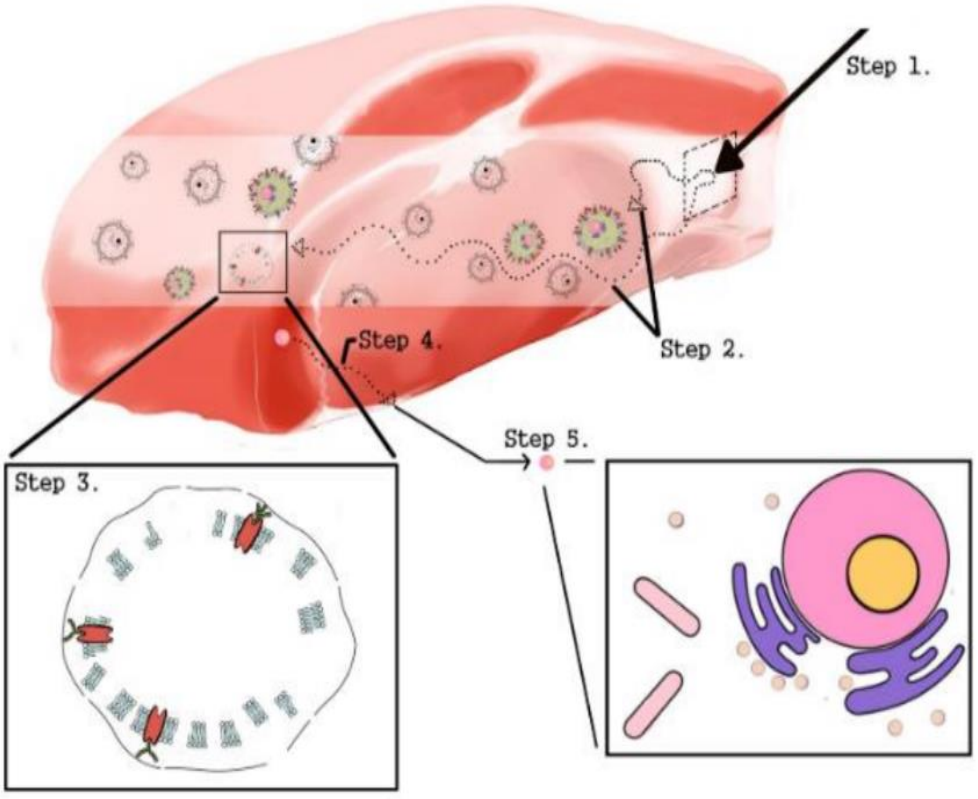


Figure 6.1 Diagram of diffusion process during enzyme reaction in adipose tissue. Step 1: Diffusion of reactant from surface. Step 2: Penetrating through channels.

Step 3: Chemical Reaction Step 4: Diffusion out through channel Step 5: Extraction. [58]

Collagen is a critical element in pharmaceutical and bio-medical industries and take important part in formation of tissues and organs because of ultimate biocompatibility with other immune system. The collagen analysis result shows that DE-ECM applying optimal extraction condition at pre-enzyme process at 30±5℃ and 3.04×10^{7}Pa was evaluated as 0.16 ㎍/㎖; 17.72%. Thus, high extracting pressure and lower extracting temperature extraction is the effective variables to produce bio-compatible DE-ECM. Application of supercritical fluid extraction and enzyme decellularization process proves that medically available production of DE-ECM, which is cost-effective, non-toxic and less time consuming is possible without any unnecessary chemicals and damage of the bio-structure. Moreover, the technology of controlling extracting pressure and extracting temperature provides optimal bio-compatibility, and it has a potential usage of supercritical fluid extraction in the DE-ECM mass production from the feature of rapid process. This experiment evaluated the feasibility of utilizing supercritical extraction technology with tissue and it is proven that it is successful.

To improve the DE-ECM efficiency in tissue engineering and medical area or any other applicable area, PTE printing technology was applied as final product formation step and proved its flexibility at tissue engineering. It could be evaluated

printable ability using high viscosity of DE-ECM bio-ink as 2D pattern which can applicable in medical patch area such as nicotine patch or vitamin patch and cosmetic sheet containing nutrition for human skin. Furthermore, using similar condition of material, actual human tooth part 3D structure was also successfully printed as expectation, and checked the possibility of 3D structure printing using DE-ECM. As a result, PTE printing technology is optimal process for bio-printing since it has strong flexibility, hygienic process, cost-effective, less time consuming and customizable function in printer. This thesis shows infinite possibility of DE-ECM as bio-ink, medical area, drug delivery system and cosmetic area. Moreover, this research solves the problem of surgery in current society and provide new opportunity in the area of non-surgery therapeutic method.

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Vita

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