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A SupraGel for efficient production of cell spheroids

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ABSTRACT **Cell spheroids are markedly more representative of the native tissue and the *in vivo* environment than traditional two-dimensional (2D) cultured cells, thus offering tremendous potential in cell biology research, tissue engineering, and drug screening. Therefore, it is crucial to develop materials and methods for efficient production of cell spheroids. However, currently developed materials, including natural and synthetic hydrogels, present drawbacks, such as undefined ingredients and imperfect biocompatibility, which hinder their widespread application. In this study, we have rationally designed biotinylated peptides that can self-assemble into supramolecular hydrogels (termed SupraGel) for 3D cell culture. The introduction of one *D*-amino acid in the peptide may decrease cell-matrix interactions, thus facilitating spontaneous cell spheroid formation. Two cancer cell lines, MCF-7 and 4T1, and intestinal stem cells (ISCs) can efficiently divide into cell spheroids when cultured in SupraGel. The reversible shear-thinning and recovery behavior of SupraGel is highly suitable for live-cell embedding and cell spheroid harvesting. The mechanical properties of SupraGel can be easily tuned by adjusting the peptide concentration, thus enabling its suitability for the 3D culture of diverse cell spheroids. We envision the significant potential of our SupraGel for applications in cell therapy, regenerative medicine, and drug screening.**

Keywords: cell spheroids, 3D cell culture, supramolecular hydrogel, peptides, self-assembly

INTRODUCTION

Cell spheroids present advantages over monodispersed cells or monolayer cultured cells in tissue engineering and regenerative medicine. For instance, research has demonstrated that cell spheroids can improve the therapeutic efficacy of stem cells by enhancing cell survival, increasing the secretion of growth factors, and retaining cellular pluripotency. For tumor cells, cell spheroids also lead to more accurate results in drug screening than two-dimensional (2D) cultured cells because of the physiologically relevant morphology and spatial arrangement [1–4]. The 3D tumor spheroids can mimic several important characteristics of tumors and the microenvironment, including phenotypic heterogeneity, growth kinetics, and cell-cell interactions. In addition, 2D cultured tumor cells often show lower chemotherapy or radiotherapy resistance than native tumors. Therefore, it is important to develop novel materials for efficiently producing cell spheroids, including cancer cell spheroids [5–10].

Among the materials reported for the production of cell spheroids, hydrogels (natural and synthetic) are the most widely used because they share similarities with the native extracellular matrix (ECM). Natural hydrogels, including the commercially available Matrigel, are composed of basement membrane extracts and naturally derived ECM constituents from animals and present drawbacks such as undefined ingredients, inconsistent stability, and potential antigenicity and immunogenicity risks. Synthetic hydrogels can overcome these limitations and have attracted increasing attention in research. Several synthetic hydrogels, including poly(ethylene glycol) (PEG), alginate, and PuraMatrix, present significant potential and have been successfully used for developing cell spheroid culture platforms. However, the high cost, complex preparation process, and imperfect biocompatibility hinder their widespread application and clinical translation [8,11–14].

Supramolecular hydrogels of self-assembling short peptides are synthetic hydrogels that are promising for drug delivery, cancer cell inhibition, and regenerative medicine [15–21]. Most peptides are biocompatible, and the peptide-based supramolecular hydrogels are thixotropic, enabling the easy incorporation of live cells into them and the 3D cell culture [22,23]. However, peptide hydrogels for cell spheroid production are rarely reported [24]. In this study, we designed a supramolecular hydrogel of a biotinylated peptide (SupraGel) for 3D cell culture and efficient cell spheroid production. SupraGel maintained cell viability within the hydrogel and promoted the spontaneous formation of cell spheroids of cancer cell lines and cancer stem cells. SupraGel could, therefore, provide a new platform for different applications related to cell spheroids, such as high-throughput screening of drugs and cell therapy.

EXPERIMENTAL SECTION

Peptide synthesis and characterization

Peptides were prepared by standard solid phase peptide synthesis (SPPS) using Fmoc-amino acids with the side chain properly protected. The synthesized peptides were characterized by high-resolution mass spectrometry (HR-MS, Agilent Technologies)

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and nuclear magnetic resonance (^{1}H NMR, Qone Instruments) (Figs S1 and S2).

Preparation of SupraGel

Peptide derivative (10 mg) was dispersed in a mixed solution of 991.23 μL phosphate buffer (PBS, pH 7.4) and 8.77 μL (1 mol L^{-1}) Na_2CO_3. The solution (1 wt%) was heated by an alcohol lamp until the powder was completely dissolved and then cooled back to room temperature for hydrogel formation.

Transmission electron microscope (TEM)

The microstructure of SupraGel was monitored by HITACHI HT7700 TEM with Hitachi TEM system (working at high voltage = 100.0 kV). Before observation, the SupraGel was coated on the copper mesh. Then the sample was stained with uranyl acetate and then examined using TEM.

Rheological tests

An Anton Paar rheometer equipped with PP25 (25 mm-diameter parallel plates) was used to test the mechanical properties of the SupraGel. Firstly, dynamic strain sweep at the frequency of 1 rad s^{-1} was conducted. Then dynamic frequency sweep in the region of 0.1 to 10 rad s^{-1} at 1% strain was performed for the gels. To verify thixotropic and self-healing characteristics of the SupraGel, the sample was tested at enhanced strains at a constant frequency of 1 Hz at 25°C. Then continuous step strain (the strain value was set to 0.1% for the first 600 s, and then increased to 50% instantly for the next 240 s; the cycle was repeated three times) measurements were performed at a constant frequency of 1 Hz at 25°C.

Characterization of secondary structure

Circular dichroism (CD) was applied to collect the conformational information of the peptide in the diluted gel. CD spectra were measured by spectroscopy (MOS-450, BioLogic) with 0.2 mm quartz cuvettes. The peptide concentration for CD testing was 0.1 wt% in PBS.

Cell cultivation

For MCF-7/4T1 culture, SupraGel was mixed with the cell suspension by gentle vortex and then transferred to a 96-well plate. After the cell-gel construct was placed in the incubator for about 10–15 min, complete medium was added on its surface. Fresh complete medium (50 μL) was changed every other day. The corresponding cell number and gel usage amount, cell suspension volume, medium addition amount and other information for different cells are listed in the Table S1.

For intestinal stem cells (ISCs) (Table S2) culture in gel, 100 μL of SupraGel was vortexed, and then mixed with 25 μL of cell suspension containing 10,000 ISCs. The mixture was pipetted into a 24-well plate and stabilized in a cell incubator for about 15 min. Lastly, 400 μL of ISC expansion medium was added on top of the cell-gel construct. The use of Matrigel followed its product instructions. It was worth noting that organoids were digested into single cells (using TrypLE^{TM} Express, 12605-028) before cell count and planking. ISC expansion medium was comprised of Advanced DMEM/F12, 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid (HEPES), Glutamax, penicillin/streptomycin, Noggin conditioned medium, B-27 Supplement (Life Technologies), etc. During the subsequent organoid culture process, 100 μL of medium was updated daily [25]. In the whole process, bubbles should be avoided as much as possible.

Immunofluorescence staining

4T1 cells were inoculated in a confocal small dish according to the SupraGel culture method and cultured for five days. The cell-gel construct was washed with PBS and fixed with 4% paraformaldehyde (PFA) for 30 min. The construct was washed again three times with 0.2% Triton X-100 (in PBS) for 30 min. After washing with PBS, it was blocked with 3% bovine serum albumin (BSA) for 1 h. Subsequently, the sample was incubated with diluted primary antibody (HIF1-α, in 3% BSA) overnight at 4°C and washed with PBS three times on the second day. The sample was then incubated with secondary antibody (Rabbit Anti-Mouse Alexa Fluro 488, in PBS) and 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI, 1 μg mL^{-1}) for 2 h at room temperature. Following extensive washing, images were taken with a confocal laser scanning scope (CLSM, Zeiss LSM710).

ISC spheroids after seven days of culture were blown down from the gel and transferred to a 24-well plate covered with glass slides, which was then placed in the incubator overnight. After cell adhesion, excess medium was removed. Suspended tissues were collected and centrifuged (1000 r min^{-1}, 4 min) to reduce sample loss. The following steps, including washing, staining etc., needed to repeat this centrifuging step. The samples were fixed with 4% PFA in PBS (4°C, 10 min) and then washed with PBS for three times. Following fixation, the samples were permeabilized with 0.2% Triton X-100 in PBS (4°C, 10 min) and blocked (5% BSA in PBS) for at least 30 min. The samples were subsequently incubated with primary antibodies (LGR5 and CK20 diluted in blocking buffer, Table S3) overnight at 4°C. After washing with PBS for three times, the samples were incubated with secondary antibodies (Alexa 488 mouse, Alexa 555 rabbit) for 3 h at room temperature. The nuclei were counterstained with DAPI for 3 min at room temperature. Following extensive washing, the stained spheroids were imaged in the confocal (Zeiss LSM900) mode.

Cell separation and extraction from gel

The cell-gel construct and culture medium were transferred from the cell culture plate to a 1.5-mL Eppendorf tube. In order to disintegrate the gel, PBS (one volume of the gel-cell construct) was added to the Eppendorf tube to wash the mixture and resuspended (900 r min^{-1}, 3 min) for three times. All steps needed to be gentle, so as not to damage the structure of spheroids. The spheroids released from SupraGel could be used for subsequent staining and other relevant experiments.

Spheroids size statistics

The imageJ was applied to measure the spheroid size. More than 100 samples were collected and counted.

Live-dead staining

MCF-7/4T1 cells were stained with Calcine AM/PI (propidium iodide) Double Stain Kit (calcine AM 2 μmol L^{-1}, PI 4.5 μmol L^{-1}, solvent DMEM; Yeasen, China). ISCs were stained with Live&Dead^{TM} Viability/Cytotoxicity Assay Kit (1:3000; US EVERBRIGHT® INC, L6023) for 10 min at 37°C. After the samples were washed with PBS for three times, CLSM was used to collect the images of the spheroids.

RESULTS AND DISCUSSION

Molecular design of the hydrogelator

Short-peptide-based hydrogelators frequently need to be capped with aromatic groups, including fluorenyl (Fmoc-) and naphthalene (Nap-) [26–29]. Besides, biotin has been used as a capping group for amino acids and short peptides to produce hydrogelators [30,31]. For the application of peptide-based hydrogels in 3D cell culture and regenerative medicine, biotin-capped short peptides may have certain advantages. Meanwhile, the π-π interaction was crucial for the formation of self-assembling short-peptide-based nanomaterials and hydrogels, and short peptides based on F, FF and FFY were widely used for the construction of supramolecular nanomaterials [32–35]. To this end, we designed biotin-FYIGSR as a potential hydrogelator for the following reasons. The pentapeptide YIGSR is from the functional motif (929–933 sequence of the β1 chain) of laminin, a major glycoprotein located in the basement membrane. It can directly bind to the 67 kDa laminin receptor, a non-integrin cell surface receptor, and regulate cell adhesion, growth, differentiation, and migration [36]. The phenylalanine (F) between biotin and YIGSR provides a hydrophobic force that drives peptide self-assembly. However, after obtaining the designed compound, we found that it could not form a hydrogel but only precipitated by the heating-cooling procedure in the PBS solution (pH 7.4, 1.0 wt%, Fig. S3a). In our previous work, the replacement of *L*-amino acid with *D*-amino acid led to improved properties of the resulting *D*/*L* peptide, including decreased aggregation tendency, enhanced drug loading, and better stability against enzyme digestion [37]. Therefore, we designed and synthesized Biotin-^{D}FYIGSR by replacing *L*-phenylalanine with its *D*-enantiomer. The Biotin-^{D}FYIGSR could form an opaque hydrogel (unpublished data), which was not suitable for 3D cell culture because of the difficulty in observing cell morphology within the hydrogel. We then designed and synthesized Biotin-^{D}FYIGSRGD by introducing a hydrophilic dipeptide GD. In addition, the tripeptide arginine-glycine-aspartate (RGD) is a functional motif that binds to the cell surface receptor integrin and regulates cell behavior. Biotin-^{D}FYIGSRGD (Comp. **1**, Fig. 1a) could self-assemble into a transparent hydrogel (1.0 wt%, Fig. 1c, inset), which was named SupraGel.

Preparation and characterization of SupraGel

Gel could not be formed when the concentration was less than 0.6 wt% (Fig. S3b, c). According to the rheological measurements, SupraGel at 0.67 wt% in PBS showed a storage modulus (G') of approximately 200 Pa at 37°C (Fig. 1b). By increasing the concentration of Comp. **1** from 0.67 to 6.0 wt%, the G' value of SupraGel reached approximately 62 kPa (Fig. S4). G' value increased along with the concentration of Comp.**1**. SupraGel at 1.0 wt% was stable for at least half a year at room temperature (20–25°C). The following cell experiments with SupraGel were based on this concentration. TEM images revealed networks of entangled nanofibers with a diameter of approximately 10 nm formed by the self-assembly of Comp. **1** (Fig. 1c).

Thixotropy and self-healing of SupraGel

SupraGel exhibited a typical shear-induced gel-sol transition behavior and rapid self-healing, which is critical for incorporating cells. The gel could transform into a solution by simply vortexing or vigorously shaking by hand (Fig. 2a), and the resulting solution could form a hydrogel again after approximately 10 min. We performed rheological measurements with the dynamic strain sweep mode to characterize the gel-sol transition (Fig. 2b and Fig. S5). The gel-sol phase transition occurred at a strain value of approximately 22%. Fig. 2c shows reversible gel-sol transitions during cyclic shearing at low (0.1%) and high (50%) strains. At 50% strain, the G' value decreased drastically from 1672 Pa to approximately 7 Pa, which was lower than the loss modulus (G''), indicating a fluid-like state. After

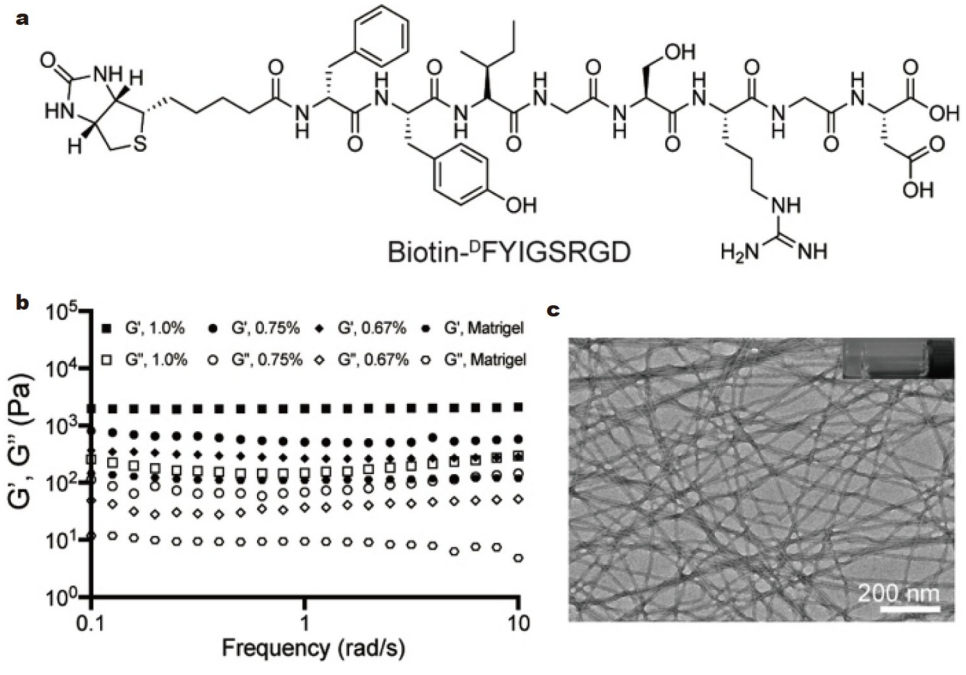

Figure 1 (a) Chemical structure of the Biotin-^{D}FYIGSRGD gelator. (b) Dynamic frequency sweep of the gel at different concentrations at 37°C. (c) TEM image of nanofibers in the gel (inset shows the optical image of SupraGel).

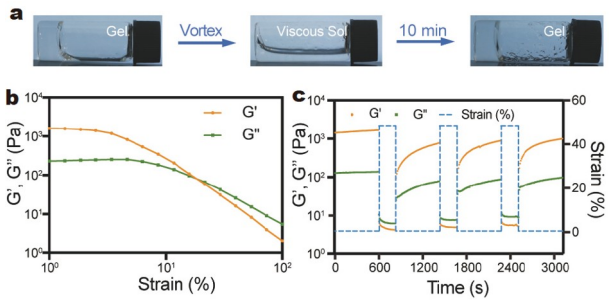

Figure 2 (a) Gel-sol transition and self-healing of SupraGel, (b) Dynamic strain sweep of SupraGel (1.0 wt%) at a frequency of 1 Hz at 25°C, and (c) cyclic dynamic time sweep at low strain (0.1%) and high strain (50%) at 25°C.

the strain was set to 0.1%, the G' instantly showed a value higher than G'' and then recovered to approximately 775 Pa within 10 min, suggesting the fast recovery property of the gel. Such shear-thinning and recovery behaviors can be repeated at least three times. Adding one volume of PBS solution to the gel would also lead to the transformation from gel to a solution. These properties suggest the potential of SupraGel for cell incorporation and separation after culture.

Production of cell spheroids by SupraGel

Next, we tested the application of SupraGel in 3D cell culture (Fig. 3a). After converting the gel to a solution by vortexing, we mixed the resulting solution with a cell suspension at 2:1 and 3:1 ratios by volume (Table S1, Fig. S6). The resulting cell-gel constructs were transferred to a 96 well plate (50 μL well^{-1}, final cell number = 3000 well^{-1}). After an incubation of approximately 15 min to stabilize the cell-gel construct, an additional 150 μL of complete cell culture medium was added on the top of it to provide nutrients for cells in the subsequent culture process (Fig. S7). After 4 h, cells were distributed uniformly in the gel without settling at the bottom of the dish (Fig. S8). Interestingly, the cells efficiently divided into small spheroids at day 3, which grew into larger spheroids with a median cross-sectional area of approximately 4610 μm^2 at day 7 (Fig. 3b). Similar to MCF-7 cells, 4T1 cells could also efficiently divide into spheroids during the 7-day 3D cell culture period, and the final median cross-

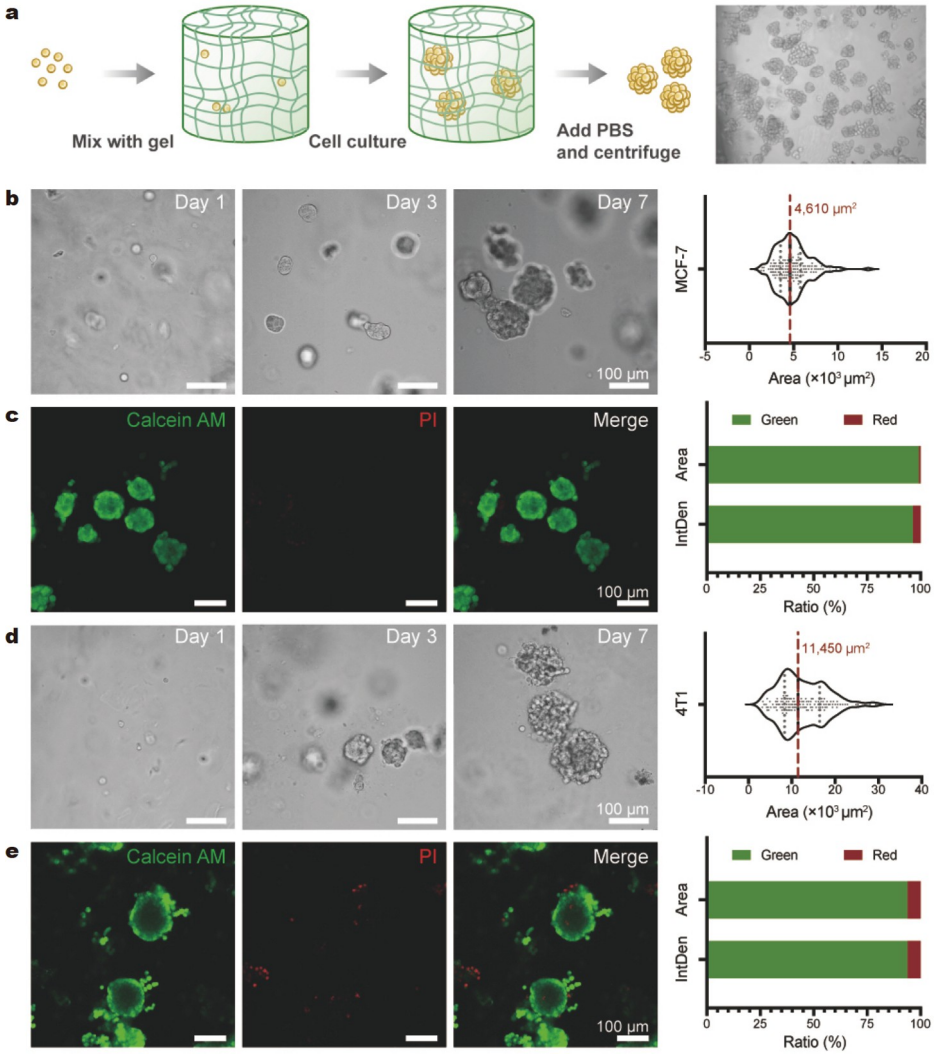

Figure 3 (a) Flowchart of spheroid formation and extraction. Representative MCF-7 (b) and 4T1 (d) spheroids after 1, 3, and 7 days. The right panel shows the cross-sectional area distribution of spheroids. Live-dead staining of 7-day-cultured MCF-7 (c) and 4T1 (e) spheroids released from SupraGel. The right panel shows the corresponding area or integrated density of green and red fluorescence. Scale bar = 100 μm.

sectional area of the spheroids was 11,450 μm^2 (Fig. 3d), which was two times larger than that of MCF-7 cells. The morphology, size, and quantity of spheroids in the two gels (final peptide concentration = 0.67 or 0.75 wt%) were similar, suggesting that both gels were suitable for the formation of spheroids of MCF-7 and 4T1 cells. The possible mechanism by which the gel assists cell spheroid formation might be related to the presence of *D*-amino acid (^{D}F) in the peptide sequence, which was also observed in our previous results [22]. The cell spheroids could be obtained by simple dilution with one volume of PBS solution and subsequent centrifugation (Fig. 3a and Fig. S9), following which they were subjected to live-dead staining. As the spheroids grew bigger, there were more dead cells in 4T1 spheroids (6.26% area, IntDen 6.26%) than in MCF-7 spheroids (0.88% area, IntDen 3.60%). However, in general, most cells in the spheroids were in a viable state (Fig. 3c, e). The SupraGel left after incubating with cells showed no significant degradation (Fig. S10). Solid tumors are typically in a hypoxic environment owing to their fast growth rate. Immunofluorescence staining revealed that most cells in the spheroids expressed HIF1-α, which co-localized in the nucleus (Fig. S11). These observations suggest that the spheroids obtained in our SupraGel shared some similarities with the solid tumors *in vivo*, which could be useful for drug screening.

Encouraged by the success of spheroid formation in cancer cell lines, we moved to the production of cancer stem cell spheroids. At the optimal peptide concentration of 0.83 wt% in the gel, we found that ISCs could grow into spheroids with a median cross-sectional area of approximately 10,000 μm^2 after 15 days of cell culture (Fig. 4a, Fig. S12). Live-dead staining results displayed a clearer spheroid structure and confirmed that SupraGel exerted minor cytotoxic effects on the cells. ISCs seeded in/on SupraGel or in Matrigel had different structures after 7 days of culture (Fig. S13). Spheroids in both SupraGel and Matrigel formed symmetric structures, whereas bud formation occurred in on-gel (2D) spheroids cultured in SupraGel. The diameter of spheroids cultured in Matrigel was larger than that of spheroids cultured in SupraGel, and the culture of MCF-7 and 4T1 encountered a similar situation as ISCs culture (Fig. S14). The median cross-sectional area of the spheroids in Matrigel was 4880 and 12,400 μm^2, respectively. However, immunofluorescence results showed that the spheroids in SupraGel showed stronger green (from Lgr5 staining) and weaker red (from CK20 staining) fluorescence. This suggests that SupraGel helped to maintain the stemness of ISCs during the formation of the spheroids (Fig. 4c), probably owing to the absence of growth factors in the pure peptide-based SupraGel.

CONCLUSIONS

In summary, a supramolecular hydrogel (SupraGel) of a self-assembling peptide derivative was developed for efficiently producing cell spheroids. The gelator was composed of biotin

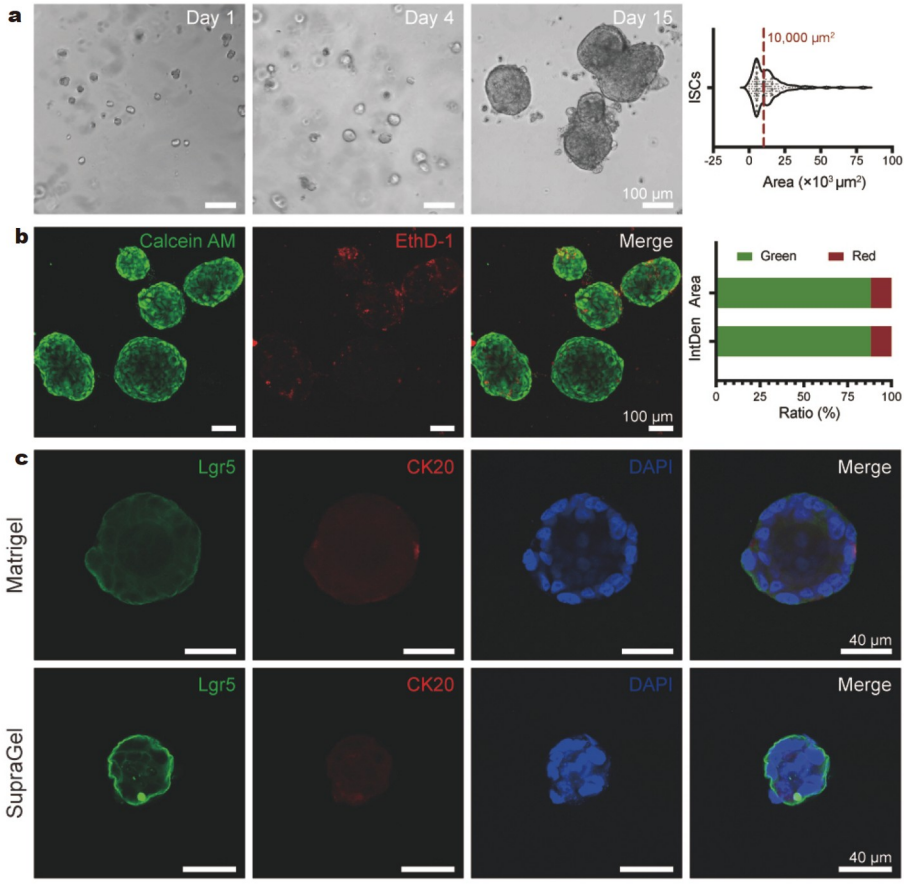

Figure 4 (a) Representative ISC spheroids after 1, 4, and 15 days. The right panel shows the cross-sectional area distribution of spheroids; scale bar = 100 μm. (b) Live-dead staining of 15-day-cultured ISC spheroids released from SupraGel. The right panel shows the corresponding area or integrated density of green and red fluorescence; scale bar = 100 μm. (c) Spheroids in Matrigel and SupraGel on day 7, stained for Lgr5 and CK20; scale bar = 40 μm.

and a short peptide with excellent biocompatibility. The mechanical strength of SupraGel could be easily tuned by the gelator concentration, making it suitable for culturing different types of cell spheroids. Because the networks inside SupraGel were formed by non-covalent interactions, they exhibited typical shear-thinning and recovery behavior, which facilitated cell incorporation and spheroid separation. Two cancer cells, MCF-7 and 4T1, and ISCs could grow into cell spheroids spontaneously in SupraGel. All cell spheroids maintained vitality and activity during long culturing periods. We envision significant potential for SupraGel in cell spheroid preparation and a range of related applications, such as studying cellular complexities and screening drugs.

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Author contributions Ai S and Yang Z conceived and designed the study. Ai S carried out the characterization of the hydrogels. Ai S and Li H designed and performed all the cellular studies with the assistance of Zheng H, Liu JM, Chen Q and Liu JF. Ai S and Gao J prepared the manuscript with the assistance of Li H. Gao J and Yang Z revised the manuscript. All the authors discussed the results and have approved the submission of the final version of the manuscript.

Conflict of interest The authors declare that they have no conflict of interest.

Supplementary information Supporting data are available in the online version of the paper.

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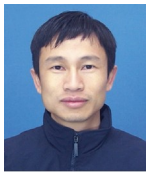

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一种高效生产细胞球的超分子水凝胶

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摘要 与传统的二维培养的细胞相比, 三维培养的细胞球更能够代表天然组织和体内环境, 因此细胞球在细胞生物学研究、组织工程、药物筛选方面具有巨大的潜力. 然而目前开发的包括天然和合成水凝胶在内的材料, 存在成分不确定和生物相容性差等缺点, 阻碍了它们的广泛应用. 在这项研究中, 我们设计了能够自组装的超分子多肽水凝胶(称为SupraGel)用于三维培养中. 在多肽中引入一个*D*构型的氨基酸可能会减少细胞与基质之间的相互作用, 从而促进自发细胞球的形成. 当在SupraGel中培养两种癌细胞系MCF-7和4T1以及肠道干细胞(ISCs)时, 细胞能够有效增殖并生长为细胞球. SupraGel的触变性以及自恢复行为非常适合活细胞的包埋以及细胞球的后续收集. 同时, SupraGel的机械强度能够通过调整多肽的浓度轻松调节, 从而使其适用于不同细胞球的三维培养. 实验结果表明SupraGel能够有效生产细胞球, 在细胞治疗、再生医学和药物筛选方面具有巨大的应用潜力.

