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Development of a tannic acid cross-linking process for obtaining 3D porous cell-laden collagen structure

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Cell-printing is an emerging technique that enables to build a customized structure using biomaterials and living cells for various biomedical applications. In many biomaterials, alginate has been widely used for rapid gelation, low cost, and relatively high processability. However, biocompatibilities enhancing cell adhesion and proliferation were limited, so that, to overcome this problem, an outstanding alternative, collagen, has been extensively investigated. Many factors remain to be proven for cell-printing applications, such as printability, physical sustainability after printing, and applicability of *in vitro* cell culture. This study proposes a cell-laden collagen scaffold fabricated *via* cell-printing and tannic acid (TA) crosslinking process. The effects of the crosslinking agent (0–3 wt% TA) in the cell-laden collagen scaffolds on physical properties and cellular activities using preosteoblasts (MC3T3-E1) were presented. Compared to the cell-laden collagen scaffold without TA crosslinking, the scaffold with TA crosslinking was significantly enhanced in mechanical properties, while reasonable cellular activities were observed. Concisely, this study introduces the possibility of a cell-printing process using collagen and TA crosslinking and *in vitro* cell culture for tissue regeneration.

1. Introduction

In tissue engineering, the cell-printing process has arisen as a promising technology with the development of 3D printer and biomaterials [1]. It enabled to build three-dimensional (3D) porous mesh structure using cell-laden bioink and locate the cells in desired structure homogeneously [2]. In printing process, controllability in pore/strut size or mechanical strength is at stake to meet different requirements of regenerating various cell types. For instance, different optimal pore sizes were reported as 5 μm for vessel regeneration, 5–15 μm for fibroblast regeneration, 20–125 μm for skin regeneration, and 100–500 μm for bone regeneration [3,4]. Therefore, printing conditions have been actively investigated to achieve precise shape-ability and sustainability using biomaterials like gelatin, alginate, and collagen [5–7].

Hydrogels, among various biomaterials, are widely used for cell-printing process due to relatively low cytotoxicity and similar structure to extracellular matrix (ECM) [8]. However, some critical obstacles should be resolved to apply hydrogels on realistic tissue engineering applications. The hydrogels often have low mechanical properties that lead to low structural sustainability, resulting in reduced pore size and the collapse among interlayers of 3D structure [9–12]. To overcome the problem, various technical methods were proposed to enhance mechanical properties of hydrogels, such as additional physical supporter using synthetic polymers, gelation methods using a controlled temperature of working plate to regulate rheological properties, and various crosslinking processes [6,7,12,13].

To date, alginate has been a typical hydrogel explored with the advantages of high processability by using the crosslinking process with calcium ions [9]. The exposure of calcium ions to alginate results in rapid gelation, and rheological/mechanical properties can be easily regulated [14]. Also, several studies had shown the regeneration of various cell types such as aortic/skeletal muscle cells, osteoblast cells, and stromal cells [15–18]. However, the alginate revealed low biocompatible properties, which does not induce effective cell attachment nor proliferation [19]. The modification with arginine-glycine-aspartic (RGD) acid peptide sequence was suggested to improve biocompatibility of alginate,

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but the process required the use of a toxic reagent, [(1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride) (EDC)] [20]. For cell-laden bioink, cells may not be completely safe from the chemical toxicity even with several cleansing processes. In this regard, collagen, a main protein of ECM possessing RGD sequences, is getting recognized as an optimal biomaterial for cell-laden bioink [21].

Since the introduction of collagen as a cell-laden bioink, many researchers have strived to develop optimal conditions to obtain mechanically stable collagen structures [22–24]. In brief, the crosslinking reagents of glutaraldehyde (GA) and EDC were widely used to enhance mechanical properties and viscosity of collagen [25,26]. However, both crosslinking reagents are cytotoxic, and the need in safe crosslinking process has been aroused [24]. In a study of Kim et al., genipin was proposed as a novel crosslinking reagent in cell-printing applications, and showed high cell viability of 93% using osteoblast and human adipose stem cells (hASC) [27]. Furthermore, polyphenol (tannic acid; TA) in various weight fractions of 1–6 wt% was premixed with collagen for fabricating hASC-laden bioink to build a 3D mesh structure [28]. High cell viability was observed, but the polyphenol chemically reacting in collagen bioink may have affected cellular activities throughout the cell culture period. The possibility of cell-printing with collagen was demonstrated, and further attempts on building a cell-laden collagen structure using non-cytotoxic crosslinking reagents are required to broaden tissue engineering applications.

Here, we propose a cell-printing with TA crosslinking process using cell-laden collagen bioink. TA, one of a non-toxic plant polyphenol, had been applied on tissue engineering as a crosslinking reagent, owing to the formation of hydrogen bonds between collagen and TA [1]. Collagen scaffolds, which were not laden with cells, were physically reinforced through the TA crosslinking process [29,30]. Based on these concepts, we used preosteoblasts (MC3T3-E1)-laden collagen (5 wt%) bioink, and the scaffold was printed with the diameter of 300 μm and pore size of 500 μm [4,31]. The collagen scaffold without crosslinking process was used as a control, and the scaffolds were crosslinked with various concentrations of TA (0.1, 0.25, 0.5, 1, and 3 wt%) for 10 min [29,30,32]. Based on the pretest, the crosslinking time of 10 min was enough despite of TA concentration (data not shown). After crosslinking process, the cell-laden collagen scaffolds using preosteoblasts (MC3T3-E1) were examined on mechanical properties and cell-release. Also, cellular activities like cell viability and metabolic activity throughout 14 days were observed.

2. Materials & methods

2.1. Materials

Mouse preosteoblasts (MC3T3-E1; ATCC, Manassas, VA, USA) and type-1 collagen (Matrixen-PSP; SKBioland, South Korea) were used as the components of the bioink for cell printing in this work. Collagen was derived from porcine tendon and neutralized by being mixed with 10 × enriched DMEM at a volume ratio of 1:1 [7]. The final neutralized weight portion of collagen was 5 wt%. After the cell-laden collagen bioink (5×10^6 cells mL^{-1}) were printed, it was crosslinked with tannic acid (TA; 0, 0.1, 0.25, 0.5, 1, and 3 wt% of concentrations in tri-distilled water; Sigma-Aldrich, St. Louis, MO, USA) for 10 min.

2.2. Rheological test

The rheological properties (storage modulus (*G*') and tangent delta (tan δ)) of the neutralized collagen-based bioink (5 wt%; cell density of 5×10^6 cells mL^{-1}) were evaluated by temperature. A rotational rheometer (Bohlin Gemini HR Nano; Malvern Instruments, Surrey, UK) equipped with cone-and-plate geometry (cone angle with 4°, diameter of 40 mm, and gap of 150 μm) was used to measure the properties. The temperature swept in range of 15–45 °C with a ramping rate of 5 °C per min with fixed strain (1%) and frequency (1 Hz) within the range of the linear viscoelastic region.

2.3. Cell-printing using cell-laden collagen and tannic acid crosslinking

The cell-laden bioink was printed in 3D structure with a dispensing system (DTR2-2210T; Dongbu Robot, South Korea) equipped with a printing nozzle with 150 μm of inner diameter. Standard 3D printing software installed in the printer was used to control the printing process and parameters as in Fig. 1. The temperature of the barrel/nozzle (5–10 °C) and the printing stage (35–37 °C), pneumatic pressure (220 ± 10 kPa), and moving speed of printing nozzle (10 mm s^{-1}) were appropriately applied to obtain a fine 3D multi-layered mesh structure. After the printing process, the 3D structure was crosslinked in tannic acid (TA) solutions with various concentrations (0.1, 0.25, 0.5, 1, and 3 wt% in tri-distilled water) for 10 min each.

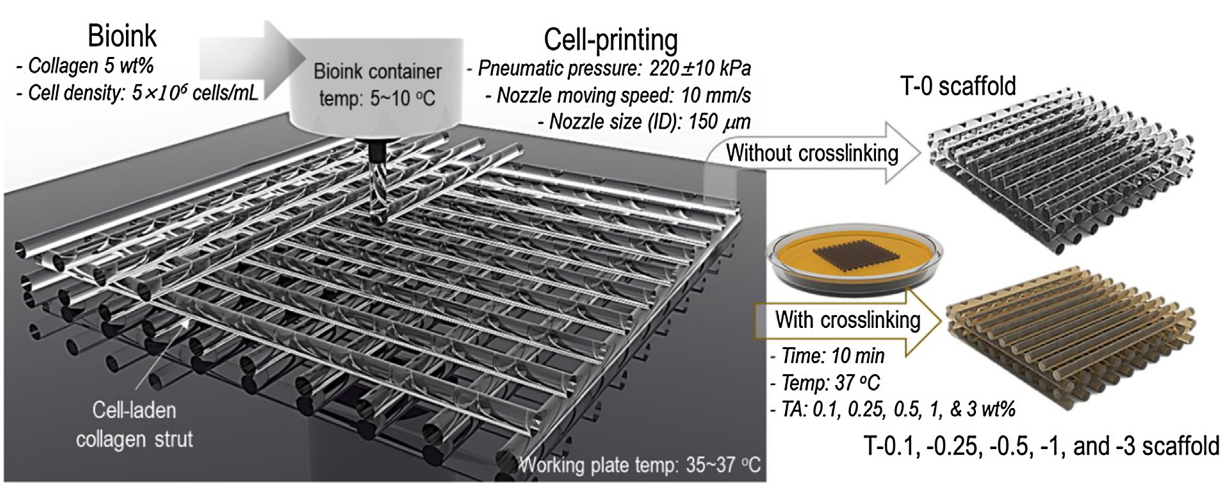

Fig. 1. Schematic images of fabricating MC3T3-E1-laden collagen scaffold without crosslinking process and with crosslinking process using different concentrations of tannic acid (0.1, 0.25, 0.5, 0.1, and 3 wt%).

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