Electronic Supplementary Material (ESI) for Biomaterials Science.

Supplementary Information for

Bulk poly(*N*-isopropylacrylamide) (PNIPAAm) thermoresponsive cell culture platform: toward a new horizon in cell sheet engineering

Andrew Choi, Kyoung Duck Seo,[a] Hyungjun Yoon, Seon Jin Han, and Dong Sung Kim *

Department of Mechanical Engineering, Pohang University of Science and Technology (POSTECH), 77 Cheongam-ro, Nam-gu, Pohang 37673, Republic of Korea

* Corresponding author: smkds@postech.ac.kr

[a]Present address: Department of Mechanical Engineering, Wonkwang University, 460, Iksan-daero, Iksan 54538, Republic of Korea

Experimental

2.1 Fabrication of bulk PNIPAAm hydrogels

A bulk PNIPAAm hydrogel was synthesized through a simple UV photopolymerization method. The monomer solution was prepared by mixing *N*-isopropylacrylamide (NIPAAm, Sigma Aldrich, USA) and distilled water in a weight (wt) ratio of 9:1 and subsequently adding 0.05 wt% of a photoinitiator, 2-hydroxy-1-[4-(hydroxyethoxy)-phenyl]-2-methyl-1-propanone (Irgacure 2959, BASF, Germany) and 1–5 wt% of a cross-linker, N,N'-methylenebisacrylamide (MBAAm, Sigma Aldrich, USA), depending on the suitable surface roughness required for each examined cell type. The prepared monomer solution was then poured into a polydimethylsiloxane (PDMS, Sylgard 184, Dow Corning, USA) mold that was replicated from a polymethylmethacrylate (PMMA, Acryl Choika, Korea) master mold previously fabricated using a laser cutter (IS350, INNOSTA, Korea) in a targeted shape of the final hydrogel. The poured solution was covered with a polyethylene terephthalate film (PET, Goodfellow, UK) and then photopolymerzied through exposure to a UV light source (LC8, Hamamatsu, Japan) at a power of 1800 W (λ = 365 nm) for 10 min. After its complete polymerization, the fabricated bulk PNIPAAm hydrogel was easily removed from the PDMS mold. During the fabrication process, the ambient condition of 25 °C was deliberately maintained considering the sensitive thermoresponsiveness of the monomer solution containing the core material, NIPAAm.

2.2 Sterilization

Before the cell culture, the fabricated PNIPAAm hydrogels underwent medium detoxification (MD) process for 5 days inside the specified culture medium according to each type of the cells later being cultured on the cell culture platform. During the process, the unreacted cross-linkers

were effectively eliminated from the hydrogels, and the essential nutrients were enriched inside them. Then, the surface of the prepared hydrogels was treated by oxygen plasma (VITA1, Femto Science, Korea) at a power of 50 W for 10 s to enhance surface hydrophilicity and cell adhesion. Finally, the hydrogels were sterilized under UV light overnight prior to their use.

2.3. Absorption of BSA contents inside of PNIPAAm hydrogel

0.2 g BSA was dissolved in 100 mL PBS solution in order to generate a 2 mg/mL BSA solution. The MD processed PNIPAAm hydrogel was immersed inside of culture medium, and 25 µl of the cell culture medium was aliquoted in 96 well plate in a time interval of 15 min and was mixed with a 200 µl working reagent. Then, the samples were incubated at a temperature condition of 37 °C. The ultraviolet absorption peak at 562 nm was then measured while the standard curve of BSA with different concentrations was established in advance.

2.4 Culture of diverse cells

As cell culture platforms, the sterilized hydrogels were pretreated with 5 µg/mL fibronectin (FN, Roche, Germany) for 2 h. Afterward, mouse myoblasts (C2C12, Korean Cell Line Bank, Korea), mouse embryo fibroblasts (NIH3T3, Korean Cell Line Bank, Korea), HUVECs (Promocell, Germany), and human epidermal cells (keratinocytes, American Type Culture Collection, USA) were cultured on the Upcell® plate (CellSeed, Inc., Tokyo, Japan) and prepared bulk PNPIAAm cell culture platforms with 1, 2, 3, and 5 wt% of the cross-linker concentrations which were denoted by PNIPAAm_cs-1, PNIPAAm_cs-2, PNIPAAm_cs-3, and PNIPAAm_cs-5, respectively. All cells were seeded at a density of 1×10^5 cells/cm^2 on the surface of the prepared cell culture platform to achieve a confluent cell sheet after 72 h of incubation at 37 °C with 5%

CO_2. C2C12 and NIH3T3 cells were cultured in high-glucose Dulbecco's modified Eagle's medium (HG-DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS, Hyclone) and 1% penicillin/streptomycin (Hyclone), whereas HUVECs and keratinocytes were cultured in endothelial cell growth medium (EGM-2, Lonza) and EpiLife™ medium with human keratinocyte growth supplement (Gibco), respectively.

2.5 Live/dead assay

All cultured cells were rinsed twice with 1 × phosphate-buffered saline (PBS, Hyclone) and stained with calcein AM (green) and ethidium homodimer-1 (red) using a LIVE/DEAD assay kit (Molecular Probes, USA) to determine the ratio of live to dead cells and indirectly observe the morphology of the cells. Their fluorescent images were acquired using a phase-contrast inverted fluorescence microscope (TS100F, Nikon, Japan).

2.6. Immunofluorescence imaging of diverse cell sheets

After 3 days of the cell culture, the cultured cell sheets were fixed in 4% paraformaldehyde solution (pH 7.4) for 15 min inside the incubator and they were permeabilized with 0.3% Triton™ X-100 (Sigma-Aldrich, USA) containing PBS solution for 10 min. The cell sheets were washed using PBS solution, and were placed in a blocking buffer for 1 h inside of the incubator. Subsequently, the cell sheets were incubated with the diluted primary antibody solution containing Type 1 collagen (1:100;Abcam, UK) or Zo-1 (1:100;Thermo Fisher Scientific, USA) for 1 h. Lastly, the nuclei counterstaining was performed with DAPI (Sigma Aldrich, USA) for 5 min. The fluorescent images were acquired using a phase-contrast inverted fluorescence microscope (TS100F, Nikon, Japan).

2.7. Detachment mechanism of the cell sheet

The detachment mechanism of the cell sheet from PNIPAAm hydrogel surfaces was investigated by treating seeded samples with inhibitors that modulate contractility or adhesion. After 3 days of cell culture, the cells were individually exposed to rho-associated protein kinases (ROCK) inhibitor, Y-27632, and 3,3'-dithiobis-sulfosuccinimidylpropionate (DTSSP) inhibitor. The cultured cell sheets were separately exposed to 50 μM Y-27632 and 2 mM DTSSP for 30 min prior to initiating their detachment.

2.8 Cell morphology analysis for evaluation of universal PNIPAAm cell culture platform ~~in bulk form (UpB)~~

The morphology (circularity and spreading area) of the cells was examined daily with the microscopic images acquired using a phase-contrast inverted fluorescence microscope (TS100F, Nikon, Japan) to analyze their attachment behavior. The perimeter and the spreading area of individual cell composing the cell sheet was measured utilizing Image J software (NIH, USA). And the circularity of the cells was calculated the formula below:

$$Circularity = \frac{4\pi * A}{(P)^2},$$

where A and P are the spreading area and perimeter of individual cells composing the cell sheet, respectively. Such formula provides a number from 0-1, where circularity value below 0.5 indicated elongated cells and the value above 0.5 indicated rounded cells.

2.9 Characterization of interior morphologies of PNIPAAm hydrogels

After being frozen in liquid nitrogen, the fabricated PNIPAAm hydrogels were dehydrated for 7 days inside a vacuum freeze-dryer (Operon, Korea) at −120 °C. The dehydrated samples were then cross-sectioned into pieces and were sputtered with Pt. Afterward, the interior morphologies of the PNIPAAm hydrogels were successfully examined with a use of field emission scanning electron microscope (FE-SEM, Hitachi, Japan) at an acceleration voltage of 20 kV. The interior morphologies of the PNIPAAm hydrogels and their degrees in porosity were further statistically analyzed by utilizing Image J software (NIH, USA). In addition, the surface hardness of the fabricated hydrogels inside of culture medium was investigated using a Nano Indenter XP (MTS, USA).

2.10 Water uptake measurement

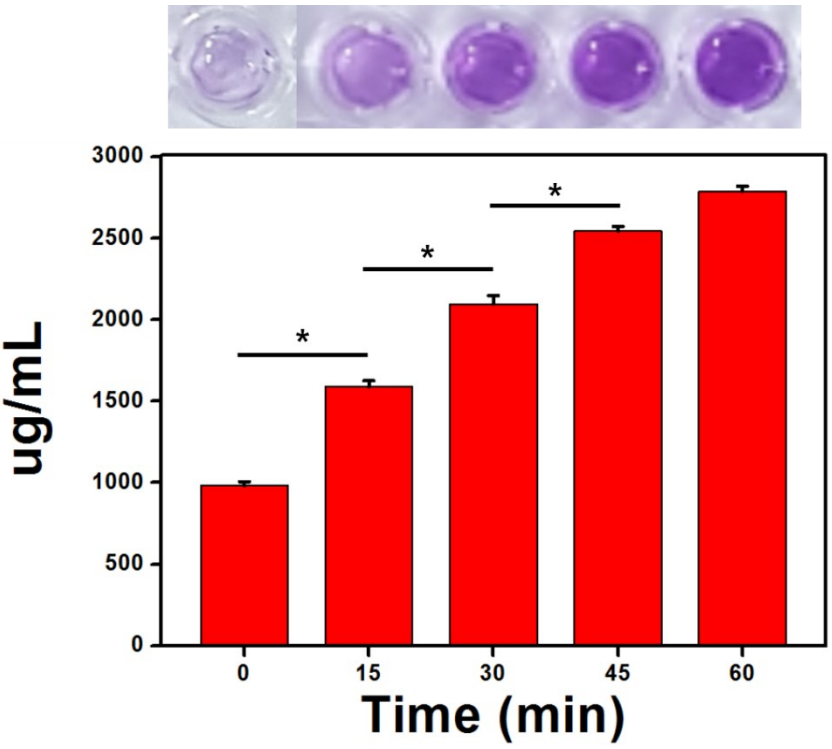
The water uptake percentages of the fabricated PNIPAAm hydrogels were measured in accordance with ASTM D 570-98. The initial weights of the prepared hydrogels before their immersion were attained, and all prepared hydrogels were soaked into the culture medium at 20 °C. In a time interval of 10 min, the samples were taken out from the medium, and each of their final weight was measured. The percentage of water uptake was evaluated using the formula below:

$$W_{\%} = \frac{W_f - W_0}{W_0} \times 100 ,$$

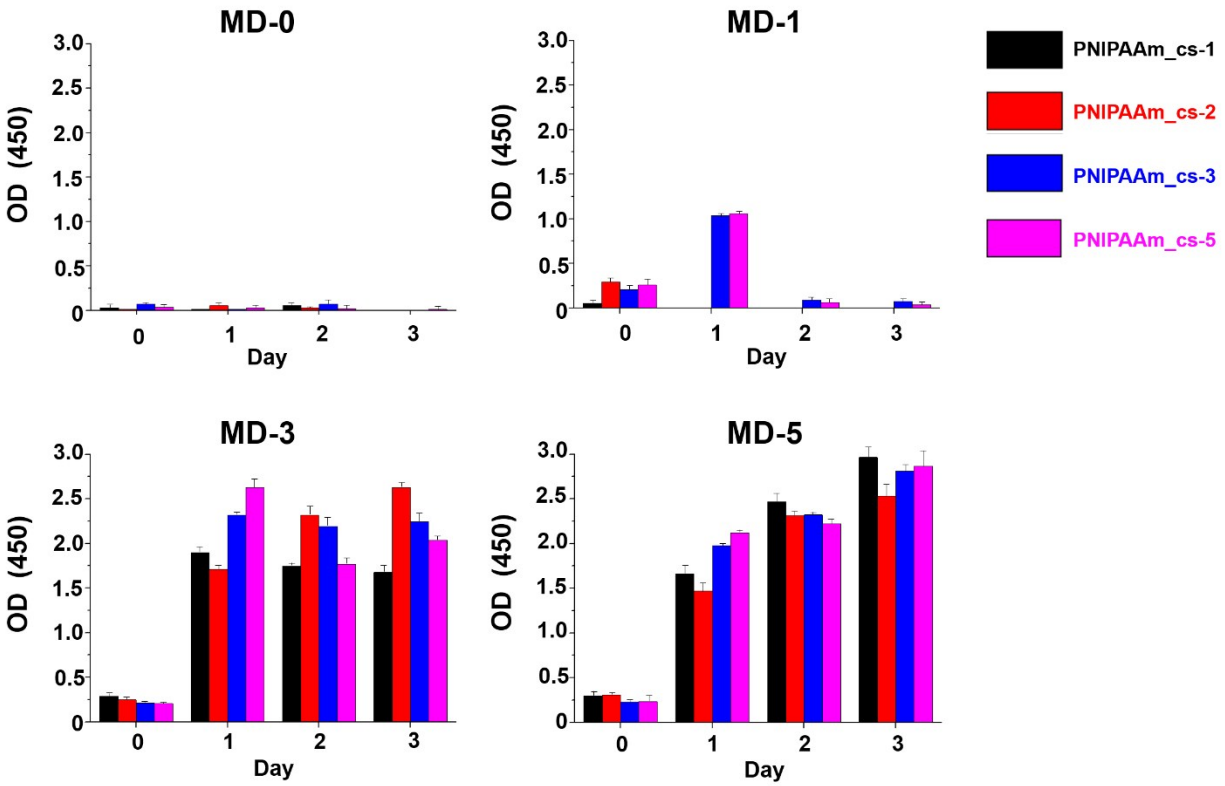
where W_0 and W_f are the initial and final weights of the hydrogel before and after the immersion, respectively.

2.11 Roughness measurement of PNIPAAm hydrogels upon a change in temperature

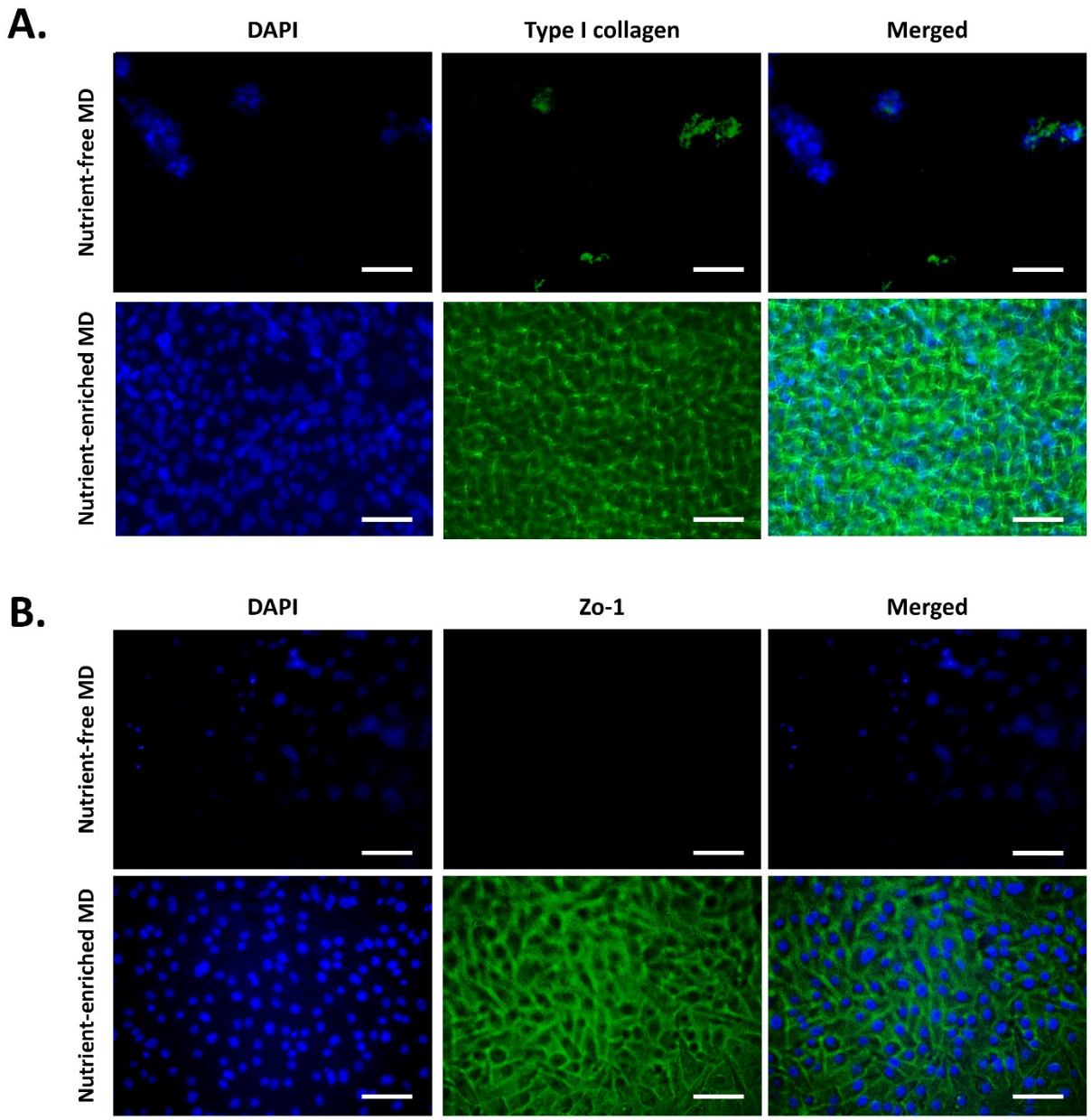
The initial surface roughness of the fabricated PNIPAAm hydrogels in dry state was examined by utilizing an atomic force microscope (XE-7, Park Systems, Korea) in a non-contact mode with a PPP-NCHR cantilever (Nanosensors, Switzerland) at a resonant frequency of 300 kHz. Furthermore, a BL-AC40TS cantilever (Olympus, Japan) at a frequency of 25 kHz was used to examine the change in a nm-scale surface roughness of the PNIPAAm hydrogels immersed in the culture medium at a temperature of 37 °C. Furthermore, the changes in a μm-scale surface roughness of the PNIPAAm hydrogels immersed in the culture medium at a temperature of 20 °C was investigated by using a 3D optical surface profiler (WykoNT110, Veeco, USA) in a time interval of 30 min. At each time interval, five different spots were randomly chosen to be examined in the area of 600 × 400 μm^2, and the measured roughness values were statistically analyzed for comparison.



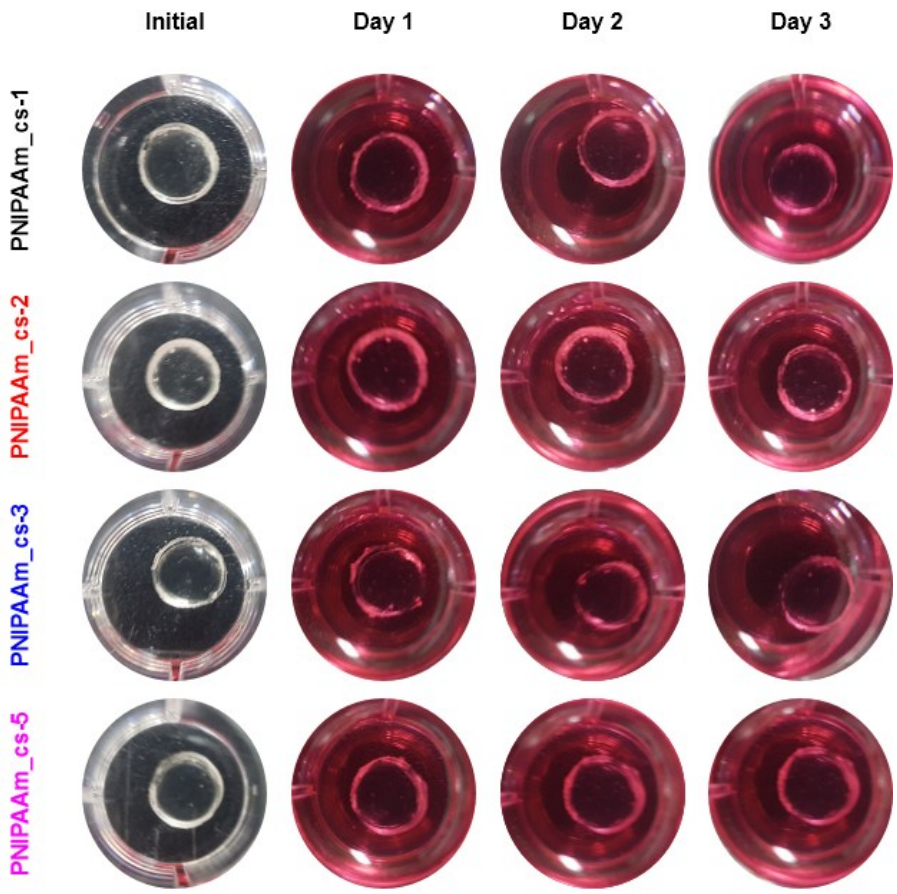
Supplementary figure 1. Release and residues profile of BSA content inside of the PNIPAAm hydrogel for an hour after the cell culture.



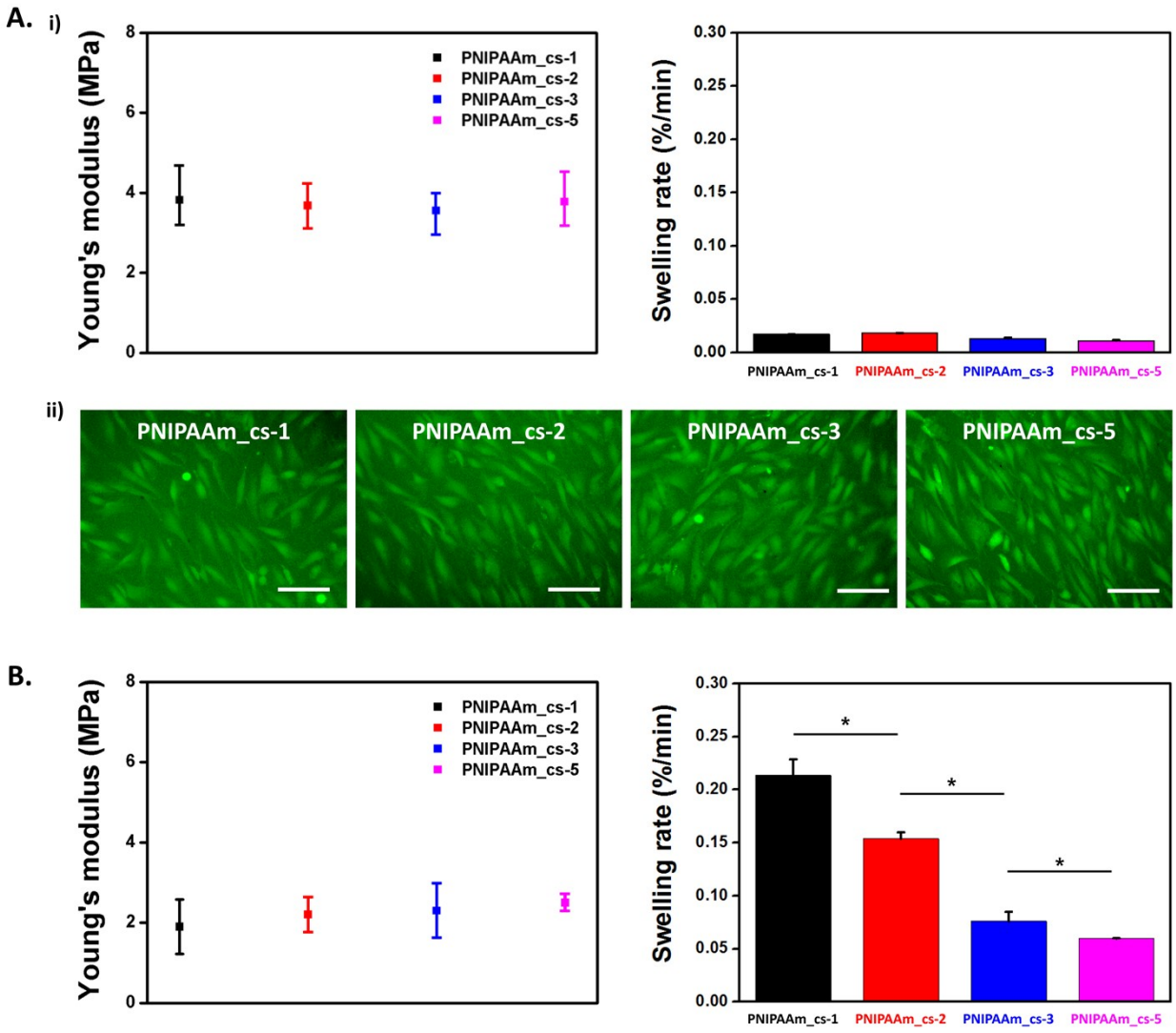
Supplementary figure 2. Proliferation of NIH3T3 cells on PNIPAAm cell culture platform after a medium detoxification process of 0, 1, 3, and 5 days



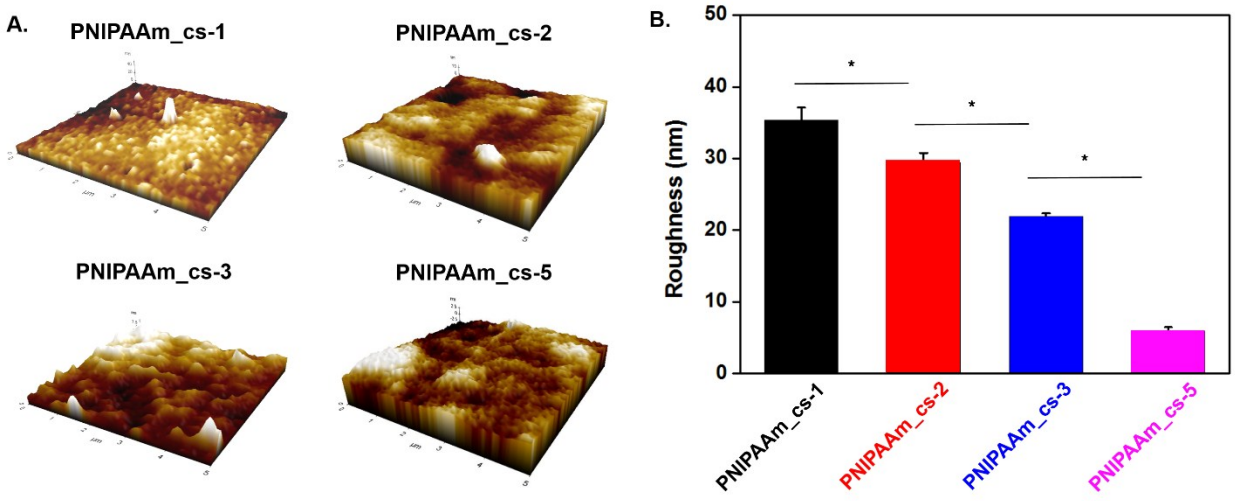
Supplementary figure 3. The accelerated maturation of the cell sheet in nutrient-enriched PNIPAAm hydrogel. (A) Characterization on ECM contents within the NIH3T3 cell sheet (scale bar: 100 μm) and (B) Characterization on intercellular junction protein of Zo-1 within the keratinocyte cell sheets (scale bar: 100 μm).



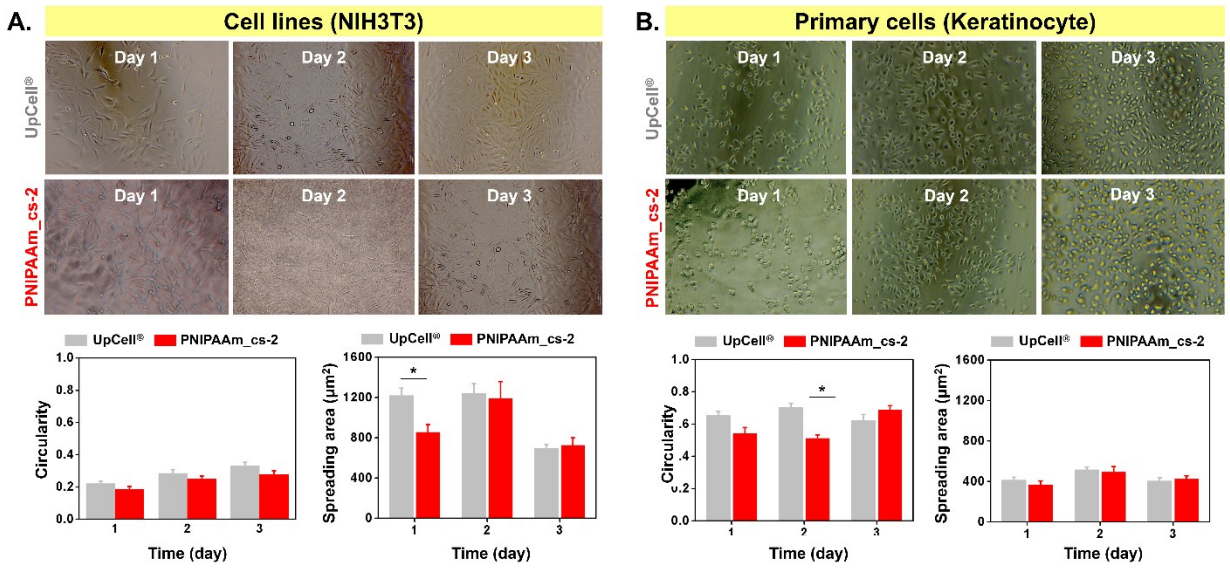
Supplementary figure 4. Stability of the PNIPAAm hydrogels in culture medium at a temperature condition of 37 °C



Supplementary figure 5. (A-i) The mechanical properties (surface hardness and swelling rate) of the fabricated hydrogels submerged in culture medium at a temperature condition of 37 °C and calcein AM-stained images (scale bar: 100 μm) of NIH3T3 cells cultured on the hydrogels. (B) The mechanical properties (surface hardness and swelling rate) of the fabricated hydrogels after they were submerged in culture medium at a temperature condition of 20 °C for 1 h.



Supplementary figure 6. Initial roughness of PNIPAAm hydrogels at a temperature condition of 37 °C. (A) AFM images of the surface of the PNIPAAm hydrogels and (B) their roughness analysis.



Supplementary figure 7. The morphologies (circularity and spreading area) of (A) NIH3T3 and (B) keratinocytes during the cultivation period of 3 days.