Transfer stamping of human mesenchymal stem cell patches using thermally expandable hydrogels with tunable cell-adhesive properties

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ABSTRACT

Development of stem cell delivery system with ability of control over mutilineage differentiation and improved engraft efficiency is imperative in regenerative medicine. We herein report transfer stamping of human mesenchymal stem cells (hMSCs) patches using thermally expandable hydrogels with tunable cell-adhesive properties. The hydrogels were prepared from functionalized four arm copolymer of Tetronic®, and the cell adhesion on the hydrogel was modulated by incorporation of fibronectin (FN) or cell-adhesive peptide (RGD). The resulting hydrogels showed spontaneous expansion in size within 10 min in response to the temperature reduction from 37 to 4°C. The adhesion and proliferation of hMSCs on FN-hydrogels were positively tunable in proportion to the amount of FN within hydrogels with complete monolayer of hMSCs (hMSC patch) being successfully achieved. The hMSC patch on the hydrogel was faced to the target substrate, which was then easily detached and re-attached to the target when the temperature was reduced from 37°C up to 4°C. We found that the transfer stamping of cell patch was facilitated at lower temperature of 4°C relative to 25°C, with the use of thinner hydrogels (0.5 mm in thickness relatively to 1.0 or 1.5 mm) and longer transfer time (>15 min). Notably, the hMSC patch was simply transferred from the hydrogel to the subcutaneous mouse skin tissue within 15 min with cold saline solution being dropped to the hydrogel. The hMSC patch following osteogenic or adipogenic commitment was also achieved with long-term culture of hMSCs on the hydrogel, which was successfully detached to the target surface. These results suggest that the hydrogels with thermally expandable and tunable cell-adhesive properties may serve as a universal substrate to harvest hMSC patch in a reliable and effective manner, which could potentially be utilized in many cell-sheet based therapeutic applications.

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1. Introduction

Stem cells derived from a variety of organs or tissues have been investigated as one of the most prominent therapeutic cell sources since they have two remarkable properties: (1) the capability of indefinite self-renewal and (2) pluripotency (the ability to generate many cell types) [1–3]. However, conventional transplantation of stem cells involves syringe-based injection following the process of detachment from the culture plate and re-suspension, which is prone to exhibit low engraftment efficiency due to rapid diffusion, limited localization to the host tissue, damage by enzymatic digestion, and shear-induced cell death. In addition, other challenges facing stem cell therapy in a functional recovery of damaged tissue include maintenance of unlimited proliferation of stem cells and selective activation of lineage-dependent cell signaling [4–7].

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In order to improve the overall therapeutic effects of stem cells, a number of delivery methods using biodegradable materials have been developed with being designed to be implantable with tunable degradation rate, mechanical properties, and cellular affinity [3,8,9]. However, biomaterials occasionally displayed several drawbacks such as insufficient cell recruitment and chronic inflammation by uncontrolled degradation of implants [10—12]. Alternatively, a confluent cell monolayer has been harvested using responsive surfaces that can be transplanted to target tissue with avoiding implantation of biomaterials [13,14].The most common method for this approach uses a poly(N-isopropylacrylamide) (PNIPAAm)-grafted surface on which the cell sheet spontaneously detaches from the surface by thermo-rheological transition of the polymeric chains at a lower temperature (20°C) [14]. In addition, enzymatically-degradable polymers (A6K (arginine6-lysine)), magnetite cationic liposomes, and physical stimuli such as electricity and light have also been employed to harvest cell sheets [15—18].

Despite its therapeutic outcomes in many types of tissue, the process to harvest cell sheet possesses several technical challenges. The reversible detachment of cell sheet on the PNIPAAm-grafted surface is sensitive to the density and thickness of the grafted polymer chains, and thus, it may be difficult to achieve consistent level of surface polymerization in laboratory conditions [19]. The varied affinity of cells onto the PNIPAAm-grafted surface may also cause problems: (1) cells with low affinity takes long culture time to reach a confluent monolayer, and (2) cells with high affinity require longer time for detachment even under reduced temperatures (reportedly requiring >30 min). Cell-instructive cues may be immobilized on the PNIPAAm-grafted surface to modulate lineage-dependent differentiation of stem cells, but at the same time, it may potentially decrease the detachment rate of the cell sheet. Other engineering tools to harvest cell sheets showed some drawbacks such as toxicity of magnetic nanoparticles or enzymes, and low versatility [16,20]. Therefore, a universal approach to effectively control stem cell function and rapidly detach the stem cell sheet is desired.

We and our colleagues previously reported transfer stamping of cell patch using thermosensitive Tetronic®-based hydrogels *in situ* polymerized with a cell-adhesive (arginine-glycine-aspartic acid (RGD)) peptide [21—23]. The cell patch transfer was achieved by rapid cleavage between cell receptors and the cell-adhesive peptide on the hydrogel upon rapid expansion of hydrogels when the temperature is reduced from 37°C to lower one. However, the culture of stem cells as a monolayer on these hydrogels was challenging likely due to weak specificity of the peptide to stem cells or change in mechanical properties of the hydrogels. In this study, we developed thermally expandable hydrogels incorporating fibronectin (FN) with tunable adhesive properties of human mesenchymal stem cells (hMSCs). The effect of FN on adhesion, proliferation, and formation of a confluent layer of hMSCs was first examined. We then investigated the effect of several parameters such as temperature and thickness of hydrogels regulating degree and rate of hydrogel expansion, and transfer time on successful detachment and re-attachment of cell patch to various model

target substrates (glass, polymeric nanofibers, and subcutaneous tissue of mouse). Finally, we showed the potential delivery of stem cell patch with being committed to osteogenic or adipogenic lineage.

2. Materials and methods

2.1. Materials

Human plasma fibronectin (FN) and purified anti-FN were purchased from BD Biosciences (Franklin Parks, NJ, USA). A synthetic peptide, GRGDGGGGGY, was custom-ordered from AnyGen (Gwangju, Korea). Anti-laminin was obtained from Abcam (Cambridge, MA, USA). Anti-connexin 43 (CX43) was purchased from Cell Signaling Technology Inc. (Danvers, MA, USA). Hoechst 33258, Vybrant™ DiD cell-labeling solution and Live/Dead viability/cytotoxicity kits were purchased from Molecular Probes (Eugene, OR, USA). Fluorescein isothiocyanate (FITC), anti-mouse IgG biotin conjugate, Mayer's hematoxylin, peroxidase from horseradish (HRP), hydrogen peroxide (H_2O_2) and fluorescamine were purchased from Sigma—Aldrich (St. Louis, MO, USA). Fetal bovine serum (FBS) was obtained from Wisent (St.-Bruno, QC, Canada), and Dulbecco's modified Eagle's medium including low glucose medium (DMEM), Dulbecco's phosphate buffered saline (PBS), trypsin/EDTA, and penicillin—streptomycin (p/s) were purchased from Gibco BRL (Carlsbad, CA, USA). Rhodamine-phalloidin was purchased from Invitrogen Corp. (Carlsbad, CA, USA). FITC-conjugated streptavidin was obtained from ebioscience (San Diego, CA, USA).

2.2. Preparation of hydrogels containing FN and peptide

Tetronic®-tyramine was synthesized and characterized as described previously [24]. Briefly, terminal hydroxyl groups of Tetronic 1307 (4-arm-polypropylene oxide—polyethyleneoxide, MW. 18,000, BASF, Germany) was conjugated with p-nitrophenylchloroformate (PNC) to form Tetronic®-PNC, which was subsequently used to synthesize Tetronic®-tyramine by coupling reaction between Tetronic®-PNC and tyramine. The hydrogels were prepared by previously described methods with minor modifications [24,25]. We prepared two separate solutions of Tetronic®-tyramine with (1) 0.1 wt% of H_2O_2 in PBS and (2) 0.025 mg/ml of HRP in PBS. The solution (2) contained defined concentrations of FN (10, 25, and 50 μg/ml for FN-10, FN-25, and FN-50 hydrogels, respectively) or cell-adhesive RGD-containing peptide (4 mg/ml). The final concentration of Tetronic®-tyramine in all groups was equally adjusted to 7.63 wt%. The two solutions were separately filled within the chamber of the dual-syringe, which were then co-injected into the space between glass plates separated by a Teflon spacer. The crosslinking reaction rapidly occurred within 1—2 min to form hydrogels incorporating FN or peptide. After the *in situ* crosslinking reaction, circular-shaped samples were obtained using a biopsy punch (8-mm diameter, Stiefel®, Verkaufsinnendienst, Germany). The code names and compositions of the hydrogel used in this study are listed in Table 1.

2.3. Characterization of hydrogels

The incorporation of FN within the hydrogels was confirmed using fluorescence-based techniques. FN hydrogels (8 mm in diameter, 0.5 mm in thickness) were immersed in 1 ml of an FITC solution (2 mg/ml in EtOH) at room temperature for 4 h and then continuously washed with EtOH for 2 h and PBS for 6 h. FITC-labeled FN hydrogels were visualized using a fluorescence microscope (Nikon TE-2000, Nikon Corp., Tokyo, Japan). Using NIH Image J medical imaging software (http://rsbweb.nih.gov/ij/, National Institutes of Health, Bethesda, MD, USA), the relative fluorescence units (RFU) of the FN distribution was quantified. The incorporated FN density on hydrogels was determined by a fluorescamine assay. Briefly, hydrogel discs (8 mm in diameter, 0.5 mm thick) were placed in a microtube containing 1 ml PBS solution under orbital shaking (RPM 250, 37°C). At designated time points, the solution was collected and immersed in another 1 ml of fresh PBS solution. The supernatant was collected and subsequently reacted with a fluorescamine solution (100 μg/ml in acetone) for 60 s under vortexing. The fluorescent intensity of the supernatant was analyzed using a spectrofluorometer (Molecular Devices, Sunnyvale, CA, USA) at an excitation and emission wavelength of 390 nm and 475 nm, respectively. A standard curve was prepared with FN solutions (0.05—50 μg/ml). The FN reaction yield was determined using the following equation: (FN reaction yield (%) = (value for the theoretically incorporated FN into the hydrogels − value from experimental FN density on the hydrogels) x 100). The Young's modulus of the FN

Table 1
Composition of FN-hydrogels.

Sample code	Component A (HRP)[a]		Component B (H_2O_2)[b]
	Tetronic®-tyramine Concentration (wt%)	Fibronectin concentration (μg/ml)	Tetronic®-tyramine concentration (wt%)
FN-0	7.6	0	7.6
FN-10	7.6	10	7.6
FN-25	7.6	25	7.6
FN-50	7.6	50	7.6

[a,b]The Final concentration of Tetronic®-tyramine in all blended solution was 7.6 wt%.