ACS APPLIED MATERIALS & INTERFACES

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Convenient Three-Dimensional Cell Culture in Supermolecular Hydrogels

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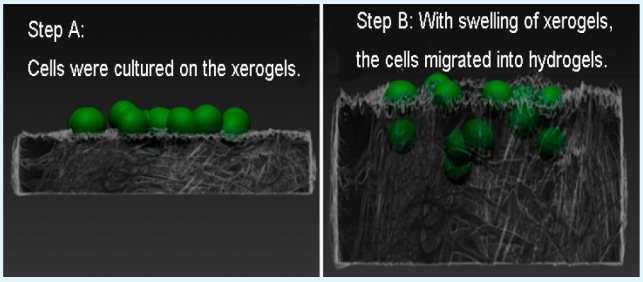
Supporting Information

ABSTRACT: A convenient three-dimensional cell culture was developed by employing high swelling property of hybrid hydrogels coassembled from C_2-phenyl-based supermolecular gelators and sodium hyaluronate. Imaging and spectroscopic analysis by scanning electron microscopy (SEM), atomic force microscopy (AFM), transform infrared (FT-IR) spectra confirm that the hybrid gelators can self-assemble into nanofibrous hydrogel. The high swelling property of dried gel ensures cell migration and proliferation inside bulk of the hydrogels, which provides a facial method to study disease models, the effect of drug dosages, and tissue culture in vitro.



KEYWORDS: *supermolecular, hydrogels, self-assemble, cell culture, migration, three dimensional*

INTRODUCTION

The conventional two dimensional (2D) cell culture systems are considerably limited in emulating complex three-dimensional (3D) microenvironments because of the lack of structural architecture and gradient,[1,2] though they have provided the base for the nascent interpretation of complex biological phenomena.[3,4] Great efforts have been made to develop different systems for constructing 3D cell culture environment, such as 3D bioprinting technology, hydrogel constructs.[5] So far, some 3D culture materials are even commercialized for fundamental cell culture studies. Among these, polymeric hydrogels are frequently used for their high water content and the adjusted biocompatible properties.[6,7] Despite of their significance in 3D cell related studies, to directly fabricate the 3D matrices with the encapsulated cells in situ is still a big challenge because of the complex fabricating procedure.[8]

Herein, by using conventional 2D culture strategy, a simple and useful 3D cell culture was achieved by employing the high swelling property of the hybrid hydrogels coassembled from C_2-phenyl-based supermolecular gelators and sodium hyaluronate (HA). The merit for choosing supermolecular gelators is that they can self-assemble into the 3D cell-gel constructs through noncovalent interactions[9−16] under external stimulations, such as pH, solvent, temperature, light, and so on,[17−22] which render supermolecular hydrogels susceptible to biodegradation. Moreover, the pore size of the 3D supermolecular hydrogel can readily respond to the mechanical forces exerted by cells as they migrate through the matrix.[9]

With incorporating HA into the hydrogels, the entangled polymer-like network structures were formed, enabling the relatively rigid and quick recovering properties. Study on the swelling property of the xerogel indicated about 90 times swelling ratio for the gels and the swollen pore sizes could be increased to tens of micrometers. By applying cells containing Dulbecco's Modified Eagle Medium (DMEM) onto the xerogels, the cells could slowly migrate into the hydrogels with the migrating distance was up to ∼65 μm, ensuring the cells culture in 3D environment (Figure 1). This method is not only easy to be operated for achieving 3D cell culture, but also avoids the negative effects from additional stimulations during cell growth. It provides a way to design the novel system for achieving simple 3D cell culture and facilitate 3D cell research that is urgently needed but usually tough target.

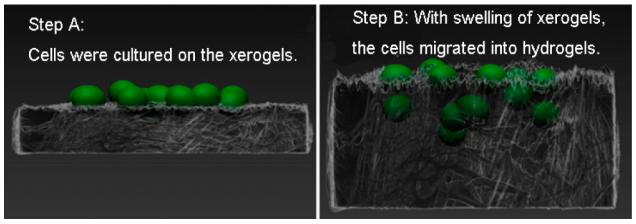


Figure 1. Schematic demonstration of the facile 3D culture strategy. Step A: cells were directly cultured on the compact thin xerogel layer. Step B: With swelling of xerogels, cells on the gels migrated from the surface into the bulk and showed 3D cell proliferation behaviors.

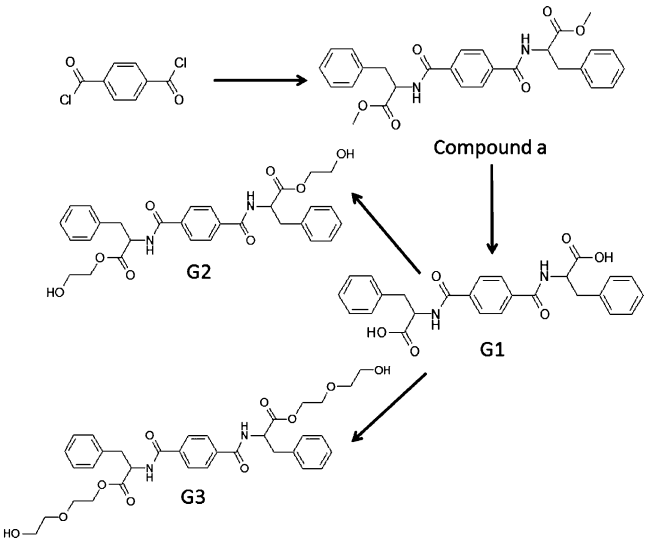
Received: March 3, 2014
Accepted: May 6, 2014
Published: May 6, 2014

   dx.doi.org/10.1021/am501275t | *ACS Appl. Mater. Interfaces* 2014, 6, 7948−7952

EXPERIMENTAL SECTION

Materials. All chemicals were purchased from Aldrich and used without further purification. In this work, a serious of C_2-symmetric benzene-based supermolecular gelators (named G1, G2, and G3) were synthesized with high yield through conventional liquid phase reaction in two or three steps according to Scheme 1. The synthesis method is described in the Supporting Information.

Scheme 1. Synthetic Route of G1, G2, and G3



Gelation. The G3-HA hydrogel with 0.3 wt % G3 and 1 wt % HA is used as an example to describe the preparation procedure. A weighed amount of gelator G3 (3 mg/mL) was suspended in 1 wt % HA solution in a septum-capped 10ml glass vial and heated (about 100°C) until the solid was dissolved, after which the solution was allowed to cool to room temperature. Hydrogel was considered to have formed when a homogeneous substance was obtained, which exhibited no gravitational flow upon inversion of the vial.

Freeze Drying. G3-HA hydrogels were kept in liquid nitrogen for 30 min, then transferred to the lyophilizer and kept there until all the frozen water in the hydrogels sublimated.

Atomic Force Microscope (AFM) Study. G3 and G3-HA solutions were diluted with about 0.1 mg/mL G3, which were rapidly pipetted onto a freshly cleaved mica surface and the samples were dried under ambient conditions. AFM measurements were carried out using a multimode NanoNavi E-sweep AFM (SII nanotechnology, Japan) in tapping mode with a scan speed of 1.7 Hz. A silicon tip/cantilever (NSC11/AlBS, μmash) was used with resonance and force constant of 60 kHz and 3.0 N/m, respectively. All measurements were performed under atmospheric pressure (ca. 30% relative humidity, 24°C). AFM images were analyzed offline using an AFM software (BinOffline500-0609) proved by the company.

Swelling Study (SR). The classical gravimetric method was used to measure the equilibrium swelling ratios of the hydrogels. The weight of vial was recorded at first. Then the weight of G3 and G3-HA xerogels (hydrogels were dried in a vacuum oven at 37°C) were measured with vial before swelling, then PBS was added into vial and xerogels were swelled at 37°C. At discrete time intervals, residual PBS on the swollen hydrogel surface was removed by filter paper. The weight of the samples in the swollen state and vial was measured. Taking the average value of three measurements for each sample, and the swelling ratio (SR, Q_t) at time t is defined as follows

$$Q_t = (W_s - W_d)/W_d$$

where W_s is the weight of hydrogel sample in the swollen state and W_d is the weight of the dried gel.

Rheological Measurements. The rheological properties of hydrogels were measured using a rotary rheometer (Gemini HRnano) with 20 mm diameter plate−plate steel geometry. The measurements were performed using a dynamic frequency sweep test in which a sinusoidal shear strain of constant peak amplitude (1%) was applied over a range of frequencies (0.01−10 Hz) at 25 °C. The strain used was within the linear viscoelastic region, as determined by dynamic strain sweep experiments.

Fourier Transform Infrared (FT-IR) Spectroscopy. FT-IR spectra of dried gels were taken using a Bruck EQUINOX55 Instrument. The KBr disk technique was used for the solid-state measurement. The samples were scanned between the wavelengths of 4000 and 400 cm^{-1} at an interval of 1.9285 cm^{-1}.

Confocal Microscopy. Confocal microscopy was carried out on a Nikon A1. Calcein was excited at 488 nm with an argon ion laser and emission was recorded through emission filter set 515/30. Equidistant Z-stack images (1 μm apart) were collected in different focal planes. Images were acquired, with a scanning mode format of 512 × 512 pixels. Each image was a 3D reconstruction of a series of Z-stack images (both for fluorescence and differential interference contrast (DIC) microscopy) using Image-Pro Plus version 6.0, a commercially available software package from Media Cybernetics.

Cell Culture. Hydrogels were statically seeded with normal human skin fibroblast (NHSF) cell (cell bank of the Chinese Academy of Sciences) and MHCC- 97L cell (Liver Cancer Institute, Zhongshan Hospital, Fudan University, Shanghai, China). Cells were maintained in Dulbecco's Modified Eagle Medium (DMEM), supplemented with 10% fetal calf serum, penicillin (100 μg/mL) and streptomycin (100 μg/mL). All cells were cultured at 37 °C in a humidified atmosphere of 5% CO2.

The viability of the cells was assessed with a fluorescent live−dead staining assay (Dojindo Corp.). The medium was removed and the constructs were washed twice in PBS. Then 200 μL of the PBS assay solution containing 2 μM calcein AM and 4 μM PI (Propidium Iodide) was pipetted onto each cell-gel construct. After 15 min incubation under humidified atmosphere of 5% CO2 at 37 °C, the labelled were then viewed under an inverted fluorescence microscope with excitation filters of 494 and 545 nm. Quantitative number of live/dead cells on hydrogels was determined by using the program ImageJ form the National Institute of Health, USA.

Cell proliferation was quantified using the Cell Counting Kit-8 (CCK-8, water-soluble tetrazolium salt) assay kit (Dojindo Corp.); 1000 cells were plated into each well of a 96-well plate, in which 10 μL of CCK-8 was added to 100 μL of DMEM. The cells were subsequently incubated for 2 h at 37 °C and the absorbance at 450 nm was measured with a microplate reader. Data shown are the means of three independent experiments.

RESULTS AND DISCUSSION

Swelling Property. Three types of C_2-phenyl-based gelators (G1, G2, G3) were synthesized (see the Supporting Information), which had excellent self-assembly abilities to form hydrogels.[23] The minimum gel concentrations were 0.2, 0.2, and 0.07 wt % for G1, G2, and G3, respectively. G3 was chosen for the following study because of its best mechanical stability and rheological property.[24] HA was incorporated into G3 in order to increase swelling ability of the hybrid hydrogels (G3-HA). With drying the hybrid hydrogels, they could form a compact thin xerogel layer and almost recover back to the original hydrogels again within 2 h after adding aqueous solution (see S-Figure 1 in the Supporting Information).

In Figure 2a, the swelling of G3 xerogels finished within several minutes and only 9 mg of water was adsorbed per milligram of xerogels. whereas G3-HA gels took about 2 h to reach equilibrium for the swelling and the amount of the adsorbed water in hydrogels (1% HA) was up to 99 mg in 1 mg of xerogels. The calculated original thickness of G3 and G3-HA xerogels were about 0.1 mm and they increased to 1 mm and 9 mm for the swollen gels, respectively. Typically, the adsorbed

dx.doi.org/10.1021/am501275t | ACS Appl. Mater. Interfaces 2014, 6, 7948−7952

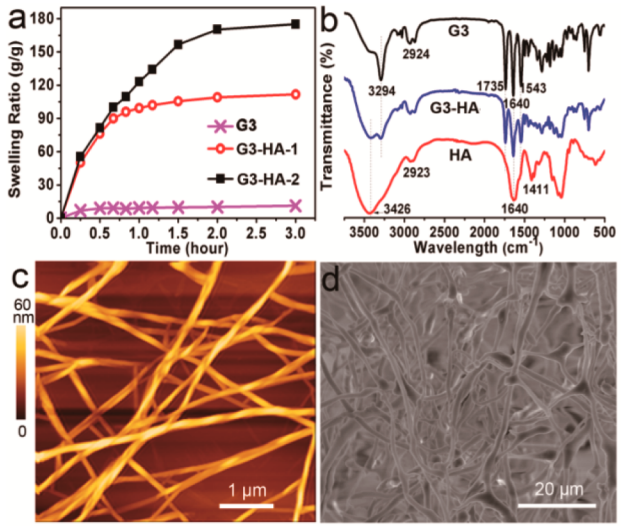


Figure 2. (a) Swelling ratio (Qt) vs time for G3, G3-HA-1 (1% HA), and G3-HA-2 (2% HA) xerogels in PBS solution; (b) FT-IR spectra of HA, G3, and G3-HA gels; (c) AFM images of the nanofibrous G3-HA hydrogels that were dried in the air at 40°C; (d) SEM image of freeze-dried G3-HA hydrogels after swelling.

water could be increased to 176 mg per mg xerogels if the amount of HA was increased to 2%, which indicated that the incorporation of HA in the hydrogels had great influence on the swelling property of hydrogels.

Transform Infrared (FT-IR) Spectra. Fourier transform infrared (FT-IR) spectroscopy was used to characterize intermolecular interactions between G3 and HA chains (Figure 2b). The bands centred at 1640 and 1411 cm^{-1} were attributed to the antisymmetric and symmetric stretching vibration of the carboxylate group of HA,[21] whereas bands at 1736, 1650, and 1543 cm^{-1} were assigned to −C═O stretching vibration and N−H bending vibration of G3 molecule. The spectrum of G3-HA exhibited a stronger and broader centred at 3420 cm^{-1}, which indicates the stretching vibration of −OH groups of HA.[25] The FT-IR spectra directly proved the coexistence of G3 and HA in the hydrogels.

Structure of Hydrogel. The nanoscopic structures of the G3-HA hydrogels were determined by atomic force microscopy (AFM) (Figure 2c). The diameters of the nanofibers were hundreds of nanometers, which were in the range of ECM nanostructures.[26,27] Typically, the thin fibers were bundled up to form large elongated fibers, leading to relatively high bending rigidity of nanostructures. From scanning electron microscopy (SEM), the xerogel thin films showed the compact nanofibers and pores were about several micrometers (see S-Figure 2 in the Supporting Information). The swollen G3-HA gels possessed much big pores and the diameters of the pores were tens of micrometers, which were similar to the size of cells (Figure 2d). With the swelling of G3-HA gels, the loose spongy structure can be formed in the inner hydrogel, which allows cell migration, whereas in the absence of HA, the swollen G3 hydrogel could not provide enough pore room for cell migration and the porous sizes are similar to those of G3 hydrogel without swelling (see S-Figure 3 in the Supporting Information).

Rheology. Rheological properties of G3-HA hydrogels were tested using rotary rheometer (Figure 3a). Frequency sweep measurements of the hydrogels suggested that the elastic modulus (G') were dependent on frequency, which is

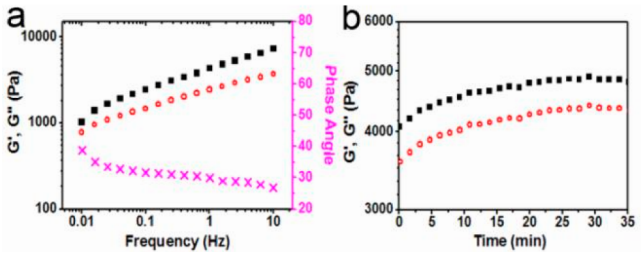


Figure 3. Rheology data of swollen G3-HA hydrogels (G3:3 mg/mL, HA: 1 wt %): (a) frequency sweep of G' (solid square), G'' (open cycle) and phase angle (cross) from 0.01 to 10 Hz, at 25°C; (b) restoration of gel moduli as a function of time after the cessation of strain treatment for gel network destruction; solid square for G', open cycle for G''.

characteristic of entangled HA network structures.[21] The G' values were larger than the G'' values over the tested frequency range, indicating the formation of solid gels with dominating elastic property.[28] The initial modulus of the reforming gel was 4010 Pa and moduli recovered to 4940 Pa after 20 min, which provide a quick recovery for the elastic hydrogels (Figure 3b). The quick reforming of hydrogels after cessation of shear was attributed to the quick relaxation time of the molecular self-assembly.

Cell Culture. Cytotoxicity of the G3 and G3-HA was studied by using live/dead cell viability and cell proliferation assay. Normal human skin fibroblast (NHSF) and human hepatoma cell lines (MHCC- 97L cells) were selected and cultured on the xerogels. All images were focused on the top of materials (PS, or G3, or G3-HA). Cells adhering on the surface of materials showed obvious 2D growth behaviors. It showed a higher density of viable cells on the G3-HA than those on polystyrene (PS) plate (control) and G3 (Figure 4a). The overall cell survival rates on both gels were also slightly higher than the rate on PS (above 87%) after 24 h culture, suggesting the biocompatibility of the xerogels.

Cell proliferation of NHSF and MHCC-97L cells on xerogels was measured by a Cell Counting Kit-8 (CCK-8) assay (Figure 4b,c). The proliferation rates for both cells on the G3-HA were

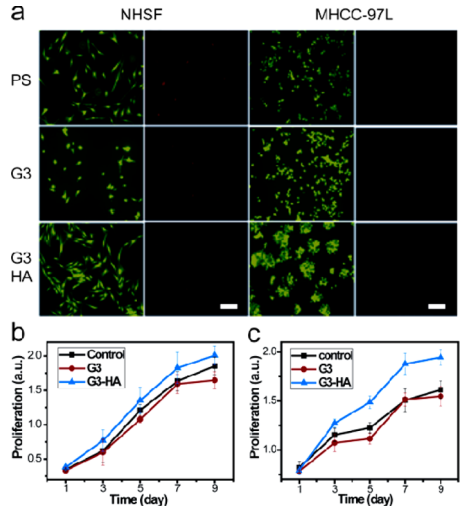


Figure 4. (a) Viability of the NHSF and MHCC-97L on PS, G3, and G3-HA (GH), respectively. Green staining indicates live cells and red staining indicates dead cells; the scale bar is 200 μm; CCK-8 assay results of (b) NHSF and (c) MHCC-97L cells on different substrate.

dx.doi.org/10.1021/am501275t | ACS Appl. Mater. Interfaces 2014, 6, 7948−7952

higher than those on G3 and control plates, which implied a fast cell growth on the G3-HA. In addition, the effect of stiffness of the hydrogel on cell morphology was studied. Cell growth morphologies on G3-HA hydrogels were also quite different from those cultured on G3 and PS surfaces. The NHSF adopted a spindle shape with fine filopodia on the G3-HA, whereas the round-shaped MHCC-97L cell clusters were observed on the G3-HA, rather than the disturbed single cells as those on G3 and PS surfaces. The results are in general agreement with formerly reported work where the mechanical properties of hydrogels can partly affect cell adhesion, spreading, and proliferation.[29]

To prove the 3D cell culture phenomena, MHCC-97L cells and chondrocyte were chosen and seeded on the G3-HA xerogels from DMEM. The swollen G3-HA gels reached equilibrium within 2 h. Laser confocal microscope was used to characterize the cell behaviors as shown in Figure 5. The 3D

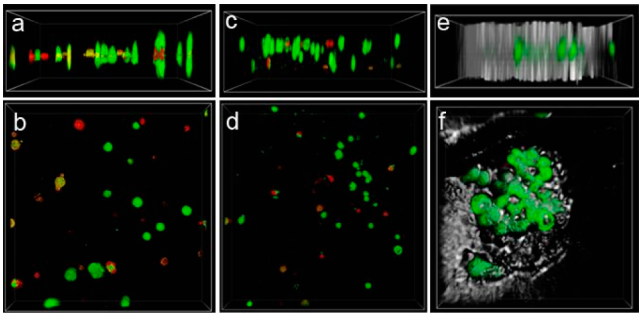


Figure 5. Three-dimensional volume rendering images of (a, b) MHCC-97L cells and (c–f) chondrocyte in G3-HA gels after 24 h culture detected by Z-stack of laser confocal microscopy with scans of 0.775 μm increments over total thickness of 74.4μm. Grey is G3-HA hydrogels.

images were generated from Z-stack of laser confocal microscopy scans of 0.775 μm increments over total thickness of 74.4 μm. The images suggested that part of MHCC-97L cells on the G3-HA gels migrated from the surface into the bulk with the swelling and the penetration depth was 65 μm for both cells. MHCC-97L cells on the surface of G3-HA gels spread, whereas cells migrating into the G3-HA gels maintained typical 3D growth morphology (round shapes) that was similar to their 3D culture in the G3 gels.[23] The similar migration behaviors of different cells were further tested (see S-Figures 4 and 5 in the Supporting Information), which clarified that the swollen G3-HA xerogels could provide enough porous space to assist different cells migration and growth in the gel matrix.

To state the contributions of the swelling for the successful 3D culture on G3-HA xerogels, we directly cultured MHCC-97L cells on the already swollen G3-HA hydrogels. As expected, the cells showed very similar 2D growth behaviours as those on G3 xerogels (see S-Figure 6 in the Supporting Information). These phenomena showed three dimensional growth behaviours for the cultured cells, which may be attributed to the swollen properties of the G3-HA xerogels. However, the maximium migration distance of the cells was limited to 65 μm, which were possibly related with the chemical structures of C_2-phenyl-based gelators and the swelling rate of the hybrid hydrogels. The work is still going on in our group.

The phenomena showed three dimensional growth behaviours for the cultured cells, which were attributed to the swollen properties of the G3-HA xerogels. It provides a facial method to study disease models, the effect of drug dosages, and tissue culture in vitro, which reduces the need for animal models.

CONCLUSIONS

In summary, a new class of hybrid hydrogels with excellent swelling property was formed from coassembly of C2-phenyl based gelator and Sodium Hyaluronate, which could provide enough space for cells migrating into 3D microenvironments, enabling the possibility to acquire 3D cell culture. The study may enrich the existing biomaterials based 3D environments and provide a supplementary system for achieving simple 3D cell culture. The abilities to facilitate 3D cell migration and proliferation in a convenient way will be crucial in various cell-related biomedical research fields, e.g., tissue repair.

ASSOCIATED CONTENT

Supporting Information

Synthetic route, SEM image, and three-dimensional volume rendering images. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

This work is supported by the NSFC (51273111, 51173105) the National Basic Research Program of China (973 Program 2012CB933803), the Program for Professor of Special Appointment (Eastern Scholar) at Shanghai Institutions of Higher Learning. Research Fund for the Doctoral Program of Higher Education of China, SRF for ROCS, SEM. Shanghai Jiaotong Medical/Engineering Foundation (YG2012MS29).

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