A Thesis for the Degree of Master of Engineering

Development of a cell-laden thermosensitive chitosan bioink for 3D bioprinting

3D 바이오프린팅을 위한 세포가 포함된 열감응성 키토산 바이오잉크 개발

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구 종 범

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Abstract

3D bioprinting is a technology used to deposit cell-laden biomaterials for the construction of complex tissue. For 3D bioprinting of cell-laden construct, many materials have been developed such as alginate, fibrin and gelatin. However, most of these hydrogels are chemically bound using crosslinkers which can cause some problems in cytotoxicity and cell viability. On the other hand, thermosensitive hydrogels are physically cross-linked by non-covalent interaction without crosslinker, facilitating stable cytotoxicity and cell viability. The typical examples of thermosensitive hydrogels are methyl cellulose, hydroxypropyl cellulose, N-isopropylacrylamide(NiPAAM), poly(ethylene glycol)/poly(propylene glycol)/poly(ethylene glycol) (PEG-PPG-PEG) and poly(ethylene glycol)/poly(lactic acid-co-glycolic acid) (PEG/PLGA). Chitosan, which has non-toxic, biocompatible and biodegradable polysaccharide composed of glucosamine and derived by deacetylation of chitin, can be used as thermosensitive hydrogels. In

chitosan thermosensitive hydrogels, gelling agent and solvent are the key factors for sol-gel transition. Yet, there is no study to investigate the comparison among these gelling agents or solvent for bioink. Therefore, the purpose of this study was to develop and compare bioinks by chitosan hydrogels for 3D bioprinting. Solvent did not affect the gel shape and gelation time. However, acetic acid had good biocompatibility compared with lactic acid and hydrochloric acid. Gelling agent was more important factor than solvent. Gel shape and gelation time were different among gelling agent. $NaHCO_3$ had greater growth rate of the storage modulus (G′) and more irregular porous structure than β-GP and K_2HPO_4. From the water soluble tetrazolium salt (WST) and live and dead assay data, it was proven $NaHCO_3$ had good effect for cell adhesion. In cell-laden construct, there was no big difference among gelling agent. However, $NaHCO_3$ had good printability compared with β-GP and K_2HPO_4.

Key words: 3D printing, Bioprinting, bioink, hydrogel, chtiosan
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List of Terms and Abbreviations

HCl	Hydrochloric acid
LA	Lactic acid
AA	Acetic acid
β -GP	β-glycerophosphate
K_2HPO_4	Potassium phosphate
$NaHCO_3$	Sodium bicarbonate
CS	Chitosan
FE-SEM	Field emission scanning electron scanning microscopy
WST	Water soluble tetrazolium salt
FBS	Fetal bovine serum
SAS	Statistical analysis system
PDLSC	Periodontal ligament stem cell
PCL	Polycaprolactone

1. Introduction

3D printing, also known as additive manufacturing, can be used to create a solid 3D structure through a layer-by-layer process. A few years ago, 3D printing is emerging as a powerful tool in many areas such as education, aerospace, consumer products, arts, food industry and manufacturing. To date, biocompatible materials of 3D printing is one of the most important research area and tools for tissue engineering and regenerative medicine. In the early years of 3D printing in tissue engineering and regenerative medicine field, 3D printing was applied to fabricate 3D scaffolds without cells. However, the development of 3D printing technology has led to form complex tissue constructs through deposit of cells and hydrogels in a layer-by-layer process that called 3D bioprinting. Cell and hydrogels have been formulated as bioink that must be printable and not toxic [1-3].

The main technologies used for 3D bioprining are inkjet bioprinting, extrusion bioprinting, laser-assisted bioprinting, digital light processing (DLP)-bioprinting [2]. Inkjet bioprinting is the commonly used type of conventional 2D inkjet printing and was the first bioprinting technology. Inkjet bioprinters use thermal or piezoelectric actuator to eject droplets of liquid onto a substrate. The advantages of inkjet bioprinters include relatively low cast, high-speed printing, but there is a risk of exposing cells and materials to thermal and mechanical stress and nozzle clogging. Extrusion bioprinter commonly has pneumatic or mechanical (piston or screw) dispensing systems, which is deposited onto a substrate. Almost commercial bioprinters are based on extrusion type. Extrusion bioprinters have a wide range of

viscosities of hydrogels that are compatibility with photo, chemical and thermal crosslinkable properties. Cell viability of extrusion bioprinter is lower than inkjet bioprinter, but extrusion bioprinting is able to deposit very high cell densities at a reasonable cost. Laser-assisted bioprinting is less common than inkjet or microextrusion bioprinting. However, laser-assisted bioprinter is increasingly being used for cell printing, tissue engineering and regenerative medicine applications. Laser-assisted bioprinter consist of a 'ribbon' structure that contain energy absorbing layer on the top and layer of bioink liquid solution suspended on the bottom. During printing, a focused laser pulses on the absorbing layer of the ribbon to create a high-pressure bubble that stimulate the bioink toward the collector substrate. In laser-assisted bioprinter, there is no mechanical stress to the cells, which results in high cell viability and nozzle-free device systems that can solve the nozzle clogging problem. Despite these advantages, there are few laser-assisted bioprinter due to high cost and complex device systems. DLP bioprinter developed by the dynamic optical projection stereolithography (DOPsL) platform that modulate the UV light and project an optical pattern. Compared to the other 3D printer, the DLP bioprinter is parallel process by projecting the entire plane of optical pattern onto the photopolymer solution. DLP bioprinter has high printing speed, high resolution and cell viability, due to these properties, recently DLP bioprinter are regarded as an attractive biprinter [1, 2].

In the 3D bioprinting, bioink such as the hydrogel or biomaterials can be printed onto substrates for cell attachments. Cells can be mixed with bioink such as collagen,

gelatin, matrigel, agarose, and alginate [4]. Materials used in the bioprinting field are based on naturally derived polymers or synthetic polymers. These materials must have printability, biocompatibility, nontoxic degradation kinetics and byproducts, suitable structural and mechanical properties for printing and material biomimicry [1]. However, most of the hydrogels are using crosslinker which are some problems in cell viability [5]. On the other hand, thermosensitive hydrogels can physically crosslinked without crosslinker that can overcome some problems in cell viability.

Chitosan, which have been widely used in tissue engineering due to its biocompatibility and osteoconductivity, can be used as thermosensitive hydrogels. In chitosan thermosensitive hydrogels, gelling agent and solvent play an important role for sol-gel transition [7]. One research suggested that hydrochloric acid (HCl), lactic acid (LA) and acetic acid (AA) are the suitable solvent for chitosan thermosensitive hydrogels [8]. Some research are reported as the gelling agent such as β-glycerophosphate (β-GP), potassium phosphate (K_2HPO_4), and sodium bicarbonate ($NaHCO_3$) [9, 10]. However, there is no study to compare among these gelling agents or solvent for 3D printing. Therefore, this thesis was attempt to compare the bioinks by gelling agent and solvent and develop the suitable bioink for 3D bioprinting.

2. Objectives

The objectives of this research was to develop three bioinks by chitosan hydrogels for 3D bioprinting and to compare the suitability and potential ability of the developed chitosan hydrogel as a bioink.

1) Fabrication and characterization bioink with chitosan.

2) Comparison of the chitosan hydrogels by solvent and gelling agent

3) Investigation of the printability of the chitosan bioink

4) Evaluation of the cell viability after the printing

5) Verification of the cell-laden construct suitable for the long term cell culture

3. Literature Review

3.1. Chitosan scaffold

Chitosan is biodegradable polysaccharide composed of glucosamine derived from the partial deacetylation of the chitin. Chitosan is very abundant biopolymer and is commonly found in invertebrates. Deacetylation degree (DD) of chitosan indicate that the number of amino groups which can be calculated as the ratio of D-glucosamine to the sum of D-glucosamine and N-acetyl D-glucosamine. Chitosan has intrinsic properties such as antimicrobial, antifungal, mucoadhesive, analgesic, haemostatic properties. For that reasons, chitosan is commonly used in the tissue engineering field as 3D scaffolds (hydrogels or sponges) or 2D scaffolds (films or fibers) [11].

3.1.1. 3D scaffold

Hydrogel and sponge are the general type of chitosan 3D scaffold in tissue engineering. Hydrogel has 3D polymer networks that can absorb large water content. These make more compatible with living cells and minimize the damage to the surrounding tissue and cells. Chemically cross-linked and physically cross-linked hydrogels are the main types of chitosan hydrogels. Chemically cross-linked hydrogels have the covalent bond between polymer chains that can more stable than physically cross-linked hydrogels. However, it requires the chemical agent (crosslinker) that might be toxic for the cell and tissue. On the other hand, physically

cross-linked hydrogels can be formed by non-covalent interactions such as electrostatic interactions, hydrophobic interactions or hydrogen bonding without chemical agent (crosslinker). This process is based on the neutralization of chitosan solution to pH value exceeding 6.2 and thus the inhibition of the repulsion of chitosan solution and then leads to the formation of a hydrogel. [7, 11]. The neutralization of chitosan solution by a polyol salt such as β-glycerophosphate (β-GP), potassium phosphate (K_2HPO_4) and Sodium bicarbonate ($NaHCO_3$) [9, 10, 12]. Physically cross-linked chitosan hydrogel is the injectable in situ thermosensitive hydrogels that are suitable to biomedical field including drug delivery and tissue engineering [7].

Chitosan sponges are the solid structures that can absorb high amount of water because of the porosity. In addition, Chitosan sponges have good cell interation and soft and flexible [11]. These sponges are mainly obtained by freeze dried methods. Chitosan sponges are mainly applied to wound healing and used as filling materials in bone tissue engineering. For example, chitosan/tricalcium phosphate (TCP) and chitosan/collagen sponges were used as scaffolds for bone regeneration [13]. Chitosan–ZnO composite sponges have antibacterial and haemostatic activities, using their potential healing in wound dressing application [14]. Cross-linked chitosan sponge was also reported [15] that it can be suitable to the loading drugs such as antibiotics and controlled release the antibiotics without decrease activity.

3.1.2. 2D scaffold

In skin tissue regeneration field, 2D scaffold such as films and porous nanofiber membranes more suitable properties for skin repair.[11]

Chitosan films typically prepared by wet casting from chitosan solution and used as wound dressing. So, there are many research reported that improve the properties of chitosan films. Plasma treatments of chitosan film improve the biocompatibility due to increase the film surface roughness [16]. Chitosan films were prepared with dialdehyde starch as a crosslinking agent that can improved mechanical and water swelling properties [17]. Inorganic particles such as silver zinc oxide nanoparticles increase antibacterials activity, and thus improved the wound healing properties [14, 18]. To increase the efficient of wound healing properties, multilayer coatings and nanoscale thin films have been investigated that various research. Langmuir–Blodgett (LB) and the layer-by-layer (LBL) deposition techniques allow to modify the chitosan films properties such as thickness, composition, morphology and roughness [19].

Electrospinning is the one of the good candidate methods for producing chitosan nanofiber. Trifluoroacetic acid (TFA) and dichloromethane (DCM) mixtures solution can produce the chitosan nanofiber. The amino groups of the chitosan create the salts with TFA that can be help to electrospinning [20]. Moreover, electrospun chitosan nanofibers are crosslinked using by glutaraldehyde vapor method. This method suggested that the brittleness of chitosan fibers was increased and decreased the chitosan fiber sliding [21].

3.2. Thermosensitive hydrogel

Hydrogels are formed by chemical or physical crosslinking that absorb amount of water without degration of their shape. In the research application, hydrogels typically used for to drug delivery and biomedical devices because of their biocompatible properties and easy control. Recently, many researchers are investigated that in situ hydrogels which are more suitable hydrogels for drug deliver and tissue engineering. In situ hydrogels can transform into gels by environment change such as temperature and pH (sol–gel transition) without chemical agent or external stimulation. Thus, in situ hydrogels are easily prepared and have high biocompatibility in in vivo environment [6].

3.2.1. Natural polymer

Chitosan, methylcellulose and hydroxypropyl cellulose are typical examples of the natural polymer based thermosensitive hydrogels.

Chitosan and glycerolphosphate disodium salt mixture solution can be a chitosan thermosensitive hydrogel at the neutral pH [12]. Many researches about chitosan hydrogels are focused on the bone regeneration. The chitosan hydrogel maintained the bioactivity of loaded bone protein and can be released bone protein. The cell viability of various cell such as chondrocytes encapsulated in the gel was above 80% [6].

Cellulose is not water-soluble polymer, but introducing hydrophilic groups such as methyl or hydroxypropyl groups, cellulose is soluble in water. The

thermosensitive mechanism of methylcellulose and hydroxypropyl cellulose are affected by concentration and balance of hydrophilic and hydrophobic group in polymer. These materials are commonly used as drug and cells delivery device in biological field [25, 26].

3.2.2. Synthetic polymer

N-isopropylacrylamide (NiPAAM) copolymers, poly (ethylene oxide)/poly (propylene oxide)/poly (ethylene oxide) (PEO/PPO/PEO) block copolymers, and Poly (ethylene glycol-b-L-lactic acid-b-ethylene glycol) (PEG–PLLA–PEG) block copolymers are commonly used as synthetic polymer based thermosensitive hydrogel.

NiPAAM solution can be a gel-like shape above 32℃ that is caused by the dehydration of the hydrophobic isopropyl groups during the coil-to-globule transition [6]. To modify the NiPAAM topology, NiPAAM incorporated with oligoNiPAAM [22] or PEG [23]. One research was found that a solution of high-molecular-weight NiPAAM/acrylic acid copolymer showed that reversible gelation above 4 wt%, without noticeable side effects [24].

The PEO/PPO/PEO block copolymers also known as Pluronic (BASF) or Poloxamer (ICI) are commercially available biomaterials. Almost researches are based on Pluronic F-127 also known as Poloxamer 407 and PEO–PPO–PEO (Mn. 4300–3770–4300) for delivery of protein/peptide drugs, such as insulin, urease, interleukin-2, epidermal growth factor (EGF), bone morphogenic protein (BMP),

fibroblastic growth factor (FGF), and endothelial cell growth factor (ECGF) [6]. Most results showed that sustained release kinetics over several hours. Pluronic F-127 hydrogels indicated that a zero-order release kinetics for interleukin-2 and urease over 8 hours [27, 28]. Tridecapeptide melanotan-I (MT-I) and mitomycin C were released from Poloxamer 407 hydrogel over 4 to 6 hours. These researches suggested that the higher the polymer concentration, the slower the release rate observed [29, 30]. However, pluronic F-127 hydrogels are easily degradation in the culture media or in vivo environment [31]. Some research substitutes the PPO of pluronic F-127 backbone to polycaprolactone (PCL) [32-34].

PEG–PLLA–PEG block copolymers have similar sol-gel transition behavior with PEO/PPO/PEO block copolymers. In PEG–PLLA–PEG block copolymers, the release profile was closely related with the hydrophobicity of the drug [38]. In this result showed that the more hydrophilic drugs were released continuously over 2 weeks, and the release rate was controlled by the concentration of the initial polymer. The more hydrophobic drugs were released over 2 months, and the release result showed a biphasic mechanism, an initial diffusion followed by a combination of degradation and diffusion at a later stage. [6]. Also PEG–PLLA–PEG block copolymers were affected by the solubilization and stabilization of water-insoluble drugs such as cyclosporine, A and paclitaxel, and various protein pharmaceuticals [39]. For example, a insulin level in the range 20–35μIU/ml was maintained for at least 2 weeks [40].

3.3. Bioink

For successful bioprinted 3D tissue like construct should possess the following properties (Figure. 1): (i) printability, (ii) biocompatibility, (iii) mechanical properties, and (iv) shape and structure [4, 37].

About the printability, during the printing, the bioink must be deposited in the substrate with suitable viscosity, shear- thinning property, short response, transition time, and suitable sol-gel transition. Biocompatibility is the basic property for tissue engineering, thus bioink must be able to support cell proliferation and adhesion, suitable degradability and do not have any toxicity. The mechanical properties must satisfy the mechanical properties of targeting tissues such as stiffness, elasticity, and strength. In addition, shape and structure must be similar with the natural tissue [4].

Table. 1. Ideal bioprinting bioink properties.

Ideal bioprinting hydrogel protperties	
Printability	Viscosity Shear-Thinning property Response and transition time Sol-gel transition stimulus
Biocompatibility	Degradability Cell binding motifs Non-toxic Non-immunogenic
Mechanical protperties	Stiffness Elasticity Strength
Shape and structure	Pore size Micro/Nano structure

One research attempts to fabricate a larger variety of hydrogel type bioinks with controllable material properties using by synthetic and natural materials. A total of 35 bioink were fabricated by devised a hydrogel fabrication method which had biocompatibility, bioprinting applicability, and rheological behavior [41]. To improve the shape and structure for complexity of extracellular matrix (ECM), one research team utilized decellularized extracellular matrix (dECM) for bioink formation. Unlike previously research report about bioinks, the dECM did not require crosslinkers and had thermosensitive property. This research suggested that dECM could allow the cells contained within to degrade the surrounding gel [36, 41]. Collagen is the major component of extracellular matrix protein, which can be form a gel at neutral pH and 37℃. Collagen consists fibrous protein that triple helix of α chains. Collagen is a fibrous protein that provides tensile strength to the extracellular matrix (ECM). Collagen is widely used for substrate coating materials and scaffold. From these properties, collagen possess potential as a bioink, so many researches attempt to apply to bioink [43]. Pluronic F127 is an attractive biomaterials for bioink because of the biocompatibility and controllable the thermosensitive gelling propertis. For example, Pluronic F127 was combined with collagen as a bioink for the neovascularization [44]. Gelatin methacrylate (GelMA) is a photocrosslinkable material that remains solid-like state between 4℃ and 22℃. GelMA can be blended with other biomaterials such as hyaluronic acidmethacrylate, silk fibroin and so on. [45, 46].

3.4. 3D bioprinting

Recently, 3D printer are applied to in many fields, especially tissue engineering and regenerative medicine field. In this field, a diversity of 3D printer are commercially used as 3D bioprinters which are required some qualification to enable the fabrication of various materials and products that can range from large reconstructive tissue like structure, and prints single cells or nanoparticles. To use 3D bioprinting, tissue engineering and regenerative medicine fields attempt the development of 3D tissue like structure that can substitute for present cell culture systems and animal models. However, tissues consist of a variety of cell types and 3D printer technique is early stage, thus some limitations for fabrication of 3D tissue models. To overcome the some limitations for the the fabrication of 3D bioprinted constructs, there are some technique such as inkjet bioprinting, extrusion bioprinting, laser-assisted bioprinting, digital light processing (DLP)-bioprinting are applied to the 3D bioprinting [47, 48].

The easiest methods of the using inkjet printer for 3D bioprtinter is modify the commercial inkjet printer. For example, HP 26 and HP G3110 were converted into 3D bioprinter system that used the commercial print component such as printer head, servo stage, ink cartridges and control software. However, commercial printer has low resolution of servo stage and low capacity of ink cartridge, which is not suitable to the 3D bioprinting [49, 50]. To solve these problems, one research team changed the servo stage to screw-based system which can be improved the resolution up to 100um. Also, to increase cartridge capacity, external ink cartridges were enlarged

and connected to multiple print heads to improve the maximum bioink volume [51, 52].

Extrusion bioprinter can be printed the high viscosity martials up to $6x10^7$ with high cell density. This property is suitable for pre-gel hydrogel solutions such as thermosensitive hydrogel, chemical crosslinkable hydrogel and photocrosslinkable hydrogel. Thus, almost commercial bioprinters are based on extrusion technology [48]. To use this advantage, extrusion bioprinter can be manufactured the multi-head 3D printer system, includes three-axis motion control with six dispensing heads with six different bioinks [53]. In addition, blood vessel-like structure was fabricated by the extrusion bioprinter with two different bioink, including the thermosensitive hydrogels [54].

Laser-assisted bioprinting are not usually used as bioprinter because of the high cost. The first prototype 3D bioprinting was developed by integrating optical laser sources with a lens [55]. Other research team were improved the laser-assisted bioprinting such as accuracy and laser throughput [56, 57].

DLP bioprinting has high resolution and faster printing speed without cell damage [58]. For example, one research reported that DLP bioprinting system can be make the 100um resolution and printing time was required within 1hour [59]. Recently, DLP bioprinting can be modified by commercial beam projector module which are not expensive and simple method. So DLP bioprinter is much more noteworthy than the before [58].

4. Materials and Methods

4.1. Preparation of the chitosan hydrogel

The Chitosan (CS) hydrogel solution was prepared essentially as described before [9, 10, 12]. A 1.67% (w/v) chitosan (YB BIO, 95% or greater degree of deacetylation) solution was prepared by stirring powdered chitosan in 0.1M aqueous acetic acid, lactic acid and hydrochloric acid at room temperature overnight. The insoluble particles in the chitosan solution were removed by filtration. The 56% (w/v) β-glycerophosphate (β-GP, Control group), 8% Potassium phosphate(K_2HPO_4) and 3.5% Sodium bicarbonate($NaHCO_3$) solution was prepared in α-MEM media as a gelling agent and sterilized using syringe filters with 0.2 mm pore size and stored at 4℃. To prepare the chtiosan hydrogel soultion, gelling agent solution was added to the chitosan solution at 9:1 ratio. Then, neutralization of pH was performed by dropwise addition of 1M NaOH solution in order to avoid precipitation of chitosan. Final pH reached above the 6.9.

Table.2. Experiment groups by solvent and gelling agent.

Samples			
Solvent variety Gelling agent =β-GP	Lactic acid	Acetic acid	Hydrochloric acid
Gelling agent variety Solvent = Acetic acid	β-GP	K_2HPO_4	$NaHCO_3$

4.2. Characterization of the chitosan hydrogel

4.2.1. Gelation time determination

Gelation time was measured as a function of time at constant temperatures of 24℃, 37℃, 50℃, and 70℃ in a water bath. The test tube inverting method was used to determine the gelation time [35]. The samples were incubated in a water bath at the predetermined temperatures, and time measurements were initiated. The flowability of the samples was observed every 30 s by tilting the tubes. The time at which flowing of the samples stopped was taken as the gelation time and the values were recorded.

4.2.2. Rheological analysis

Rheological analysis was performed with an advanced rheometric expansion system (ARES, Rheometric Scientific, UK). The changes in the elastic (storage) modulus (G′) and the viscous (loss) modulus (G″) were recorded as function of temperature (25 - 50℃) at a fixed frequency of 1 Hz. The temperature was varied with a constant heating rate of 1 ℃/min.

4.2.3. Field emission Scanning electron microscopy (FE-SEM)

The shapes and surface morphology of chitosan hydrogels were observed by field emission scanning electron microscope (SUPRA, 55VP). The chitosan hydrogels were prepared at 37℃ for 3h and then dipped into distilled water at 37℃ for 1 day. Next, samples were lypholized overnight (ilShinBioBase, FD8508).

4.3. Cell viability of the chitosan hydrogel

4.3.1 Cell culture

Periodontal Ligament Stem Cells (PDLSCs) cultured with culture media comprised of 1% antibiotics, 10% FBS, α-MEM. Fetal bovine serum (FBS), Trypsin-EDTA, Antibiotic-Antimycotic, Phosphate buffered saline (PBS), Alpha Minimum Essential Medium (α-MEM) were purchased from Welgene.

4.3.2. Water Soluble Tetrazolium Salt (WST) assay

Biological effect of chitosan hydrogels was examined by water soluble tetrazolium salt (WST, DAEILLAB, EZ-CYTOX) assay. Periodontal Ligament Stem Cells (PDLSCs) cultured with culture media comprised of 1% antibiotics, 10% FBS, α-MEM. The 0.5ml hydrogel solutions were pipetted into 24-well plates (Nunc, 142475) and allowed to gel at 37°C for 1 h prior to cell culture. 250μL of medium containing PDLSCs was pipetted into each well. Cells were seeded at 20,000 cells/well. After 1, 3, 5 and 7 days, viable cells were determined using microplate reader.

4.3.3. Live and dead assay

Cells were seeded on the chitosan hydrogels in 24-well plates at a density of 20,000 cells per well and incubated for 1 and 7days, after which the viable cells were stained with the live and dead cell assay (Abcam, ab115347) and then cell morphology was observed.

4.4. Printing of cell-laden construct

4.4.1. 3D-Bioprinting of PCL & chitosan bioink construct

Bioprinting system developed in our lab that can be adjust the set value for the materials and has stepper moter to drive xyz-stage and syringe pump to print the bioink. Bioprinter was used for the printing of the cell-laden construct. Polycaprolactone (PCL, polyscience, Mw 43000) was printed as frameworks at the 65℃ of the head temperature. The PDLSCs were encapsulated at a concentration of 5 x10^5 cells/ml into chitosan hydrogel solution. The lattice type structure is thickness of 2mm and 8-layer height. At the end of the printing, the cell-laden construct was gelated by incubating at 37℃.

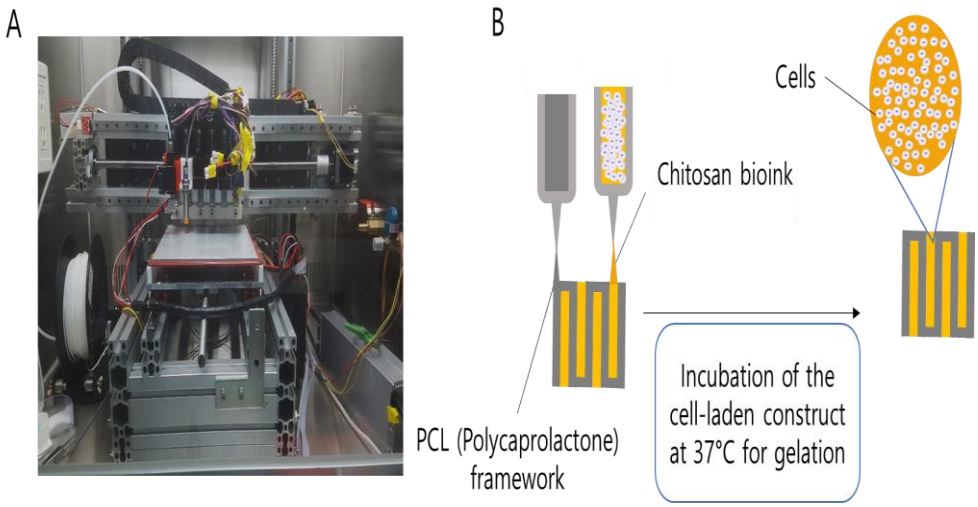


Figure. 1. Printing process of cell-laden constructs with chitosan bioink. (A) Custom made of the 5 nozzle 3D bioprinter. (B) Schematic representation of chitosan bioink printing and gelation step.

4.4.2. Cell viability of cell-laden construct

During the printing cells were under a relatively high shear-stressed environment. Therefore, we verified the viability of cells after the printing. Cells were stained with the live and dead cell assay (Abcam, ab115347) and then cell morphology was observed at day 1 and day 7.

4.5. Statistical data analysis

Statistical analysis was carried out using the statistical analysis system (SAS) for Windows v9.4 (SAS Institute, Inc., USA). Statistical significance between control and treatment groups was compared with one-way ANOVA at $*p<0.05$. The data were reported as the mean ± standard deviation, n=5.

5. Results and Discussion

5.1. Preparation of the chitosan hydrogel by solvent

Figure. 2. showed that the chitosan hydrogel solution transformed to a gel state after incubation at 37℃. The sol-gel transition was marked when the solution was becoming turbid. All groups easily can be formed hydrogel and there is no big difference among them.

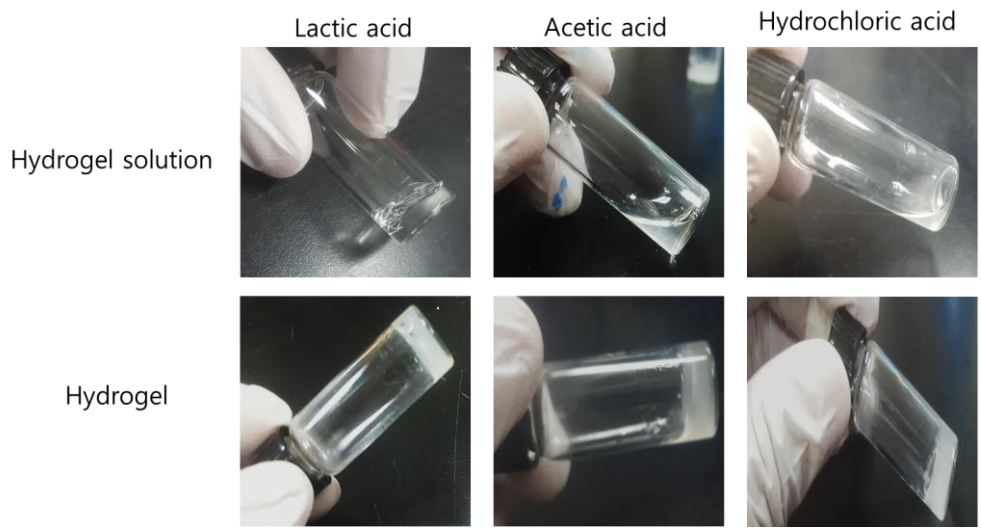


Figure. 2. Overview of chitosan hydrogel solution and chitosan hydrogel by solvent.

5.2. Characterization of the chitosan hydrogel by solvent

5.2.1. Gelation time determination

Figure. 3. showed that the gelation time decreased exponentially with increasing temperature. Gelation time of chitosan hydrogels by solvent indicated that there are almost same properties. Thus, solvent is not a key factor for the gelation time.

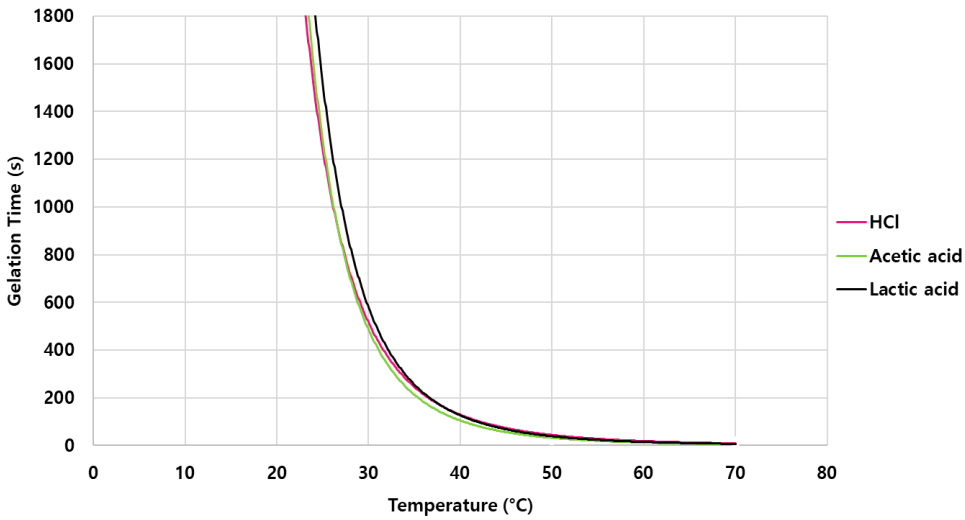
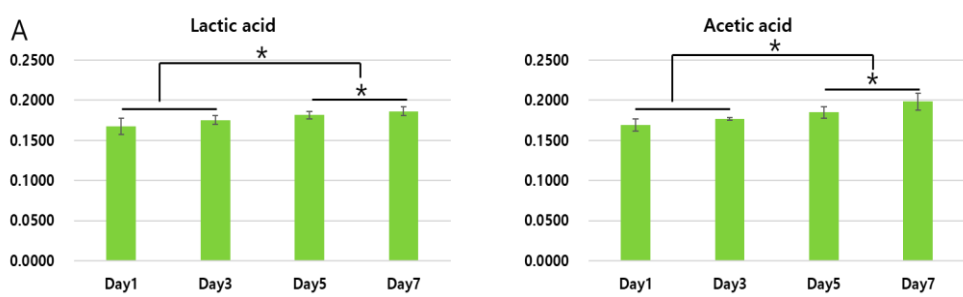


Figure. 3. The gelation time of the chitosan hydrogel solution as a function of different solvent. The test tube inverting method was used to determine the gelation time.

5.3. Cell viability of the chitosan hydrogel by solvent

5.3.1. Water Soluble Tetrazolium Salt (WST) assay

To evaluate the effect of the chitosan hydrogel on the PDLSCs via WST assay and cell morphology was observed by a microscope. Figure.4, the result of WST showed that there is no significantly difference after day 5. However, PDLSCs on the chitosan hydrogel dissolved in acetic acid significantly increased cell proliferation after day 7 ($p < 0.05$). Therefore, this result indicated that acetic acid has higher viability compared to the lactic acid and hydrochloric acid. In neutralization step, NaOH and each acid solution were formed a salt, sodium lactate, sodium acetate and sodium chloride, respectively .These salts are not toxic when interacting with the cells [8]. There is no research supported this result, however, this result showed that sodium acetate is suitable for the long-term cell culture condition. Thus, when we did the experiment about the effect of gelling agent, we used acetic acid as solvent in the experiment.



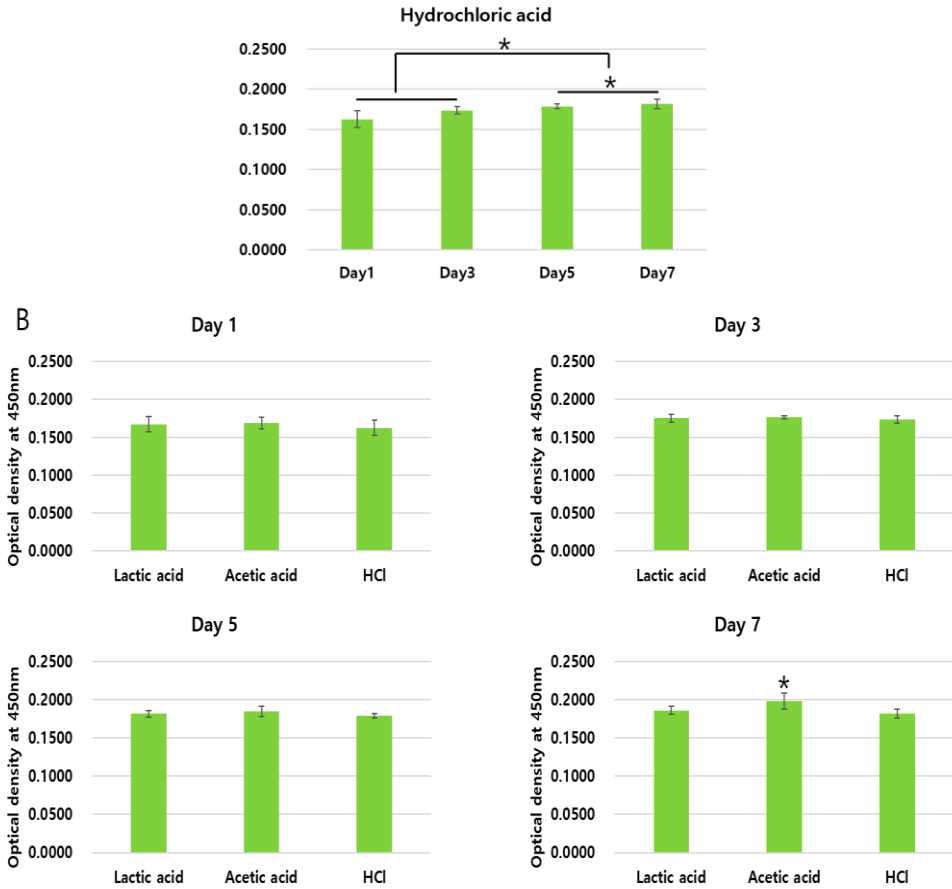


Figure. 4. Viability of PDLSCs (Periodontal Ligament Stem Cells) via indirect culture by the WST (Water Soluble Tetrazolium Salt) assay. Each value represents the mean ± SD (n =5 per each group). *Statistically significant difference compared to other groups ($P < 0.05$).

5.4. Preparation of the chitosan hydrogel by gelling agent

Figure. 5. showed that the chitosan hydrogel solution transformed to a gel state after incubation at 37℃. CS/K_2HPO_4 observed the large size precipitation that might be induce nozzle clogging during the 3D printing. On the other hand, CS/β-GP and CS/$NaHCO_3$ showed that small size precipitation compared to the CS/K_2HPO_4.

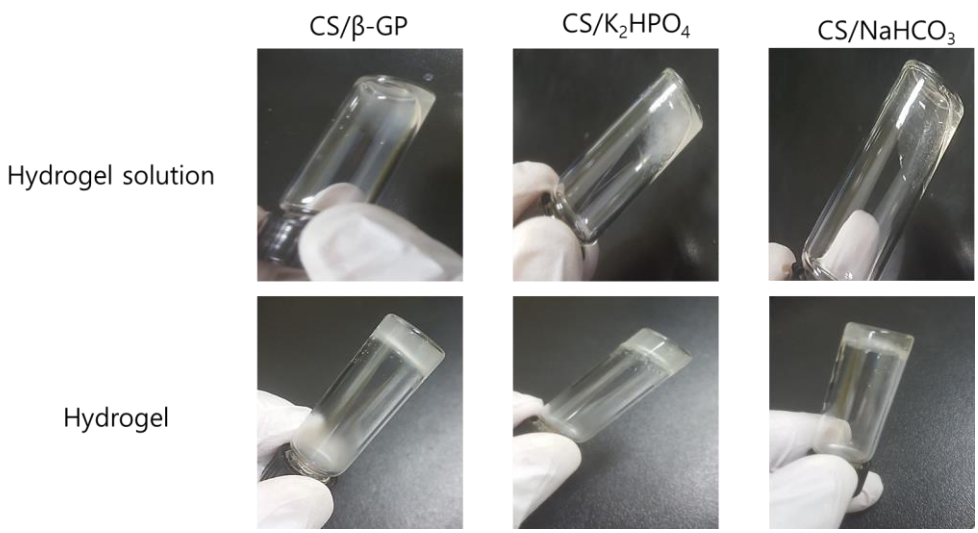


Figure. 5. Overview of chitosan hydrogel solution and chitosan hydrogel by gelling agent.

5.5. Characterization of the chitosan hydrogel by gelling agent

5.5.1. Gelation time determination

Figure. 6. showed that the gelation time of CS hydrogel by gelling agent. At higher temperatures, the shortest gelation time observed in this experiment was 20s. CS/K_2HPO_4 had the fastest gelation time and CS/$NaHCO_3$ had slowest gelation time. These result indicated that CS/$NaHCO_3$ decrease the risk of nozzle clogging during the printing because of the slowest gelation time.

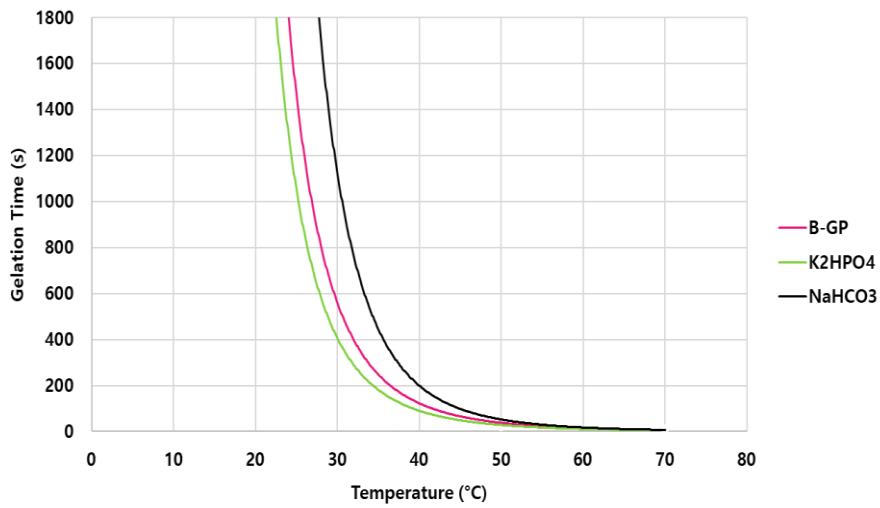
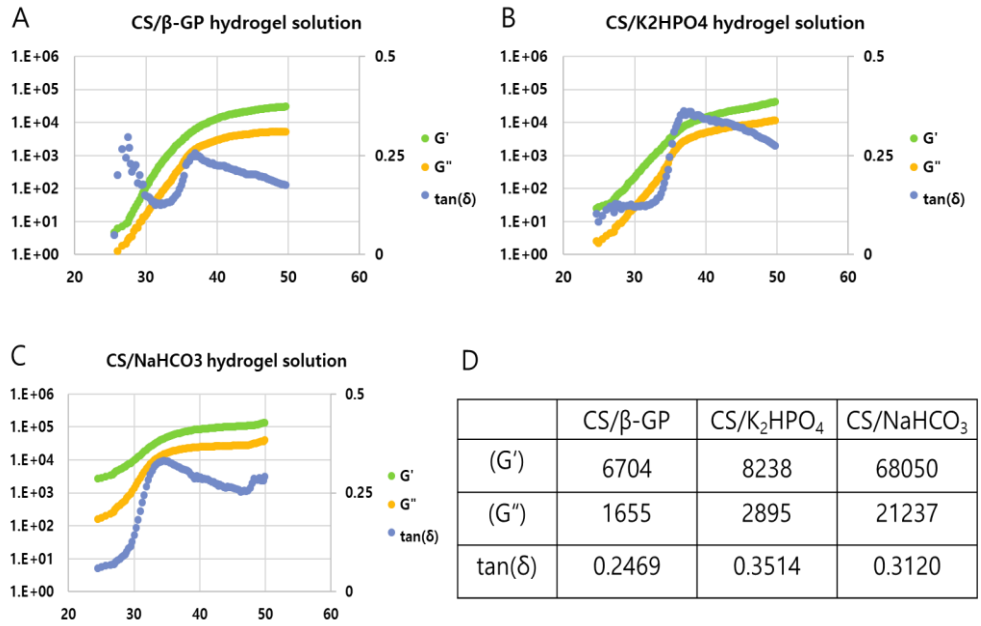


Figure. 6. The gelation time of the chitosan hydrogel solution as a function of different gelling agent. The test tube inverting method was used to determine the gelation time.

5.5.2. Rheological analysis

With increasing temperature from 25℃ to 50℃, all samples had a rapid increase of the storage modulus (G′) and decrease of tan(δ), showing the phase transition of the chitosan solution to the chitosan hydrogel around 37℃. In extrusion-based bioprinting, the ink viscosity property has up to 6 $x10^7$, thus all experimental groups are suitable for cell printing [48]. In figure. 8. (C), CS/$NaHCO_3$ hydrogel has the highest rate of the storage modulus (G′) at 37℃ compare to the CS/β-GP hydrogel and CS/K_2HPO_4 hydrogel. At the room temperature (25℃) CS/$NaHCO_3$, hydrogel also has the highest rate of the storage modulus (G′). This result showed that storage modulus (G′) of the chitosan hydrogel are affected the gelling agent. In addition, concentration of the $NaHCO_3$ is only 3.5% that is the lowest concentration compare to the β-GP and K_2HPO_4. Thus, $NaHCO_3$ is more effective and economically gelling agent for chitosan hydrogel.



D

	CS/β-GP	CS/K_2HPO_4	CS/$NaHCO_3$
(G′)	6704	8238	68050
(G″)	1655	2895	21237
tan(δ)	0.2469	0.3514	0.3120

Figure. 7. Rheological analysis of the chitosan hydrogel solution. The evolution of the storage modulus (G′) and the loss modulus (G″) of the chitosan hydrogel solution with ranged from 25℃ to 50℃; (A) CS/β-GP hydrogel solution; (B) CS/K_2HPO_4 hydrogel solution; (C) CS/$NaHCO_3$ hydrogel solution; The value of the rheological analysis at 37℃. (D) The value of the storage modulus (G′), the loss modulus (G″) and tan(δ) at the 37℃

5.5.3. Field emission scanning electron microscopy (FE-SEM)

Figure. 8. showed that the structure of the chitosan hydrogel. The inner structure of the chitosan hydrogel showed interconnected pores which were irregular porous structure. Pores size greater than 20–100μm can help cell penetration and pores size above 100μm related with the neovascularization, because it can provide large space [60]. Therefore, the chitosan hydrogels may support cell dispersion and neovascularization. CS/K_2HPO_4 had small pores size, but CS/β-GP and CS/$NaHCO_3$ had larger pores size. Thus, CS/β-GP and CS/$NaHCO_3$ might be more support cell dispersion and neovascularization than CS/K_2HPO_4.

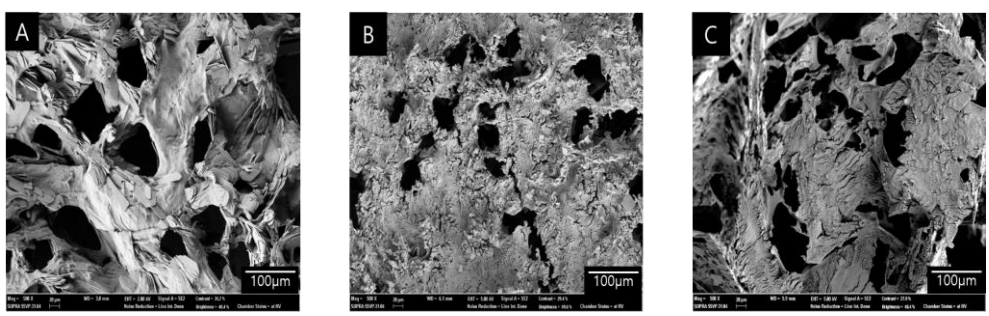
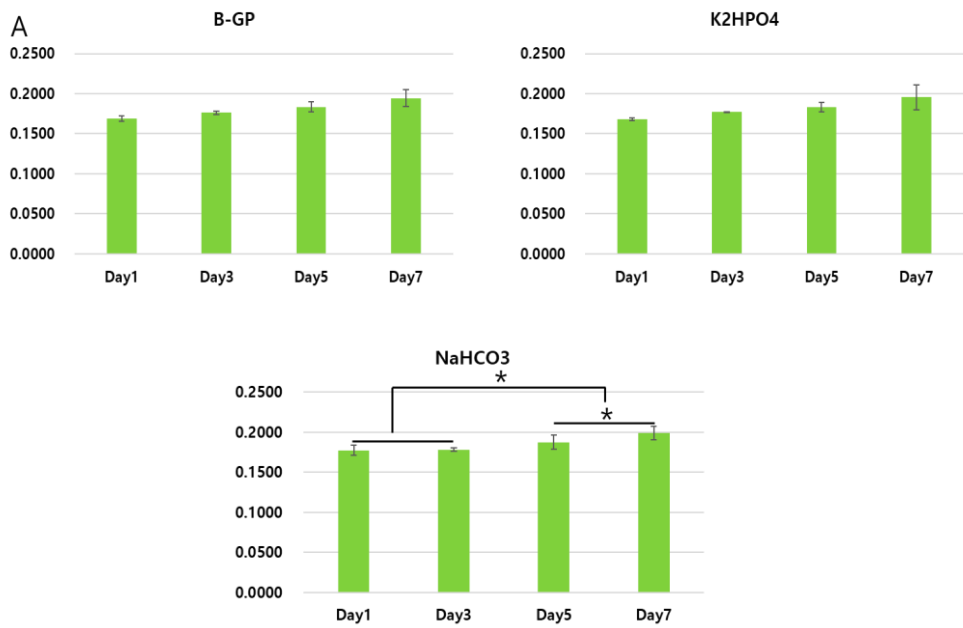


Figure. 8. SEM micrographs on the surface of the chitosan hydrogel; (A) CS/β-GP hydrogel (B) CS/K_2HPO_4 hydrogel (C) CS/$NaHCO_3$ hydrogel. The samples were prepared by dipping chitosan gels in distilled water at 37°C for 1 day, followed by lyophilization overnight. (MAG: X500)

5.6. Cell viability of the chitosan hydrogel by gelling agent

5.6.1. Water Soluble Tetrazolium Salt (WST) assay

The cytotoxicity of chitosan hydrogel study using PDLSCs demonstrated that chitosan hydrogels are cytocompatible. At the day 1, CS/$NaHCO_3$ hydrogel showed that high cell adhesion rate compared to other groups. However, after day 7 there is no significant difference among the gelling agent. The chitosan hydrogel solution is therefore a potential candidate for bioink due to its biocompatibility.



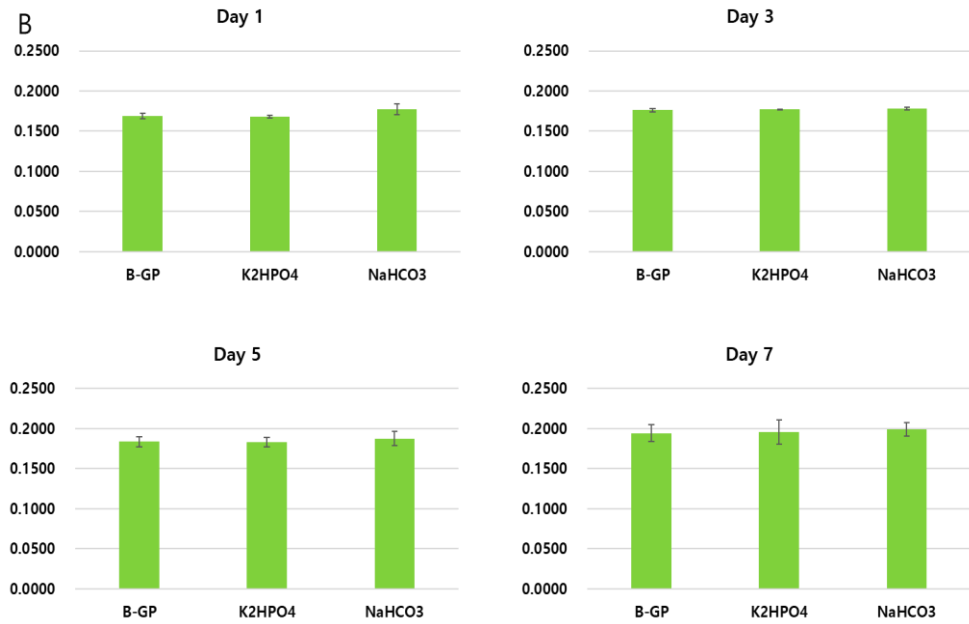


Figure. 9. Viability of PDLSCs (Periodontal Ligament Stem Cells) via indirect culture by the WST (Water Soluble Tetrazolium Salt) assay. Each value represents the mean ± SD (n =5 per each group). *Statistically significant difference compared to other groups ($P < 0.05$).

5.6.2. Live and dead assay

Figure. 10. showed the microscope image of the Live and dead assay stained PDLSCs. The result demonstrated that cells encapsulated in chitosan hydrogel were viable in a round morphology and well dispersed. After 7 days incubation, cells were more dispersed than day 1 incubation and cell viability were increased. This result suggests that chitosan hydrogel can encapsulate the cells without any cytotoxicity and spread the cells well for the incubation. Concentration of gelling agent is the important factor for the cell viability [60]. Therefore, a 56% (w/v) β-GP, 8% K_2HPO_4 and 3.5% $NaHCO_3$ solution is suitable for the cell culture condition.

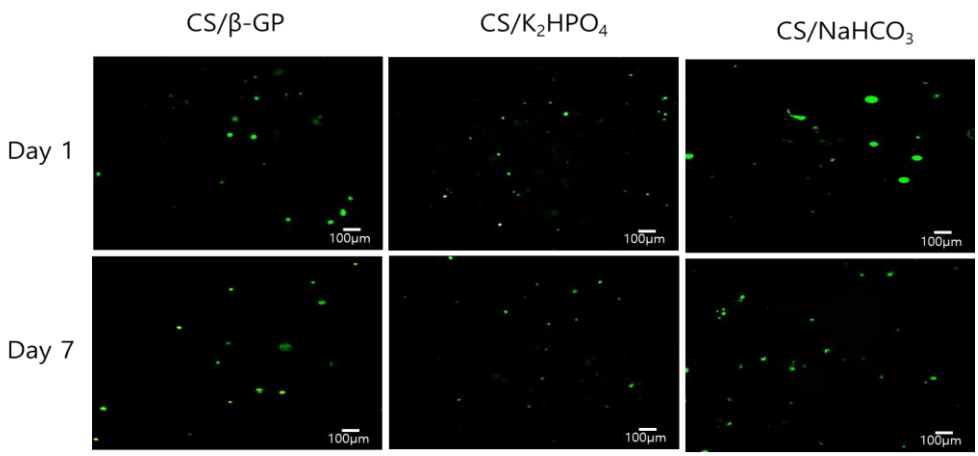


Figure. 10. Live and dead cell assay results of PDLSCs encapsulated in the chitosan hydrogel (green: live cells; red: dead cells). (MAG: X100)

5.7. Printing of cell-laden construct

5.7.1. 3D-Bioprinting of PCL & chitosan bioink construct

As figure. 11. showed that we fabricated the grid structure that encapsulated cells inside the chitosan bioink. Cell-laden chitosan pre-gel solution was placed in between the PCL framework on the petri dish. For 3D bioprinting of cell-laden bioink, one important problem is that the material should come out of the nozzle clogging. Typically, three kinds of gelling agent have almost same printability. However, the gelation time results suggested that, $NaHCO_3$ had the shortest gelation time, thus it might be a help for the printability without nozzle clogging.

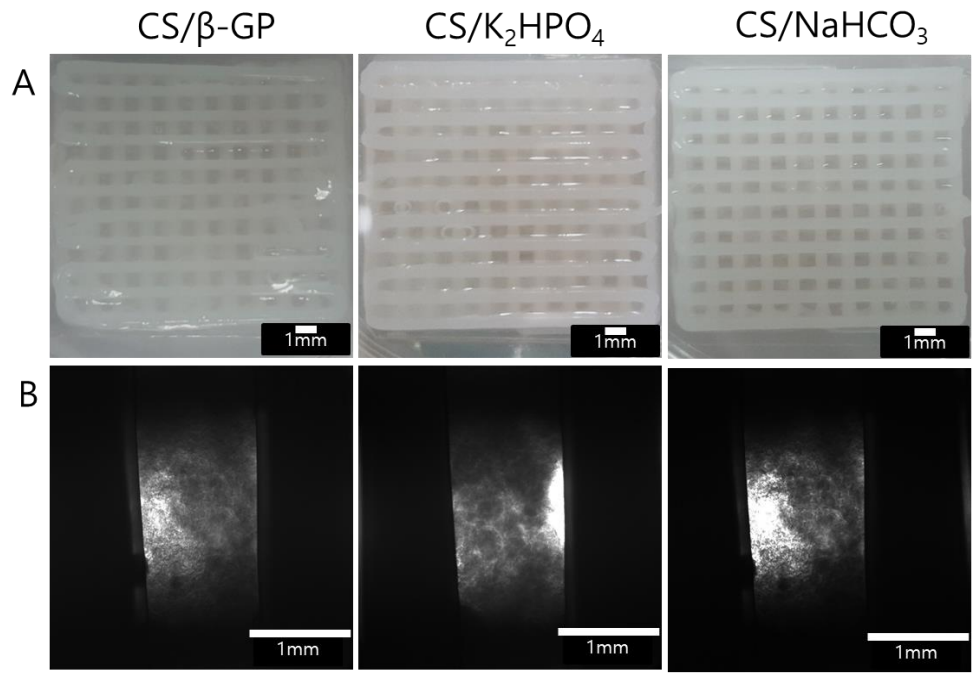


Figure. 11. The gross appearance of 3D PCL and chitosan bioink construct (A) Conceptual 3D structure made up of cell-laden chitosan bioink. (B) Microscopic image of the bioprinted structure using PCL and PDLSCs encapsulated in the chitosan bioink (MAG: X40).

5.7.1. Cell viability of cell-laden construct

In figure.12, at day 1 showed that the lattice type structure printed from PCL and chitosan bioink can encapsulate PDLSCs and maintain the high cell viability during the cell printing. Also at day 7 indicated that PCL and chitosan bioink construct are suitable for the long term cell culture without any side effect by gelling agent.

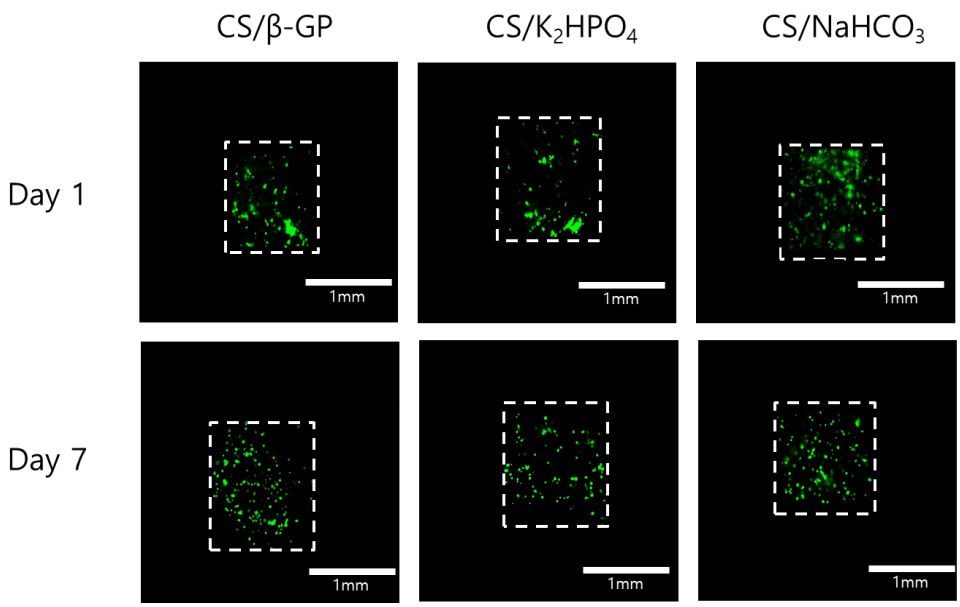


Figure. 12. Live and dead cell assay results of printed cells (green: live cells; red: dead cells). (MAG: X40)

6. Conclusions

The objectives of this research was to develop the bioinks by chitosan hydrogels by solvent and gelling agent for 3D bioprinting and to compare the cell viability and potential ability of the developed chitosan hydrogel as a bioink.

1) Solvent did not affect for the gel shape and gelation time.

2) Acetic acid had good biocompatibility compared with lactic acid and hydrochloric acid.

3) Depending on gelling agent, gel shape and gelation time were different among gelling agent. Gelling agent was more important factor than solvent.

4) During the increase temperature for 25℃ to 50℃, all experiment groups had dramatically increase of the storage modulus (G′). However, $NaHCO_3$ had the greater growth rate of the storage modulus (G′) compared with the β-GP and K_2HPO_4.

5) $NaHCO_3$ had greater growth rate of the storage modulus(G′) and more irregular porous structure than β-GP and K_2HPO_4

6) From the water soluble tetrazolium salt (WST) and live and dead assay data, it was proven $NaHCO_3$ had good effect for cell adhesion.

7) In cell-laden construct, there is no big difference among gelling agent except for printability.

8) Because of the longest gelation time, $NaHCO_3$ had good printability compared with β-GP and K_2HPO_4.

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3D 바이오프린팅을 위한 세포가 포함된 열감응성 키토산 바이오잉크 개발

서울대학교 대학원

바이오시스템·소재학부 바이오시스템공학전공

구 종 범

초 록

3D 바이오 프린팅은 세포가 함유된 하이드로겔을 적층방식으로 가공하여 복합 조직을 만드는 기술이다. 그러나 대부분의 하이드로겔은 가교제를 사용하여 화학적으로 결합하기 때문에 독성을 유발할 위험성이 있다. 반면에 열감응성 하이드로겔은 가교제가 필요 없기 때문에 더 생체친화적이라고 할 수 있다. 키토산은 열감응성 하이드로겔로써 응용이 가능할 뿐 아니라 생체적합성과 골분화능력이 뛰어난 것으로 알려져 있다. 키토산을 열감응성 하이드로겔로 만들 때에는 용매와 겔화제가 중요한 역할을 하는 것으로 알려져 있다. 그러나 아직까지 키토산을 바이오잉크로써 이용하기 위해여 용매와 겔화제의 영향을 연구한 결과는 거의 없는 실정이다. 따라서 본 연구에서는 키토산을 이용하여 용매와 겔화제에 따라 여러 바이오잉크를 개발하고 그

바이오잉크의 잠재적 효능을 알아보고자 한다. 용매에 따른 영향을 알아보는 실험에서는 겔의 모양의 양상과 겔이 되는 시간에는 큰 영향을 주지 않는 것으로 나타났으며 세포독성 실험 결과 아세트산이 가장 적합한 것으로 나타났다. 두번째로, 겔화제에 따른 영향에서는 β-GP 와 K_2HPO_4 에 비해서 $NaHCO_3$ 가 좀 더 적합한 것으로 나타났다. 먼저 겔화 되는 시간을 측정한 결과, 모든 그룹에서 온도가 높아짐에 따라 급격하게 시간이 줄어드는 것으로 나타났으나 K_2HPO_4 가 가장 빠르고 $NaHCO_3$ 가 가장 느리게 겔이 되는 것을 알 수 있었다. 상대적으로 겔화되는데 걸리는 시간이 오래 걸리는 $NaHCO_3$ 가 프린팅할 때 노즐이 막히는 문제에 자유로울 수 있을 것으로 보였다. 점탄성측정 결과를 살펴보면, $NaHCO_3$ 가 가장 높은 저장탄성률을 보였으며 이러한 특성은 겔이 되어서도 높은 것으로 나타났다. 전자현미경 사진을 통해서 표면을 관찰한 결과, 불규칙한 여러 구멍으로 구성되어 있는 것으로 나타났으며 $NaHCO_3$ 와 β-혜가 상대적으로 다양한 크기의 구멍으로 하이드로겔을 구성하고 있는 것으로 나타났다. Water soluble tetrazolium salt 와 live and dead 실험 결과, 유의적인 세포독성은 나타나지 않았지만 $NaHCO_3$ 가 세포부착능력이 가장 뛰어난 것으로 나타났다. 키토산 바이오잉크와 PCL 을 같이 프린팅하여 live and dead cell assay 를 실시한 결과 특이적인 세포 독성은 나타나지 않았다. 결론적으로 키토산 바이오잉크는

아세트산을 이용하여 $NaHCO_3$ 를 겔화제로 사용하는 것이 가장 적합한 것으로 나타났다.

핵심어: 3D 프린터, 바이오프린터, 하이드로겔, 키토산, 바이오잉크