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# Osteogenic and angiogenic potentials of the cell-laden hydrogel/mussel-inspired calcium silicate complex hierarchical porous scaffold fabricated by 3D bioprinting

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ABSTRACT

3D printing has been popularly used in the bone tissue engineering, as many of the biomaterials for this field of study can be prepared for and produced from this additive manufacturing technique. In this study, we strategized a solvent-free processing to fabricate the polydopamine-modified calcium silicate (PDACS)/poly-caprolactone (PCL) scaffold with Wharton's jelly mesenchymal stem cells (WJMSCs) incorporated with human umbilical vein endothelial cells (HUVEC)-laden hydrogel. The PDACS/PCL/hydrogel 3D scaffold yielded a Young's modulus of the 3D scaffolds as high as 75 MPa. In addition, the vascular morphogenesis and cellular behaviors regulated by our hybrid scaffolds were also intricately evaluated. Furthermore, the HUVEC in the bioink exhibited higher levels of angiogenic biomarkers and showed potential for the formation of complex vascular networks. Higher levels of bone formation proteins were also observed in our composites. Such a hybrid of synthetic materials with cell constituents not only enhances osteogenesis but also stimulates vessel network development in angiogenesis, presenting the fact that 3D printing can be further applied in improving bone tissue regeneration in numerous aspects. We believe that this method may serve as a useful and effective approach for the regeneration of defective complex hard tissues in deep bone structures.

## 1. Introduction

Effectively and uneventfully regenerating defective hard tissue in deeper and complex bone tissue is a complicated problem for clinicians [1]. In the past decade, the 3D printing method has been developed to fabricate more ideal structure scaffolds with better control of pore morphology, pore size and porosity [2]. Therefore, 3D printing is an emerging fabrication method in tissue engineering that has made significant progress toward the regeneration of transplantable tissues. The 3D printing method must be used to achieve complex and 3D tissue in order to realize its full potential as the future of regeneration medicine [3]. In recent years, various different methods of 3D-printing have been developed to create scaffolds by using traditional biomaterials such as poly lactic acid (PLA) [4], poly-caprolactone (PCL) [5, 6], hydroxyapatite, and tricalcium phosphate [7]. Thus, 3D bioprinting systems function by dispensing material in a layer-by-layer fashion to fabricate various structures of customizable shapes, pore sizes and internal architectures [8]. These methods also involve working with multiple dispensing printing heads, allowing for the generation of complex structures using multiple material types and different types of cells, including ceramics [9, 10], polymers [11], cell-laden hydrogel [12], or both [13].

Ceramics, such as calcium phosphate-based biomaterials have been successfully used as bone replacement materials for > 20 years, as these materials have excellent biocompatibility [14]. However, calcium silicate (CS)-based powder exhibits far better biodegradation and osteoconduction characteristics than calcium phosphate-based materials. In fact, our previous studies have shown that CS-based materials have the

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ability to produce a calcified bone-like apatite layer that already supersedes that of phosphate [15]. Other recent in vivo and in vitro experiments have also showed that the ion release kinetics of CS-based materials harnessed excellent osteogenesis and odontogenesis results, supporting the attachment, proliferation, and differentiation of various types of cells [16–18]. The release of Si plays many role in the cellular regulation of various types of cells, such as inhibiting osteoclastogenesis in macrophages and enhancing angiogenesis in human periodontal ligament cell [19]. However, CS-based materials are not printable and can't be applied to fabricate porous scaffolds directly with 3D printing technologies. They usually need to be mixed with other materials such as PLA and PCL to improve printability [20]. PCL is a highly biocompatible and biodegradable biomaterial with so many advantages that it has already been approved for various medical and drug delivery devices, and is already extensively used for tissue regeneration due to its cost-effectiveness, and durability [21]. PCL-based composites promote mineralization and tissue formation and can be a good base for engineering hard-tissue regeneration. It has also been approved by the FDA for several clinical applications [22]. The high mechanical strength and low degradation rate of PCL give PCL-based biomaterials several advantages for application in hard tissue engineering, which requires long term implantation [23]. PCL is a promising material and it can be incorporated with different polymers and other inorganic materials. This strategy had been extensively harnessed to design scaffolds with appropriate properties in bone tissue engineering. Recently, scientists discovered the important role that 3,4-dihydroxy-L-phenylalanine (DOPA) has in mussels with extraordinary robust adhesion to rocky surfaces [24]. Following this discovery, dopamine (DA), an analogue of DOPA, has emerged as a promising material for modification of various substrates [4, 25]. The materials-independent polydopamine (PDA) coated could be easily and quickly acquired through base-triggered oxidation and polymerization of DA, and the PDA ad-layer serves as a platform for post-modification which includes spontaneous deposition of metal and bioceramics as well as covalent immobilization of various adhesive proteins [26, 27]. Surface hydrophilicity and bioactive functional groups improve cell behaviors on the self-assembled PDA/calcium-phosphate ceramic nano-layer [28].

In recent years, the hybrid bioprinting technology has been a promising approach, allowing us to simultaneously use the cell-laden hydrogels for development of tissue engineered constructs [29]. Thus, with this method of bioprinting we design and print a 3D tissue architecture with homogeneous cell distribution, without the need for cell seeding. Bioprinting has been used widely in soft tissue engineering. For example, blood vessel, cartilage restoration is now being considered for its potential application in bone tissue regeneration. In the process of bone tissue regeneration, human umbilical vein endothelial cell (HUVEC) and other endothelial cell contribute to osteogenic through secretion of various kind of regulatory molecules such as vascular endothelial growth factor (VEGF) [30], osteoprotegerin (OPG) [31], and bone morphogenetic protein (BMP-2) [32]. These growth factors and cytokine cause an imbalance between osteoblast cells and osteoclast cells activities that promotes bone remodelling by replacing the old bone tissues with new bone tissues [33]. Selective differentiation of human mesenchymal stem cells (hMSCs) can provide further understanding regarding this important progenitor of various tissue types and the potential of novel therapeutic methods for the restoration of injured or unhealthy tissue [34].

In this study, considering the importance of HUVEC in osteogenesis, we incorporated cell-laden gel with CS scaffold as a composite to promote bone and vascular tissue formation in bone regeneration. In addition, we use the piezoelectric needle to print the Wharton's jelly mesenchymal stem cells (WJMSC) directly on the surface of the PDA-modified CS with PCL. The osteogenic properties will be evaluated by quantitative measurement of osteogenic protein in order to explore the capabilities and qualifications of PDACS/PCL/cell-laden composite scaffold for composite scaffolding in bone tissue engineering.

## 2. Materials and methods

### 2.1. Preparation of PDACS/PCL scaffold

Reagent grade CaO, $SiO_2$, $Al_2O_3$, ZnO were purchased form Sigma-Aldrich and mixed in with a weight ratio of 65%, 25%, 5%, and 5%, respectively. The oxide mixture is then sintered at 1400 °C for 2 h; after which, the sintered product is ball-milled into fine calcium silicate (CS) powder. The CS powder was immersed in 4 mg/mL of dopamine (Sigma-Aldrich, St. Louis, MO, USA) in tris-base buffer (pH 8.5) and stirred for 12 h at room temperature. After 12 h, the solution was filtered and washed with absolute alcohol for 3 times. The residue of polydopamine-modified CS (PDACS) is collected and dried in an oven for 12 h. Then, the CS/PCL and PDACS/PCL matrix was produced using the thermal pressing method. First, reagent grade PCL (Mw = 43,000–50,000, Polysciences, Warrington, PA) was placed in a 150 °C oven for 2 h. Then, CS or PDACS powders were suspended in absolute alcohol and slowly dropped into PCL while mixing. After mixing, the composites were placed in a 100 °C oven, until they became printable materials (CS/PCL = 5/5). The concept for this device was based on a precision three-axis positioning system (BioScaffolder 3.1, GeSiM, Grosserkmannsdorf, Germany). The composite pastes were loaded into a syringe and dispensed through a steel nozzle at 95 °C by applying a pressure of 500 kPa. The materials were printed in 500 μm lines with a line height of 500 μm, and a total of seven lines were printed in parallel with a gap diameter of 500 μm. Between the composites lines, the scaffold was plotted layer-by-layer until it reached up to 16 layers. The phase composition of the scaffolds was analyzed using X-ray diffractometry (XRD; Bruker D8 SSS, Karlsruhe, Germany) at a scanning speed of 1°/min. Then, the N compositions of the PDACS/PCL scaffolds were characterized with an electron spectroscope for chemical analysis (ESCA, PHI 5000 VersaProbe, ULVAC-PHI, Kanagawa, Japan). The water contact angle for each scaffold was determined at room temperature. Briefly, each scaffold was placed on the top of a stainless-steel base, and a drop of DMEM (50 μL) was placed on the surface of each specimen, and the image was taken by the camera after 30 s had elapsed. The resulting images were analyzed using ImageJ (National Institutes of Health) to evaluate the water contact angle. The compressive strength of scaffolds was measured on an EZ-Test machine (Shimadzu, Kyoto, Japan) at a loading rate of 1 mm/min. The maximal compression load at failure was obtained from the recorded load-deflection curves. The microstructure of scaffolds before and after its immersion into DMEM were investigated by a scanning electron microscope (SEM; JSM-6700F, JEOL) under the lower secondary electron image mode at a 3 kV acceleration voltage.

### 2.2. Preparation of bioscaffold

The 3D constructs contained PDACS/PCL and cell-laden hydrogel is composed of 16 layers, each of which possesses strands (0.5 mm in diameter) of PDACS/PCL bio-paste and HUVEC-alginate-gelatin bioink. The hydrogel contained 30% Type B gelatin, 5% alginate, and Ca-free Dulbecco's modified Eagle medium (DMEM; Caisson). They were prepared under UV-sterilized condition. In order to visualize the cytoskeleton organization, the HUVEC and WJMSCs were transformed using the CellLight Actin-RFP and CellLight Actin-GFP BacMam 2.0 system (Invitrogen) according to the supplier's instructions, respectively. Then, the RFP-HUVEC cells were mixed and suspended with hydrogel in the culture at a concentration of $1 \times 10^7$ cells/mL. To generate the composite construct, the cell-alginate solution and molten PDACS/PCL were dispensed alternately in each layer. First, the PDCS/PCL bio-paste was layered by a nozzle with the diameter of 0.5 mm at a printing speed of 0.883 mm/s under 450 kPa of extrusion pressure. Then, the HUVEC-alginate-gelatin bio-inks were then printed on the spaces between the PDACS/PCL strands at a dosing speed of 10 mm/s at the extrusion pressure of 20 kPa. After the completion of one layer, the Wharton's