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Injectable biodegradable hydrogels with tunable mechanical properties for the stimulation of neurogenesic differentiation of human mesenchymal stem cells in 3D culture

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ABSTRACT

We report an injectable hydrogel scaffold system with tunable stiffness for controlling the proliferation rate and differentiation of human mesenchymal stem cells (hMSCs) in a three-dimensional (3D) context in normal growth media. The hydrogels composed of gelatin-hydroxyphenylpropionic acid (Gtn–HPA) conjugate were formed using the oxidative coupling of HPA moieties catalyzed by hydrogen peroxide (H_2O_2) and horseradish peroxidase (HRP). The stiffness of the hydrogels was readily tuned by varying the H_2O_2 concentration without changing the concentration of polymer precursor. We found that the hydrogel stiffness strongly affected the cell proliferation rates. The rate of hMSC proliferation increased with the decrease in the stiffness of the hydrogel. Also, the neurogenesis of hMSCs was controlled by the hydrogel stiffness in a 3D context without the use of any additional biochemical signal. These cells which were cultured in hydrogels with lower stiffness for 3 weeks expressed much more neuronal protein markers compared to those cultured within stiffer hydrogels for the same period of time.

1. Introduction

Cell therapies represent a promising way to treat a variety of diseases and injuries in the area of regenerative medicine and tissue engineering [1–3]. The use of human mesenchymal stem cells (hMSCs) in cell therapies may be advantageous owing to their high accessibility and ease of handling. They have been reported to differentiate into multiple cell lineages, including osteoblasts, chondroblasts, adipocytes, neurons, skeletal myoblasts, and cardiac myocytes [4] and have played progressively prominent roles in tissue engineering due to their relative ease of isolation and no tumorigenic potential *in vivo* [5]. Recently, there has been increasing recognition that substrate mechanics [6] and the topography of the extracellular microenvironment [7] can modulate the tissue cell phenotype in a way similar to biochemical signals. When hMSCs are cultured on collagen-coated polyacrylamide gels with different stiffness, hMSCs are differentiated to neuronal, muscle, and bone cells as the stiffness of the gel is increased [8]. These results imply potential applications for hMSCs in tissue regeneration could be gained with a suitable material system that provides for the culture and differentiation of cells in a three-dimensional (3D) context, as cells behave more physiologically in a 3D environment compared to 2D surfaces. In addition, it is essential to administer the cells to the precise location for tissue repair and regeneration [9].

The use of hydrogels as scaffolds for cultivating cells in a 3D environment is attractive, because hydrogels have high permeability for oxygen, nutrients and other water-soluble metabolites through their high water-content matrix, which is an excellent environment for cell growth and tissue regeneration [10]. Furthermore, the use of injectable hydrogels as scaffolds in tissue engineering is advantageous compared to preformed hydrogels because they are able to fill any shape or defect; they can be easily formulated with cells by simple mixing, and do not require a surgical procedure to be implanted or in the case of biodegradable ones, to be removed. Another important requirement for an injectable hydrogel system is for the hydrogel to be formed rapidly after injection, to prevent the undesirable diffusion of the gel precursors and cells to the surrounding tissues [11]. However, a major drawback of existing injectable hydrogel systems concerns the control of the gelation rate. The means of controlling the gelation rate is limited to varying the gel precursor and/or crosslinker concentration, which inevitably changes the stiffness of the hydrogel and leads to the undesirable control of the cell growth and differentiation in hydrogels. Also, such systems would require a lot of optimization in order to achieve the appropriate growth rate and control over the differentiation of cells, because different

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concentrations of precursor polymer would be used to control the gelation rate. Using different concentrations of the polymer precursor may cause changes in the interaction between polymer chains and cells, especially when the polymer chains are conjugated with cell adhesive ligands such as Arg-Gly-Asp (RGD) peptides. Here, we demonstrate how this limitation can be overcome through the enzyme-mediated oxidation crosslinking reaction of gelatin-hydroxyphenylpropionic acid (Gtn–HPA) conjugates. Recently, the independent tuning of the stiffness and the gelation rate of an injectable hydrogel has been achieved by utilizing a simple solution mixture of constant concentrations of hyaluronic acid-phenol conjugates, hydrogen peroxide (H_2O_2) and horseradish peroxidase (HRP) [12]. The hydrogels were formed through the oxidative coupling of phenol moiety, which was catalyzed by H_2O_2 and HRP [12–14]. The H_2O_2 and HRP determined the hydrogel stiffness and the gelation rate of the injectable hydrogel, respectively.

Neural tissue engineering is an emerging research area for the treatment of injuries of central nervous system (CNS) [15–17]. Hydrogel scaffolds have been recognized as an attractive strategy for the regeneration of damaged tissue in CNS and used to release trophic factors, bridge spinal cord defects and enhance cell infiltration [15–23]. In addition, stem cell-based tissue regeneration strategies have been widely investigated for the treatment of spinal cord and traumatic brain injuries [16]. It has been reported that 3D culture of murine embryonic and neural stem cells in hydrogel shows the enhanced neuronal differentiation [24–27]. However, it has been suggested that controlled neural differentiation of hMSCs might become an important source of cells for cell therapy of neurodegenerative diseases, since hMSCs are more easily harvested and effectively expanded than neural stem cells [28]. From these perspectives, we consider that the design of an injectable hydrogel system that differentiates hMSCs to neuronal cells would be important for the treatment of central nervous injuries. We herein report enzyme-mediated Gtn–HPA hydrogels with tunable mechanical strength for controlling cell growth and neurogenesis of hMSCs in 3D (Fig. 1).

2. Materials and methods

2.1. Materials

Gelatin (Gtn) (MW = 80–140 kDa, pI = 5) and horseradish peroxidase (HRP) (100 units/mg) were obtained from Wako Pure Chemical Industries (Japan). 3,4-Hydroxyphenylpropionic acid (HPA), *N*-hydroxysuccinimide (NHS), 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC·HCl), type I collagenase (246 units/mg), Triton X-100, bovine serum albumin (BSA), anti-β-tubulin, anti-neurofilament light chain (NFL) and anti-microtubule associated protein 2 (MAP2) were purchased from Sigma-Aldrich (Singapore). Anti-neurofilament heavy chain was obtained from Zymed (USA). Human mesenchymal stem cells (hMSCs) were provided by Cambrex Bio Science Walkersville, Inc. (USA). Mesencult human basal medium supplemented with mesencult human supplement was purchased from Stem Cell Technologies (Canada). Fetal bovine serum (FBS), calcein acetoxymethyl ester, 4′,6-diamidino-2-phenylindole (DAPI) and fluorophore-conjugated secondary antibodies were provided by Invitrogen (Singapore). HRP-conjugated secondary antibodies were purchased from GE Healthcare (Singapore). Actin/focal adhesion stain kit were provided by Millipore (Singapore). Phosphate buffer saline (PBS, 150 mM, pH 7.3) solution was supplied by media preparation facility in Biopolis (Singapore).

2.2. Synthesis of Gtn–HPA conjugate

3,4-Hydroxyphenylpropionic acid (HPA) was used to synthesize Gtn–HPA conjugates by a general carbodiimide/active ester-mediated coupling reaction in distilled water [29]. HPA (3.32 g, 20 mmol) was dissolved in 250 ml of mixture of distilled water and *N*,*N*-dimethylformamide (DMF) (3:2). To this *N*-hydroxysuccinimide (3.20 g, 27.8 mmol) and 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (3.82 g, 20 mmol) were added. The reaction was stirred at room temperature for 5 h, and the pH of the mixture was maintained at 4.7. Then, 150 ml of Gtn aqueous solution (6.25 wt.%) was added to the reaction mixture and stirred overnight at room temperature at pH 4.7. The solution was transferred to dialysis tubes with molecular cut-off of 1000 Da. The tubes were dialyzed against 100 mM sodium chloride solution for 2 days, a mixture of distilled water and ethanol (3:1) for 1 day and distilled water for 1 day, successively. The purified solution was lyophilized to obtain the Gtn–HPA.

2.3. Rheological measurement

Rheological measurements of the hydrogel formation were performed with a HAAKE Rheoscope 1 rheometer (Karlsruhe, Germany) using a cone and plate geometry of 35 mm diameter and 0.945° cone angle. The measurements were taken at 37 °C in the dynamic oscillatory mode with a constant deformation of 1% and frequency of 1 Hz. To avoid slippage of samples during the measurement, a roughened glass bottom plate was used. The solutions of HRP and H_2O_2 with different concentrations were added sequentially to an aqueous solution of Gtn–HPA (2 wt.%, 250 μl in PBS). The solution was vortexed and then immediately applied to the bottom plate. The upper cone was then lowered to a measurement gap of 0.024 mm and a layer of silicon oil was carefully applied around the cone to prevent solvent evaporation during the experiment. The measurement parameters were determined to be within the linear viscoelastic region in preliminary experiments.

2.4. Enzymatic degradation of Gtn–HPA hydrogels

For the preparation of slab-shaped Gtn–HPA hydrogels, lyophilized Gtn–HPA was dissolved in PBS at a concentration of 2 wt.%. 6 μl of HRP was added to 1 ml of Gtn–HPA solution to give a final concentration of 0.15 units/ml. Crosslinking was

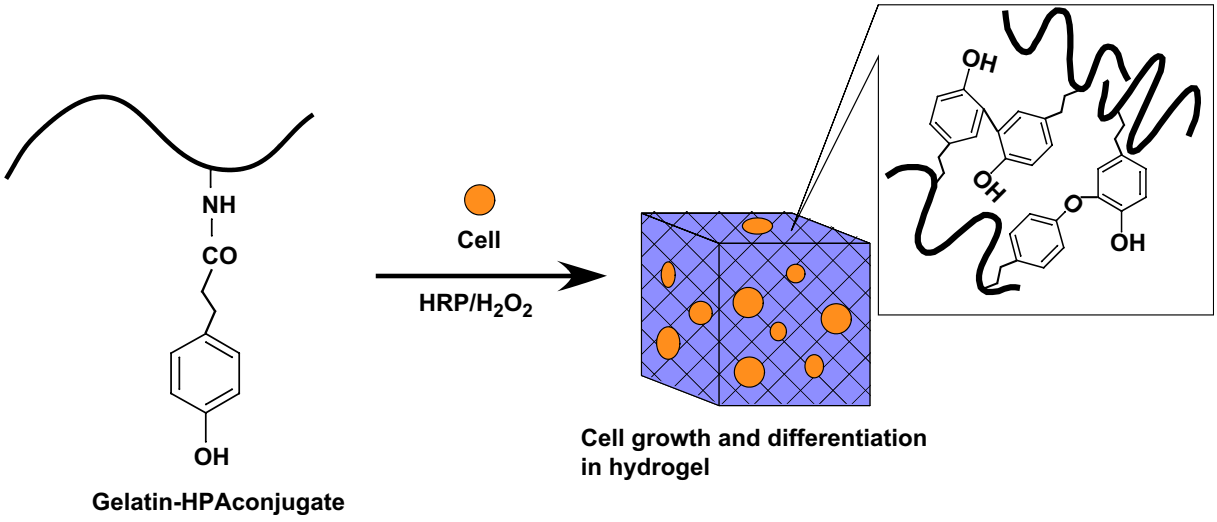

Fig. 1. *In situ* forming of Gtn–HPA hydrogel by an enzyme-catalyzed oxidation for 3D cell growth and differentiation.