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Wide-range stiffness gradient PVA/HA hydrogel to investigate stem cell differentiation behavior

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ABSTRACT

Although stiffness-controllable substrates have been developed to investigate the effect of stiffness on cell behavior and function, the use of separate substrates with different degrees of stiffness, substrates with a narrow range stiffness gradient, toxicity of residues, different surface composition, complex fabrication procedures/devices, and low cell adhesion are still considered as hurdles of conventional techniques. In this study, a cylindrical polyvinyl alcohol (PVA)/hyaluronic acid (HA) hydrogel with a wide-range stiffness gradient (between ~20 kPa and ~200 kPa) and cell adhesiveness was prepared by a liquid nitrogen (LN_2)-contacting gradual freezing–thawing method that does not use any additives or specific devices to produce the stiffness gradient hydrogel. From an *in vitro* cell culture using the stiffness gradient PVA/HA hydrogel, it was observed that human bone marrow mesenchymal stem cells have favorable stiffness ranges for induction of differentiation into specific cell types (~20 kPa for nerve cell, ~40 kPa for muscle cell, ~80 kPa for chondrocyte, and ~190 kPa for osteoblast). The PVA/HA hydrogel with a wide range of stiffness spectrum can be a useful tool for basic studies related with the stem cell differentiation, cell reprogramming, cell migration, and tissue regeneration in terms of substrate stiffness.

Statement of Significance

It is postulated that the stiffness of the extracellular matrix influences cell behavior. To prove this concept, various techniques to prepare substrates with a stiffness gradient have been developed. However, the narrow ranges of stiffness gradient and complex fabrication procedures/devices are still remained as limitations. Herein, we develop a substrate (hydrogel) with a wide-range stiffness gradient using a gradual freezing–thawing method which does not need specific devices to produce a stiffness gradient hydrogel. From cell culture experiments using the hydrogel, it is observed that human bone marrow mesenchymal stem cells have favorable stiffness ranges for induction of differentiation into specific cell types (~20 kPa for nerve, ~40 kPa for muscle, ~80 kPa for cartilage, and ~190 kPa for bone in our hydrogel system).

1. Introduction

The development, structural/functional maintenance, and regeneration of tissues/organs in the body are regulated in a sophisticated manner by a variety of biochemical and biophysical microenvironments [1–3]. Since the beginning of the concept of biochemistry in the 18th century [4], biochemical cues including growth factors, cytokines, and hormones have been studied extensively in order to discover their pivotal roles in living organisms. The acquired knowledge has become a notable resource for understanding various diseases and injuries as well as for finding appropriate therapeutics. Although the roles of biophysical cues on biological processes are less known than those of biochemical cues, it is also postulated that the biophysical properties (e.g., stiffness) of the extracellular matrix (ECM) influence the behavior and function of cells that are in direct and continuous contact with the ECM. In general, it is accepted that a substrate with a certain tissue-like stiffness provides an optimal environment for target cell differentiation and tissue formation [5]. In the body, the stiffness ranges of the ECM in different tissues vary between several kPa to more than a few hundreds kPa [6–8]. Since the first finding of a cell

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traction force, which was a fundamental concept for elucidating the effect of the stiffness of a substrate on the behavior of cells [9], many investigators have proven the effect of stiffness on cell behaviors [10–20]. Recently, various techniques to fabricate a substrate with a stiffness gradient have been developed for more systematic studies. They include controlled dipping of a substrate into a crosslinking solution [21], photopolymerization based on microfluidics (or gradient makers) [22–25], photopolymerization based on various masks (gradually darkening or sliding masks) [20,23,26–28], controlled height of the substrate [29], and temperature gradients during curing step [30]. However, the toxicity of residues (i.e., monomers, initiators, precursors, crosslinkers, etc.) [31–34], narrow ranges of stiffness gradient [29], different surface composition caused by different densities of precursors/crosslinkers along the gradient [21], complex fabrication procedures/devices, and low cell-adhesive surface (deficiency of specific interaction between synthetic polymeric surfaces and cells [35]) are still considered as hurdles of conventional techniques that need to be overcome for evaluation of the stiffness effect on cell behavior.

Herein, we report the fabrication of a substrate (hydrogel) with a wide-range stiffness gradient and cell adhesiveness using a gradual freezing–thawing method with a polyvinyl alcohol (PVA) and hyaluronic acid (HA) mixture solution (Fig. 1(A)). This method does not include toxic materials or specific devices to produce a stiffness gradient hydrogel. It is known that the freezing of a PVA aqueous solution can induce polymer crystallites (act as physical crosslinkers among PVA chains) as a result of ice formation in the amorphous region, and the repeated freezing–thawing of the solution can lead to the growth of the crystallites and transform the hydrogel without toxic chemicals (i.e., monomers, crosslinkers, precursors, etc.) [36]. The physical properties depend on the crosslinking (crystallite) density of the PVA hydrogel, which is influenced by the freezing temperature and time, polymer concentration, and the number of freezing–thawing cycles [35–37]. In our previous study [38], we demonstrated that the gradual freezing and thawing of a PVA aqueous solution can lead to gradual crosslinking density along the freezing direction, thereby producing a hydrogel with a stiffness gradient (stiffness ranges, 1–24 kPa). However, we also recognized that more wide-ranged stiffness gradient and cell compatible substrates should be developed to cover the stiffness range of the most tissue types (0.1–100 kPa [29]) and compensate the low cell adhesiveness of PVA hydrogel itself. Therefore, the main purpose of this study was (1) to fabricate a hydrogel with a wide-range of stiffness gradient that cover the stiffness values of most tissues in the human body as well as enhance cell adhesiveness for use as a tool to investigate biophysical effects on cell behavior and function; and (2) to determine the optimal substrate stiffness ranges for stem cell differentiation into pivotal cell types in the body such as nerve, muscle, cartilage, and bone cells. HA is a high molecular weight polysaccharide and one of the major components of the ECM in the body, and it plays an important role for cell growth, migration, and renewal [39–41]. HA was used as a cell-compatible additive for incorporation in the PVA hydrogel as a semi-interpenetrating polymer network (semi-IPN) to regulate the stiffness ranges and improve cell adhesiveness of the PVA hydrogel with a stiffness gradient.

2. Materials and methods

2.1. Fabrication of stiffness gradient PVA/HA hydrogel

PVA (Mw 89–98 kDa; hydrolysis, 98%; Aldrich, USA) and HA (MW, ~4800 kDa; Genewel, South Korea) were used to fabricate a stiffness gradient hydrogel. Cylindrical PVA/HA hydrogel with a stiffness gradient was prepared by a gradual freezing–thawing method [38]. In brief, 35 mL of 5 wt% PVA and 2 wt% HA aqueous solution was poured into a polypropylene (PP) mold (height, 80 mm; inner diameter, 25 mm). The mold, fixed at a clamp stand, was put in direct contact with a surface of LN_2 for 30 min at room temperature to enable gradual freezing of the whole PVA/HA solution from the bottom (LN_2 side) to the top (gradual freezing step); and then the frozen PVA/HA solution was stored at room temperature for 6 h (thawing step). This gradual freezing–thawing procedure was repeated for 10 cycles. The number of freezing–thawing cycles was optimized from our preliminary study (data not shown). The prepared cylindrical PVA/HA hydrogel (height, 70 mm; diameter, 25 mm) was cut into 5 mm transverse sections, and each section was characterized in terms of substrate stiffness.

2.2. Measurement of compressive modulus

The PVA/HA hydrogel sections (diameter 25 mm, thickness 5 mm) were placed between load cells (50 kgf) with round metal plate (Ø 40 mm) equipped at an universal testing machine (AG-5000G, Shimadzu). The specimens were compressed up to 80% strain of the specimen thickness (crosshead speed of 5 mm/min) and the compressive modulus (stiffness) was determined from the stress–strain curves.

To examine the stability of HA entrapped in the PVA/HA hydrogel in aqueous environment, the PVA/HA hydrogel sections were incubated in a phosphate buffered saline (PBS; pH ~ 7.4) at 37 °C for up to 4 weeks. After 2 and 4 weeks incubation, their compressive modulus (stiffness) change (possibly caused by the leaching-out of HA from the PVA/HA hydrogel) was also investigated using the same procedure above.

2.3. Measurement of crystallinity

The crystallinity [based on melting temperature (T_m) and heat of melting (ΔH_m)] of the prepared PVA/HA hydrogel sections were characterized using a differential scanning calorimetry (DSC) (DSC 2910, TA Instruments, USA). Each 2 mg freeze-dried sample was loaded in an aluminum pan. The sample was equilibrated at 25 °C and then heated from 25 °C to 250 °C (heating rate, 10 °C/min). The T_m and ΔH_m values of the PVA/HA hydrogel sections were determined from the melting peak of the DSC curves, and the crystallinity of the hydrogel sections was determined by dividing the ΔH_m of each section by the ΔH_m of a 100% crystalline PVA (138.6 J/g) [42].

2.4. Cell culture

The human bone marrow mesenchymal stem cells (*h*BMSCs; provided by Dongguk University (Prof. G.I. Im), South Korea) were isolated from three patients (age range, 37–64 years; mean age, 50 years) under approval from the Institutional Review Board of Dongguk University. Selected sections from the cylindrical PVA/HA hydrogel with a stiffness gradient [diameter, 25 mm; thickness, 5 mm; stiffness ranges, ~20 kPa, ~40 kPa, ~70 kPa, ~80 kPa, ~110 kPa, ~160 kPa, and ~190 kPa (7 sections)] were placed in 12-well polystyrene (PS) plates (Corning, USA). The *h*BMSCs at the third passage in a cell culture medium [Dulbecco's modified Eagle's medium/Ham's F-12 (DMEM/F-12; Gibco) containing 1% antibiotics (Gibco, USA) and 10% FBS (Gibco)] were seeded on the PVA/HA hydrogel sections (cell density, 1×10^6 cells/section), and the cells on the hydrogel sections were incubated at 37 °C in a humidified atmosphere with 5% CO_2 for up to 4 weeks. The culture medium was changed for fresh medium every third day. Cell adhesion and growth behavior on the hydrogel sections were determined by

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