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Responsive Small Molecular Hydrogels Based on Adamantane−Peptides for Cell Culture

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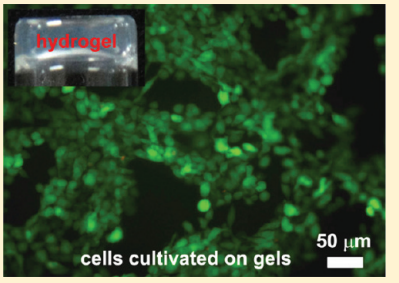
Supporting Information

ABSTRACT: The development of responsive small molecular hydrogels that can be applied for recovery of cells postculture attract extensive interests for researchers in fields of cell biology, stem cell differentiation, and tissue engineering. We report in this study several responsive small molecular hydrogels based on adamantane−peptides whose gel to clear solution phase transition can be achieved by addition of β-cyclodextrin (β-CD) derivatives. The small molecular hydrogels are formed by our recently developed method of disulfide bond cleavage by glutathione (GSH). Mouse fibroblast 3T3 cells attach and grow well at the surface of hydrogels. Furthermore, 3T3 cells postculture can be recovered from the gels by the addition of a β-CD derivative due to formation of clear solutions by the adamantane−β-CD interaction. The culture on hydrogels and recovery process do not cause obvious effects on behaviors of 3T3 cells. The results shown in this study indicate that small molecular hydrogels based on adamantane−peptides have great potentials in research fields where further analysis of cells is needed.



INTRODUCTION

Small molecular hydrogels,[1−4] formed by the self-assembly of small molecules (usually molecular weight less than 2000) via noncovalent interactions, have attracted extensive research interests in recent years because of their excellent biocompatibility and rapid responsiveness by external stimulus.[5−15] They have shown promising potential applications in controlled drug delivery,[16−19] environmental protection,[20] tissue engineering,[21−23] etc. For the application of small molecular hydrogels in tissue engineering, especially in cell culture, peptide-based small molecular hydrogels have been widely investigated and demonstrated suitable for culture of different cells by several groups. For example, peptide hydrogels developed by Zhang and co-workers,[24−26] peptide amphiphile hydrogels developed by Stupp and co-workers,[27,28] and multidomain peptide hydrogels developed by Hartgerink and co-workers[29,30] are well suited for cell culture and stem cell controllable differentiation. Short peptide-based hydrogels have been applied for the culture of chondrocytes and primary human dermal fibroblasts by Ulijn and co-workers[31,33] and our group.[32] Coassembly of peptide-based hydrogels developed by Collier and co-workers[23,34,35] can support the growth of endothelial cells. β-Hairpin peptide hydrogels,[36] sugar-based hydrogels,[37] and amino acid-based hydrogels[38] also show potentials in cells culture, controlled drug release, and tissue engineering.

It will be greatly beneficial to develop hydrogels that can be converted to clear solutions by an external biocompatible stimulus, because hydrogels with this unique property can facilitate separation and further analysis of cells postculture. In order to achieve this goal, thixotropic and photoresponsive hydrogels have been investigated recently.[39−41] However, most of the hydrogels can only change to viscous solutions by external stimulus (mechanical force and light) and it is difficult for cells to be totally separated from them postculture by centrifugation. In this study, we report several small molecular hydrogels based on adamantane−peptides that show a rapid response (that is, gel to clear solution phase transition) by the addition of β-cyclodextrin (β-CD) derivatives. With this unique property, they can be applied for cell culture and cell recovery postculture.

EXPERIMENTAL SECTION

Materials and Methods. Rink amide-AM resin, Fmoc-OSu, and other fluorenylmethyloxycarbonyl (Fmoc) -amino acids were obtained from GL Biochem (Shanghai, China). β-Cyclodextrin (β-CD) was purchased from Bio Basic Inc. 2-Hydroxypropyl-β-cyclodextrin (HP-β-CD, M_w 1617) and methyl-β-cyclodextrin (M-β-CD, M_w 1331) were purchased from Aladdin (Beijing). Live/dead viability/cytotoxicity kit was purchased from Invitrogen [0.01 M of phosphate-buffered saline (PBS) solution was used]. Other chemical reagents and solvents were obtained from Alfa (China) and used as received unless noted.

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^{1}H NMR (Bruker ARX 300) was used to characterize the synthesized compounds. Electrospray ionization mass spectrometry (ESI-MS) spectrometric analyses were performed at the Thermo Finnigan LCQ AD System. Transmission electron microscopy (TEM) was done on a Tecnai G2 F20 system, operating at 200 kV. The ratio of precursor (compound K2), hydrogelator, and dimer of hydrogelator was determined on an LCMS-20AD instrument (Shimadzu). Rheology test was performed on an AR 2000ex (TA Instruments) system by use of 40 mm parallel plates at a gap of 500 μm. Dynamic strain sweep was first performed and the strain value within the plateau was used for the following dynamic frequency sweep for each gel.

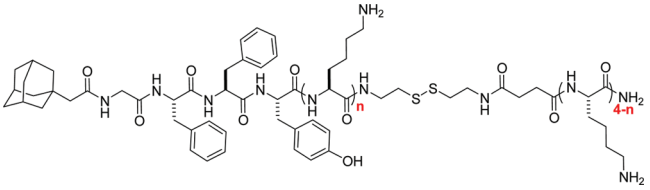
TEM Sample Preparation. TEM samples were prepared as follows: a carbon-coated copper grid (from Zhongjingkeyi Technology Co. Ltd., Beijing) was vertically dipped into the hydrogels for 5 s, and then it was placed in a desiccator overnight before the measurement.

Peptide Synthesis and Purification. All the peptide derivatives were prepared by solid-phase peptide synthesis (SPPS) with rink amide-AM resin. The first amino acid was loaded on the resin at the C-terminal with loading efficiency about 0.4 mmol/g. Piperidine (20%) in anhydrous *N*,*N*′-dimethylformamide (DMF) was used during the deprotection of Fmoc group. Then the next Fmoc-protected amino acid was coupled to the free amino group by use of *O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HBTU) as the coupling reagent. The growth of the peptide chain was according to the standard Fmoc SPPS protocol. After the last coupling step, excessive reagents were removed by five DMF washes for 1 min every time (5 mL/g of resin), followed by three steps of washing with dichloromethane (DCM) for 1 min (5 mL/g of resin). The peptide derivatives were cleaved from the resin by a mixture of 95% trifluoroacetic acid, 2.5% double-distilled (dd) water, and 2.5% triisopropylsilane for 30 min and then dried via rotary evaporation, followed by diethyl ether precipitation. The crude products were purified by preparative reverse-phase HPLC (Lumtech).

Hydrogel Formation. Peptides were prepared at a concentration of 10 mg/mL in PBS, and then 1.0 equiv of Na_2CO_3 was added to neutralize the carboxylic acid groups on peptides. Gels would form after mixing with reductant solution [2.0 equiv of dithiothreitol (DTT) or glutathione (GSH) to the peptide] within 1 min at room temperature (22−25 °C) (freshly prepared DTT or GSH solutions with pH value of 7.4 were used, and final concentration of precursors of gelators was 5 mg/mL).

Cell Culture on K2 Gels. NIH 3T3 (mouse embryonic fibroblast cells) were used in this study. NIH 3T3 cells were cultured in complete Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS (fetal bovine serum), 100 units/mL penicillin, and 100 μg/mL streptomycin. For 2D cell culture on hydrogels, the peptide powders, Na_2CO_3, and GSH were sterilized by UV light in the superclean worktable for 1 h before hydrogel formation. The hydrogels were prepared following the above procedures. After the addition of GSH, 50 μL of the solution was transferred into the well in a noncoating 96-well plate immediately. For the purpose of buffer exchange, 1 h after the formation of the hydrogels, 100 μL of complete medium was added on the top of the hydrogels and the medium was replaced by a new one after 30 min. This procedure was repeated three times. Then 100 μL of 3T3 cell suspension in complete medium containing 1.0×10^4 cells was pipetted onto the hydrogels. The 96-well plate was maintained in a 37 °C/5% CO_2 incubator.

Scheme 1. Chemical Structures of Precursors of Small Molecular Hydrogelators We Designed (Ada-GFFYK$_n$-ss-K$_{(4-n)}$-CONH$_2$, n = 0−3)



Cell Counting of NIH 3T3 Cells on K2 Gels. After NIH 3T3 cells were separated from K2 gels by use of M-β-CD in PBS buffer solutions, the proliferation rate of cells on K2 gels was determined by cell counting with a hematocytometer. Cells separated from three wells of 96-well plate were combined as one sample, and the result represents the mean value of three times.

Live/Dead Assay. Viability of the cells cultured on the hydrogels was tested by the live/dead assay according to the manufacturer's instructions. Specifically, the cell−gel constructs were washed twice with PBS for 20 min each. Staining reagent (100 μL) containing 2 μM calcein AM and 4 μM EthD-1 was then added onto the cell−gel constructs. After 30 min of incubation in a 37 °C/5% CO_2 incubator, cells were observed by use of a Nikon Eclipse TS100 inverted fluorescent microscope with excitation filters of 450−490 nm (green, calcein AM) and 510−560 nm (red, EthE-1).

Cell Recovery. To identify the proliferation ability of NIH 3T3 cells separated from K2 gels, we separated the cells from the cell−gel constructs by use of a β-CD derivative, M-β-CD. The cell−gel constructs were prepared by the above 2D culture procedure. After 3 days of culture, the cell−gel constructs were washed twice with PBS for 5 min each. M-β-CD (10 mM) in PBS buffer solutions (30 μL for each well) was then added onto the cell−gel constructs. The resulting cell suspensions were combined, pipetted several times, and then transferred into Eppendorf tubes, followed by centrifugation for 5 min at room temperature at 1000 rpm. Afterward the supernatant was discarded and the cells were washed with 100 μL of fresh M-β-CD PBS solution again. To disperse the cell clusters formed after the 3-day culture period, a trypsinase (0.25%)−ethylenediaminetetraacetic acid (EDTA) (0.02%) solution needed to be used in the second centrifuge step. The individual cells collected by centrifugation were counted and reseeded on normal tissue culture plates at a density of 10 000 cells/well. In order to compare the morphology and proliferation rate of cells collected from gels and normal cells (collected from conventional tissue culture plate), the live/dead assay and a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay were performed.

RESULTS AND DISCUSSION

Molecular Design. In our previous paper, we developed an adamantane−peptide derivative of Ada-GFFpY-OMe that could be catalyzed by a phosphatase to form nanospheres in aqueous solution. The nanospheres could be disassembled into clear solutions by β-cyclodextrin (β-CD).[42] We also reported on the first example of using the disulfide bond as a cleavable linker to control molecular self-assembly and the formation of hydrogels.[43] On the basis of these two previous studies, we hoped to form nanospheres with positive charge from Ada-GFFYK-ss-KKK by

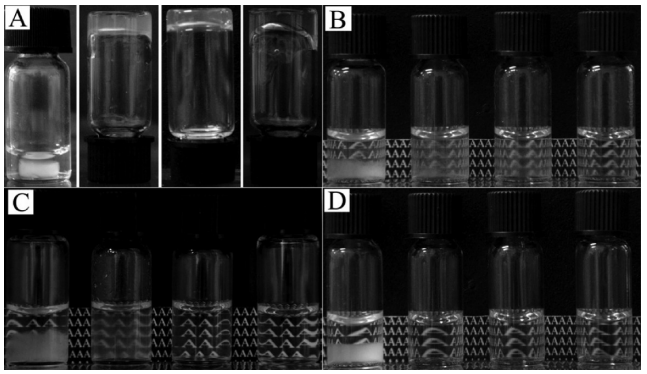


Figure 1. Optical images of (A) hydrogels formed by treating PBS solutions containing 0.5 wt % precursors with 2 equiv of DTT at 10 min and (B−D) solutions formed by treating the hydrogels in panel A with 4 equiv of (B) β-CD, (C) 2-hydroxypropyl-β-CD, and (D) methyl-β-CD. (The compounds were K0, K1, K2, and K3 from left to right in each panel).

disulfide bond reduction. However, we accidentally found that Ada-GFFYK-ss-KKK formed small molecular hydrogels after addition of reductants. Since gels formed by Ada-peptides could show response to β-CD, we opted to developed gels based on Ada-peptides for cell culture and cells recovery from cell−gel constructs for further culture or analysis. Therefore, we designed the molecules Ada-GFFYK$_n$-ss-K$_{(4-n)}$-CONH$_2$ in Scheme 1. They have several features: (1) The Ada portion provides the hydrophobic interaction that helps to extend the supramolecular chain, and it can form a tight complex with β-CD or its derivatives that might dramatically change the solubility of the gelators. (2) The FFY portion has been demonstrated to be an efficient self-assembled peptide. (3) Lysine (K) can enhance cell adhesion and adjust the balance between hydrophobicity and hydrophilicity (n = 0, 1, 2, and 3 for compounds K0, K1, K2, and K3, respectively). (4) The disulfide bond can connect hydrogelators and hydrophilic parts to increase the solubility of precursors of gelators and it can be cleaved by reductants, thus leading to the formation of hydrogels.

Hydrogel Formation. After the successful synthesis by solid-phase peptide synthesis and purification by HPLC, the gelation ability of compounds K0−K3 was tested. These four compounds had good solublilities in PBS buffer solutions at the concentration of 0.5 wt % and could form hydrogels immediately upon the addition of dithiothreitol (DTT) or glutathione (GSH) (K0−K3 gels for compounds K0−K3, respectively), which proved the success of our design. However, the four gels exihibited different behaviors: as shown in Figure 1A (left to right: K0, K1, K2, and K3 gels, respectively). K0 gel shrank and formed a precipitate after 10 min at room temperature (22−25 °C) due to the formation of more and more amounts of very hydrophobic compound of Ada-GFFY-SH that lacked the lysine (K) with an amine group. K1 gel was stable over weeks but was opaque and had a small amount of precipitates. K3 gel was totally clear but weak and had difficulty keeping its shape in the invert tube due to the presence of three Ks after disulfide bond reduction. Only K2 gel could maintain a transparent gel appearance and was stable for longer than 3 months. These observations indicated that different numbers of lysine residues (K) on the gelators could tune the balance between the hydrophilicity and hydrophobicity of the resulting gelators and affected their gelation behaviors.

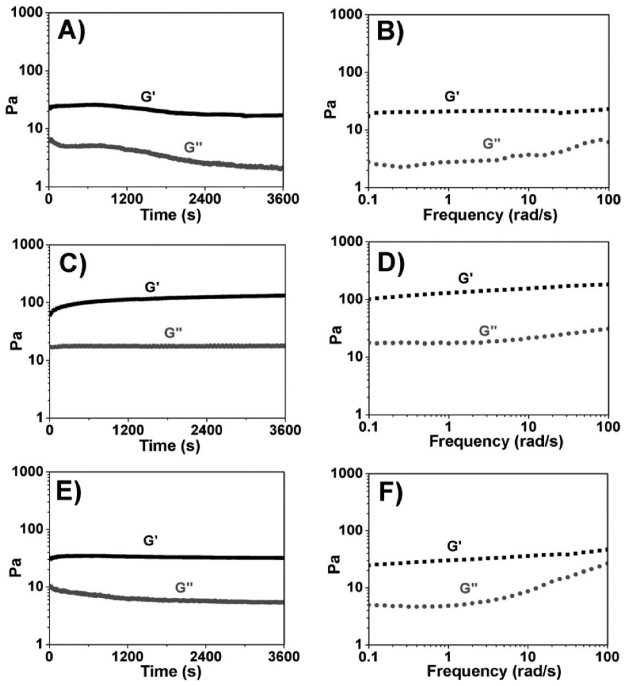


Figure 2. (A, C, E) Dynamic time sweep of treating different solutions containing 0.5 wt % precursors with 2 equiv of DTT at the strain of 2% and frequency of 2 rad·s^{-1}: (A) compound K1, (C) compound K2, and (E) compound K3. (B, D, F) Dynamic frequency sweep at the strain of 2% for (B) K1 gel, (D) K2 gel, and (F) K3 gel.

Responsiveness to β-CD Derivatives. Hydrogels that could be changed to clear solutions can be applied for cell collection post-cell culture. Therefore, we tested the responsiveness of K0−K3 gels to addition of β-CD (Figure 1B) and its derivatives [2-hydroxypropyl-β-CD (HP-β-CD) in Figure 1C and methyl-β-CD (M-β-CD) in Figure 1D]. A 400 μL PBS solution containing 10 mM β-CD or its derivatives was first added to each gel (200 μL, 3.44 mM gelator), and then the mixture was pipetted for 1 min. The results in Figure 1B indicated that K0−K3 gels formed precipitates (K0 and K1) or changed to turbid solutions (K2 and K3) after the addition of β-CD. For HP-β-CD, only K3 gel could be converted to a clear solution; the other three gels formed precipitations or turbid solutions. We then switched the HP-β-CD to M-β-CD with a better solubility (Figure 1D); K1−K3 gels could be converted to clear solutions, and only K0 gel formed a precipitate. The different responsiveness behaviors of the gels to β-CD and its derivatives were due to the different hydrophobicity of the gelators and different solubility of β-CD and its derivatives (<18.5, >750, and >1000 mg/g of water for β-CD, 2-HP-β-CD, and M-β-CD, respectively). The results also indicated that M-β-CD had the best ability to convert gels to clear solutions.

Rheology. Rheological measurements in a dynamic time sweep mode were first used to characterize the kinetics of the hydrogelations. Freshly prepared PBS solution containing 0.5 wt % precursor and 2.0 equiv (compared to precursor) of DTT was transferred immediately to the rheometer at a strain of 2% and a frequency of 2 rad·s^{-1}. As shown in Figure 2A,C,E, G' values of all three gels rapidly dominated their corresponding G'' values, suggesting that the hydrogelations happened immediately after the addition of DTT. Both G' and G'' values reached plateaus within 10 min, which could be interpreted by the results shown in

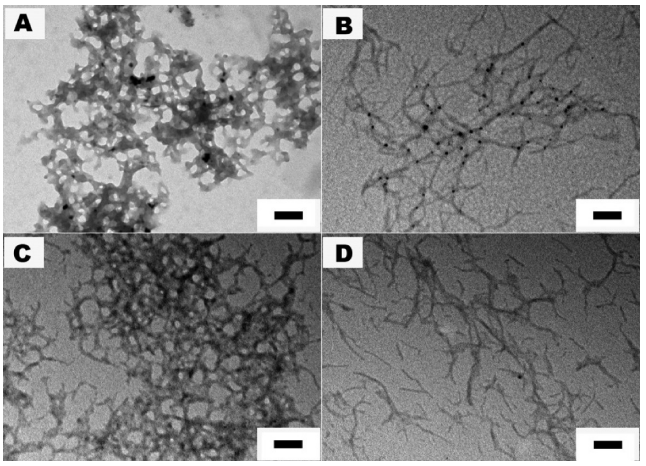


Figure 3. TEM images of (A) K0, (B) K1, (C) K2, and (D) K3 gels (scale bar = 200 nm).

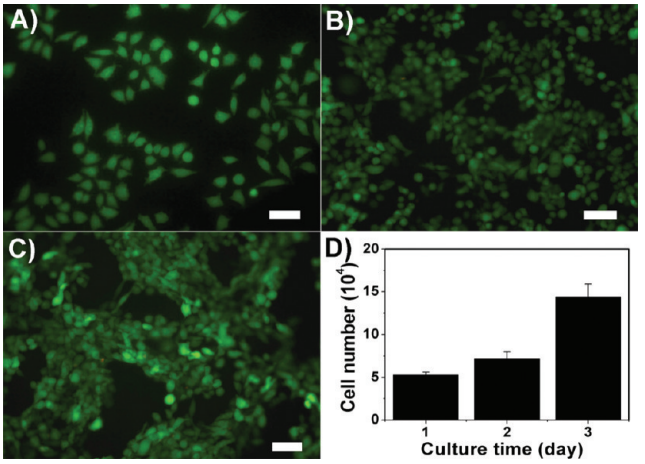


Figure 4. Live/dead assay of NIH 3T3 cells cultured on K2 gel at (A) day 1, (B) day 2, and (C) day 3 (live cells are shown in green and dead ones in red; the scale bars in panels A−C represent 50 μm). (D) Proliferation rate of NIH 3T3 cells on K2 gel determined by cell counting.

Table S-1 in Supporting Information that compound K2 was nearly totally converted to its corresponding gelator just 1 min after the addition of 2.0 equiv of DTT. The slight decrease of both G' and G'' in K1 gel was probably due to the formation of small amount of precipitates in the gel. Compared with the G' values recorded in Figure 2panels B (20 Pa for K1 gel), D (100 Pa for K2 gel), and F (25 Pa for K3 gel), K2 gel possessed the biggest one, which was consistent with the optical images in Figure 1A that K1 gel possessed a small amount of precipitates and K3 gel was mechanically weak and had difficulty keeping its shape in the invert-tube.

Transmission Electron Microscopy. Transmission electron microscopy (TEM) was used to characterize the morphology of self-assembled structures in the hydrogels. As shown in Figure 3A, K0 gel exhibited irregular short fiber structures with sizes of 35−80 nm and the fibers formed large aggregates with sizes of 1−3 μm, thus leading to the shrinkage of K0 gel. K1, K2, and K3 gels exhibited similar fibril structures with sizes of 150−450, 100−200, and 110−260 nm, respectively. When the morphology

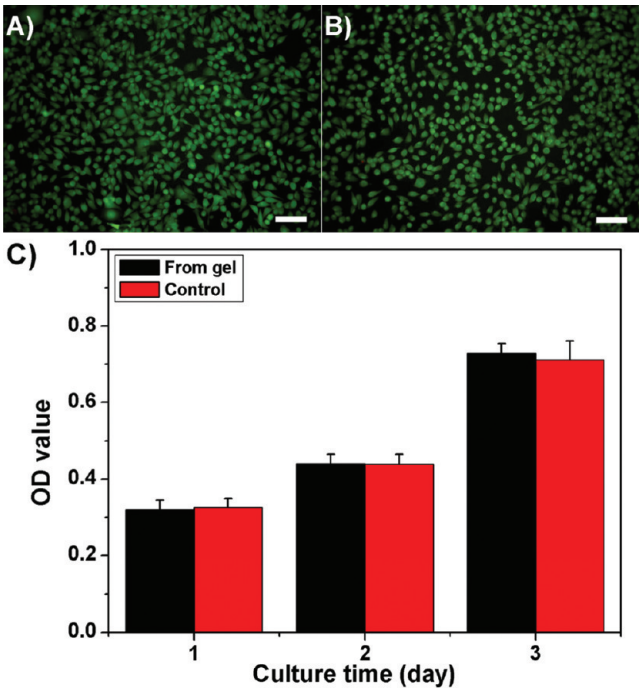


Figure 5. Morphology of NIH 3T3 cells cultured on tissue culture plates at day 3: A) 3T3 cells separated from K2 gel and (B) normal 3T3 cells (the scale bars in panels A and B represent 100 μm). (C) Proliferation rates of normal NIH 3T3 cells (cells collected from conventional tissue culture plates) and 3T3 cells separated from K2 gels determined by a MTT assay.

of the self-assembled structures in the hydrogels is compared, K2 gel exhibited the most uniform nanofibers that formed a dense network within the gel. This observation was consistent with the results observed in Figure 1A, that K2 gel had the best stability, and the rheological measurement shown in Figure 2, that K2 gel was mechanically stronger than the other two gels.

Live/Dead Assay and Proliferation. Since K2 gels were transparent and stable and could be converted to clear solutions by M-β-CD, we opted to test them for cell culture and cell recovery postculture. Mouse fibroblast NIH 3T3 cells were seeded on top of K2 gels at the density of 10 000 cells/well in a 96-well plate and then a live/dead assay was performed at different time scales. As shown in Figure 4A−C, 3T3 cells attached well and adopted spindle or polyhedron shapes at the surface of the gel. Most of the cells were alive, as indicated by most of the cells showing a green color [live and dead cells could only be stained by fluorescent dyes of calcein AM (emits green light) and EthD-1 (emits red light), respectively]. The cell density kept increasing during the 3 day culture period. We then recovered cells from gels by the method described in the following section and used manual cell counting to quantify the proliferation rate of cells on hydrogels. As shown in Figure 4D, the cell density at day 3 was 179% higher than that at day 1 on top of gel (cells in three wells are combined as one sample), which was a little bit higher than that of cells cultivated on conventional tissue culture plates (128% in Figure 5C). These observations indicated that K2 gel was suitable for 3T3 cell culture in 2D environments.

Morphology and Proliferation of Cells Separated from Gels. We then tried to collect cells postculture from cell−gel constructs. PBS buffer solutions were needed to first wash cell−gel constructs to remove DMEM culture medium. Otherwise,

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components in culture medium might also form complexes with M-β-CD and gels could not be converted to clear solutions. After PBS washing, PBS solutions containing 10 mM M-β-CD were used to separate cells from cell−gel constructs. For cells cultured on gels for 1 day, individual cells were observed, as indicated in Figure 4A. Individual cells could be separated from cell−gel constructs by treatment with only M-β-CD solutions following a centrifugation. However, cells cultured for more than 2 days connected with each other and formed clusters (Figure 4C). Besides washing with M-β-CD solutions, trypsinase (0.25%)−EDTA (0.02%) solutions were also needed to divide cells clusters into individual cells.

The individual cells obtained from cell−gel constructs by the above procedures were then counted and reseeded in normal 96-well tissue culture plates. The morphology and proliferation rate of cells from gels were compared with those of normal 3T3 cells. As shown in Figure 5panels A (cells from gels) and B (normal cells), cells from gels exhibited similar behaviors to normal cells: both kinds of cells possessed similar morphologies and proliferation rates during the 3 day culture period. These results indicated that the single culture procedure on K2 gels(and single separation procedure for cell recovery did not cause obvious effects on the behavior of 3T3 cells.

CONCLUSION

In summary, we have developed several small molecular hydrogels based on Ada-peptides that can response to β-CD and derivatives of β-CD. The hydrogels are formed by disulfide bond reduction by DTT or GSH, which was biocompatible for encapsulations of cells and drugs. K2 gels from Ada-GFFYKK-ss-KK were transparent and stable over months and could be converted to clear solutions by M-β-CD. Therefore, the possible applications of K2 gels in cell culture and cell recovery post-culture were tested. The results indicated that 3T3 cells grew well and divided efficiently on top of gels. The cells could be easily collected by washing cell−gel constructs with M-β-CD solutions and then centrifuging. The collected cells behaved the same as normal cells on tissue culture plates. The results reported in this study suggest the great potential of Ada-peptide gels in applications where further cell analysis is needed, such as stem cell differentiation.

ASSOCIATED CONTENT

Supporting Information. Additional text, nine figures, and one table showing synthesis and characterization of compounds K0−K3; dynamic strain sweep of K1, K2, and K3 gels; and analysis of molar percentage of precursor, hydrogelator, and dimer of hydrogelator by LC-MS. This material is available free of charge via the Internet at http://pubs.acs.org/.

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