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Short communication

Inkjet-based multilayered growth factor-releasing nanofilms for enhancing proliferation of mesenchymal stem cells *in vitro*

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ABSTRACT

We report the preparation and characterization of inkjet-based basic fibroblast growth factor (bFGF)-containing nanofilms on a flexible PET substrate. bFGF and heparin (HEP) were assembled by inkjet-based layer-by-layer (LbL) assembly driven by electrostatic interactions. The bFGF/HEP nano-assembly surface coatings were formed *via* alternating printing adsorption of positively charged bFGF and negatively charged HEP; the process was monitored by UV–vis spectroscopy and quartz crystal microbalance. The bFGF release profile could be controlled by altering the number of layers of printed LbL films. Mesenchymal stem cells, which are capable of extended proliferation *in vitro*, require a continuous supply of bFGF for proliferation. However, enhancing mesenchymal stem cell proliferation by continuous supplying bFGF is difficult, even with medium replacement, because of the instability of bFGF. Here, we established a novel system for releasing bioactive bFGF from a modified surface by using an inkjet-based nanofilm fabrication method.

Introduction

Mesenchymal stem cells (MSCs), a type of multipotent stromal stem cells [1–3], can differentiate into a variety of cell types, such as osteoblasts (bone cells) [4], myocytes (muscle cells) [5], chondrocytes (cartilage cells) [6], and adipocytes (fat cells) [7]. It has also been suggested that these cells can be widely used in various biomedical applications, including cell-based therapies in fields ranging from medicine for orthopedic treatment [8] to transplantation for regeneration of various organs/tissues [9–14]. Before MSCs can be widely used in various studies and treatments, the labor and financial costs of cell culture should be reduced and the proliferation of MSCs should be enhanced.

MSC proliferation can be enhanced by some cytokines, such as platelet-derived growth factor [15] and transforming growth factor β-1 [16]. Basic fibroblast growth factor (bFGF), also known as FGF-2, plays critical roles in maintaining the pluripotency and self-renewing activity of various stem cells, including MSCs [17–19]. bFGF performs its biological functions by binding to and activating FGF receptors, a subfamily of cell surface receptor tyrosine kinases [20]. bFGF has been shown to promote the proliferation of MSCs and the formation of cartilage and bone [21]. However, bFGF is highly unstable under normal culture conditions [22,23]: bioactivity of bFGF in physiological environments decreases below 50% just twelve hours ago [24,25].

For decades, many studies have attempted to stabilize bFGF in specific environments [26–29] and have characterized the stability of bFGF [30–32]. Well-organized and multilayered film structures at the nano-meter level have been used to prevent the denaturation of bioactive molecules under aqueous conditions and develop controlled-release bioactive molecules for cell culture. Among the well-known nanofilm fabrication methods, layer-by-layer (LbL) assembly involves alternating layers of oppositely charged materials with rinsing steps onto any surface [33,34]. This method has widely been used for biomolecule delivery applications and is useful for incorporating bioactive molecules because high temperature, high pressure, and complicated treatments are not necessary [35–42].

Even though LbL methods are able to fabricate functional nanofilms on the any surface, bio-active molecules can be contaminated by contact between substrate and solutions. Inkjet-based fabrication of nanofilms is useful for the development of MSC culture systems. A drop-on-demand, piezoelectric inkjet printing technique generates ink droplets without a heating element, as the heating element can damage bioactive molecules while ejecting ink droplets [43]. The inkjet technique is a non-

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contact and economical method [44]. In particular, the inkjet printing technique is very useful for the use of expensive biomolecules because ink drop placement into drop small droplets with accurate volumes (1.5–2 pL) can be tightly controlled. Nanofilms prepared by inkjet printing may be useful for improving cell culture systems.

In this study, we report the preparation of inkjet-based nanofilms composed of bFGF and heparin (HEP) bilayers. The printed nanofilms were generated for the continuous release of bFGF by utilizing the LbL assembly. These films can be used in a variety of cell culture applications. In order to verify the potential of practical applications, we investigated the effect of bioactive bFGF from the printed LbL films on the proliferation of bone marrow stem cell (BMSCs) from rabbit. To release a large amount of bFGF and minimize the negative effects of substrates, we prepared bFGF/HEP nanofilms on a flexible PET substrate, rolled the nanofilms, and placed the nanofilms in cell culture plates. Our results suggest that rabbit BMSCs have a high proliferative capacity on printed LbL bFGF/HEP films *in vitro*.

Experimental

Materials

Heparin sodium salt from porcine intestinal mucosa and glycerol solutions (86–89%) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Basic fibroblast growth factor (bFGF, recombinant human protein) was obtained from Gibco® Life Technologies (Grand Island, NY, USA). bFGF enzyme-linked immunosorbent assay kits were obtained from R&D Systems (Minneapolis, MN, USA). To maintain biological activity of the molecules, we used a deionized water:glycerol (7:3 v/v) mixture solvent as ink (viscosity; 2.4 mPa s). Glycerol, which is widely used as a polyol to stabilize proteins, induces protein compaction, reduces protein flexibility, affects both native and non-native protein aggregation, as well as recovers protein activity following refolding/reoxidation [45–48]. The concentrations of bFGF and heparin ink solution were 1 μg/mL and 1 mg/mL, respectively.

Instrumentation: inkjet printing system

The printer used in this paper is a drop-on-demand piezoelectric inkjet photo printer (Model: Epson R290, Nagano, Japan). It can be readily available a commercial instrument which has a function of CD print. The printer was equipped with a 6-nozzle line, including black, yellow, light magenta, magenta, light cyan, and cyan color reservoirs. The nozzle diameter was set to approximately 25 μm, with a droplet volume of 1.5–2 pL. Because of the commercial instrument, not customized instrument, we should make materials solutions of specific viscosity (1–20 cP) to work properly.

Film construction and treatment of prepared films with inkjet system

Multilayer films were assembled LbL on a silicon wafer, quartz glass, and flexible propylene film by alternatively inkjet printing the substrate. The printing of the solution was controlled with the Epson CD Print software and Photoshop CC. First, the positively charged bFGF solutions in the black cartridge were printed onto the substrates. Second, the substrate was rinsed under a stream of deionized water three times and dried with an air blower. Third, the negatively charged HEP solutions in the cyan cartridge were printed onto the substrates. Finally, the second step was repeated, thus fabricating one bilayer.

Characterization of printed LbL films

The concentration of HEP in the films was measured by UV–vis spectroscopy (Jasco V-670, Oklahoma City, OK, USA). Absorption intensity is related to the buildup of the LbL film. Adsorption and deposition from the inkjet multilayered $(bFGF/HEP)_n$ films were evaluated using the quartz crystal microbalance (QCM, QCM200,

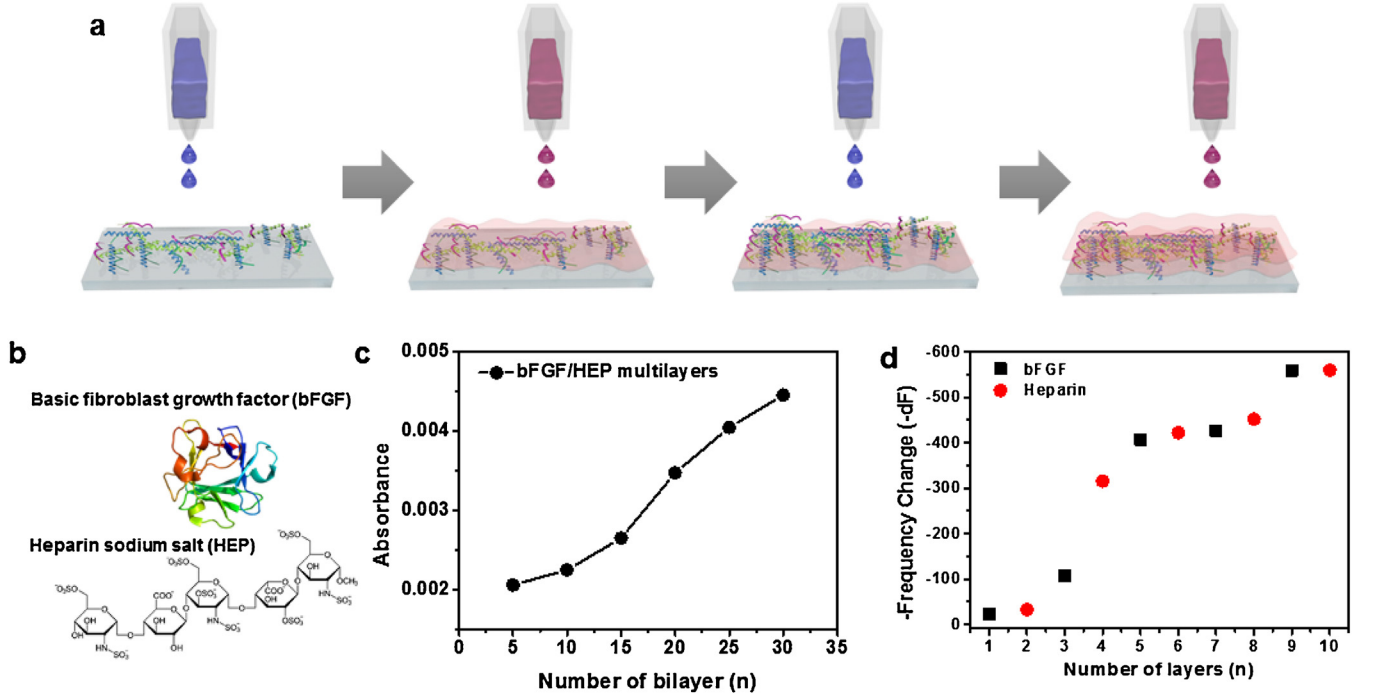

Fig. 1. (a) Inkjet-based layer-by-layer (LbL) process for printing LbL basic fibroblast growth factor (bFGF)/heparin (HEP) films. (b) Chemical structures of bFGF and HEP silica samples used in the present study. (c) UV–vis absorption spectra of printed LbL $(bFGF/HEP)_{n(n = \text{numberofbilayers})}$ films. Absorbance at 195 nm *versus* number of bilayers. (d) Frequency changes in printing LbL films prepared from bFGF and HEP by QCM with the number of deposited bilayers.

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