Supporting Information

Formation of stem cell aggregates and their differentiation on surface-patterned hydrogels based on poly(2-hydroxyethyl methacrylate)

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Expansion of human mesenchymal stem cells

For subculturing, the cells were detached from T125 tissue culture flask by incubating with trypsin-EDTA for 2 min at 37 °C and 5% CO₂. Complete culture media (double the volume of trypsin-EDTA) was added to deactivate trypsin and the cell suspension was centrifuged at 2000 rpm for 2 min. The cell pellet was dispersed in new growth media, mixed with Trypan blue (Thermo Fisher Scientific) and the cell count was obtained using a Countess® automated cell counter (Thermo Fisher Scientific). The cells were divided into T175 flasks and cultured at 37 °C and 5% CO₂ in the humidified incubator until 70% confluent, to produce enough cells for the experiments.
Staining protocols

Rhodamine phalloidin and DAPI

The cells were fixed with 10% neutral buffered formalin for 30 min, permeabilized with Triton® X-100 in 0.1% BSA in PBS for 15 min and stained with Rhodamine phalloidin (1:1000 dilution in 0.1% BSA and 0.1% Tween® 20 in PBS) for 2 h at room temperature in the dark. Each step was followed by two rinsing steps in 0.1% BSA in PBS. The nuclei were counterstained with DAPI (1:5000 dilution in 0.1% BSA and 0.1% Tween® 20 in PBS) for 5 min at room temperature in the dark.

Oil Red O staining

First, 0.3% Oil Red O stock solution, prepared in isopropanol, was diluted with distilled water to generate a 0.18% working solution. Then the fixed cells were incubated in 60% isopropanol for 5 min followed by staining with the Oil Red O solution for 15 min at room temperature. Finally, the cells were washed with distilled water until the water became clear.

Alcian blue staining

At the end of the experiment, the cells were fixed with 10% neutral buffered formalin for 2 h and washed with DI water. Then, the samples were incubated in the Alcian blue staining solution, prepared in 3% acetic acid solution (pH 2.5), for 4 h in the dark at room temperature. The samples were washed with the destaining solution (3% acetic acid) thrice. After each wash, the samples were left in DI water for 20 min and finally stored in PBS. Bright-field images of each sample were obtained from the Olympus-IX83 inverted microscope.
**Immunostaining**

For immunostaining experiments, the cells were fixed and then digested in Pronase E solution for 20 min for epitope unmasking. After the Pronase E digestion, the cells were permeabilized with 0.1% Triton® X-100 in PBS for 15 min, rinsed with PBS, followed by incubation in 10% goat serum in PBS for 1 h. Then the samples were incubated in primary antibody solutions (anti-collagen type II primary antibody solution (COL II), 1:10 dilution in 1% goat serum or IgG, 1:2000 dilution in 1% goat serum) for 3 h at room temperature. After rinsing with PBS twice, the samples were incubated in the secondary antibody (FITC-conjugated goat anti-mouse IgG) solution (1:50 dilution) in PBS with 0.05% Tween® 20 for 2 h at room temperature. The samples were washed with PBS three times, counterstained with DAPI for 5 min, and imaged by the Olympus-IX83 inverted microscope using GFP and DAPI channels.

**Preparation of samples for Scanning Electron Microscopy (SEM) analysis**

After the completion of the experiment, the hMSCs cultured on hydrogels samples were fixed in 2% glutaraldehyde in 0.2 M cacodylate buffer for at least 2 h followed by three washes (15 min each) in 0.1 M buffered wash solution (cacodylate buffer with sucrose). Then, the samples were fixed with 1% OsO₄ in 0.2 M cacodylate buffer for 1 h followed by three buffer washes (15 min each). After fixation, the samples were dehydrated in a concentration series of absolute ethanol and water (50%, 70%, 90%, 95%, and 100%). Here the samples were left in each solution for 15 min and three times (15 min each) in 100% ethanol. The dehydrated samples were transferred to critical point drying holders and critical point dried. Next, the samples were mounted on aluminum stubs, coated with Au/Pd for 1 min, and imaged from an Environmental Scanning Electron Microscope (FEI Quanta 600 FE – ESEM).
The effect of media change on retention of cell aggregates on the hydrogel samples

Cell aggregates are loosely attached to the surface due to the hydrophilicity of the hydrogel and can be easily washed away during media exchange. To evaluate the loss of cell aggregates from media exchange, media was replaced either by aliquots (Method A) or completely (Method B) using a micropipette (seeding density was $3.1 \times 10^4$ cells/cm$^2$ and the cells were cultured for five days by refreshing media every other day). In Method A, a portion of spent media (250 µL) was removed and replaced with the same volume of new media ensuring that the hydrogel surface was covered throughout the entire time. The process was repeated 3-4 times to make sure that the spent media was replaced with sufficient new media. In Method B, the spent media was completely (1 mL at once) removed before adding new media. Bright-field images were taken before each media change. After five days, the aggregates were counted on each sample and compared to the number of aggregates present at day 1. Two methods were statistically compared using a Student T-test ($p < 0.05$).
Figure S1. The effect of media change on retention of cell aggregates on hydrogel samples; Method A – media was changed in aliquots so that the hydrogel surface was covered by media entire time, Method B – spent media was completely removed before adding new media.

Figure S2. The graph of %weight loss for poly(HEMA/DMAEMA/TEGDMA) hydrogel as a function of time.

Figure S3. (a) Bright-field and (b) fluorescent images of a cell aggregate on a hydrogel surface bearing 2.05 µm pillars; actin and nuclei were stained with Rhodamine phalloidin and DAPI, respectively.
**Figure S4.** Light microscopy images of Oil Red O-stained, large cell aggregates formed on hydrogel samples bearing 6 µm pillars treated with normal culture medium at different seeding densities (a) $1.6 \times 10^5$ cells/cm$^2$ and (b) $2.6 \times 10^5$ cells/cm$^2$.

The red color appeared on the edges of the cell aggregates can be an indication of positive adipogenic differentiation and the black-brown color in the center might be due to the poor penetration of light through the aggregate caused by the thickness of the aggregate.

**Table S1.** Contact angle measurements of hydrogel samples.

<table>
<thead>
<tr>
<th>Hydrogel Sample</th>
<th>Contact Angle (°)</th>
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<tbody>
<tr>
<td>Blank</td>
<td>55.464 (±4.387)</td>
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<tr>
<td>2.05 µm pillars</td>
<td>67.113 (±0.850)</td>
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<tr>
<td>4.91 µm pillars</td>
<td>71.968 (±1.652)</td>
</tr>
</tbody>
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Table S2. Contact angle hysteresis measurements of hydrogel samples.

<table>
<thead>
<tr>
<th>Hydrogel Sample</th>
<th>Advancing Angle (°)</th>
<th>Receding Angle (°)</th>
<th>Hysteresis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blank</td>
<td>73.896 ± (9.310)</td>
<td>54.809 ± (4.930)</td>
<td>19.088 ± (5.059)</td>
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<tr>
<td>2.05 µm pillars</td>
<td>84.904 ± (2.647)</td>
<td>36.279 ± (2.991)</td>
<td>48.625 ± (3.453)</td>
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<tr>
<td>4.91 µm pillars</td>
<td>87.471 ± (2.232)</td>
<td>42.679 ± (7.116)</td>
<td>44.793 ± (7.785)</td>
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