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Sliced Magnetic Polyacrylamide Hydrogel with Cell-Adhesive Microarray Interface: a Novel Multicellular Spheroid Culturing Platform

Ke Hu[1]†, Naizhen Zhou[1]†, Yang Li[1], Siyu Ma[1], Zhaobin Guo[1], Meng Cao[1], Qiying Zhang[1], Jianfei Sun[1,2], Tianzhu Zhang[1,2*], and Ning Gu[1,2*]

[1] State Key Laboratory of Bioelectronics, Jiangsu Laboratory for Biomaterials and Devices, School of Biological Sciences and Medical Engineering, Southeast University, Nanjing, China

[2] Collaborative Innovation Center of Suzhou Nano-Science and Technology, Suzhou Key Laboratory of Biomaterials and Technologies, Suzhou, 215123, China

ABSTRACT

Cell-adhesive properties are of great significance to materials served as extracellular matrix mimics. Appropriate cell-adhesive property of material interface can balance the cell-matrix interaction and cell-cell interaction and promote cells to form three-dimensional structures. Herein, a novel magnetic polyacrylamide (PAM) hydrogel fabricated *via* combining magnetostatic field induced magnetic nanoparticles assembly and hydrogel gelation was applied as a multicellular spheroids culturing platform. When cultured on the cell-adhesive microarray interface of sliced magnetic hydrogel, normal and tumor cells from different cell lines could rapidly form multicellular spheroids spontaneously. Furthermore, cells which could only form loose cell aggregates in classic 3D cell culture model (such as hanging drop system) were able to be promoted to form multicellular spheroids on this platform. In the light

of its simplicity in fabricating as well as effectiveness in promoting formation of multicellular spheroids which was considered as a prevailing tool in the study of the microenvironmental regulation of tumor cell physiology and therapeutic problems, this composite material holds promise in anti-cancer drugs or hyperthermia therapies evaluation *in vitro* in the future.

KEYWORDS: magnetic hydrogel, cell-adhesive microarray interface, cell-matrix interaction, cell-cell interaction, multicellular spheroids, 3D cell culture

1. INTRODUCTION

Hitherto, monolayer cell culture model has provided many important results to interpret biological phenomena. However, researches show that cell behaviors are often unnatural when excised from native three dimensional (3D) tissues and cultured on cell culture plates or Petri dishes[1,2]. Findings in stem cell differentiation elucidate acute disparities in cell functions between 2D and 3D cell culture[3]. Tumor cells culture in 2D plate also display lower resistance to radiotherapy and chemotherapy compared with tumor cells *in vivo*[4]. These deficiencies of traditional 2D culture models lead growing numbers of researchers switch to develop novel materials for developing synthetic extracellular matrix (ECM) analogs[5-9]. These matrices are designed based on one or more structural or functional features of ECM to promote cells to form 3D structures[10]. Multicellular spheroid, an important *in vitro* 3D model for both stem and tumor cell research, is usually generated by preventing cells adherent to matrix therefore favoring cell-cell interaction[11]. Most matrices developed for generating multicellular spheroids were focused vitally on the anti-cell adhesive interfacial properties [12,13].

The degree and distribution of cell adhesive sites on the interface of matrix will greatly affect the cell behavior. Recent studies have indicated that a strong cell-matrix interaction between cells and 2D substrates induces cellular features that differ from those growing *in vivo*[14]. It might be due to the competition between cell-cell and cell-matrix interaction which is greatly affected by the cell adhesive environment[15,16]. We believe it's essential to balance cell-cell and cell-matrix interaction by controlling the interfacial adhesion properties for mimicking the ECM to build *in vitro* culture models. Researchers also found that multicellular hepatocyte spheroids could be formed when cultured in different natural materials by controlling the cell adhesive properties[17].

Due to their ability to simulate the nature of most soft tissues, hydrogels capture numerous characteristics of the architecture and mechanics of the native cellular microenvironment. Recently, applying hydrogel-based composite materials as cell culture matrices draws the attention of many researches. Among the diverse preparation strategies, the combination of nanomaterial and hydrogel holds promise of providing superior functionality[18,19]. Interfacial properties of materials for cell culture have been implicated to play increasingly important roles on a wide spectrum of cellular functions[20]. The heterogeneous interface of nanomaterial-hydrogel composite could affect cell behaviors and mimic ECM to a certain extent.

In our previous work, we have fabricated a novel magnetic hydrogel with anisotropic properties and controllable enhancement of magnetothermal effect when placing in the alternating magnetic field[21]. In this study, we applied the slice of this multifunctional magnetic hydrogel as 3D cell culture matrix. Cell adhesive assemblies array combined with anti-cell adhesive substrate enhance the cell-cell interaction and promote the spontaneous formation of multicellular spheroid by both epithelial and cancer cells (Figure 1). This composite material could provide a new platform for different applications such as anticancer drugs or hyperthermia therapies models *in vitro*.

2. MATERIALS AND METHODS

2.1 Materials

Polyglucose sorbitol carboxymethyether encapsulated Fe_3O_4 magnetic nanoparticles (Fe_3O_4@PSC MNPs) were provided by Jiangsu Key Laboratory for Biomaterials and Devices. Millipore-quality water (18.25 $M\Omega cm^{-1}$), prepared with a Milli-Q Plus water system, was the only used water throughout experiments. Human embryonic kidney 293A (stably transfected with enhanced green fluorescent protein encoding gene (EGFP)) were established and provided by Jiangsu Key Laboratory for Biomaterials and Devices. Breast cancer cell line MCF-7 and ovarian cancer cell line SK-OV-3 were purchased from Chinese Academy of Science Shanghai cell bank. Unless stated, all reagents were purchased from Aladdin Industrial Inc.

2.2 Preparation of sliced magnetic hydrogel

The preparation of anisotropic magnetic hydrogel has been described previously[18]. Briefly, Fe_3O_4@PSC MNPs, acrylamide monomer (87 mg), N, N'-methylene-bis-acrylamide (9 mg), ammonium persulfate (2.4 mg), and tetraethylethylenediamine (0.2 μL) were mixed in 1 mL water first. The mixed solution was poured into a PTFE module after ultrasonic agitation for 10 min and then subjected to a magnetostatic field. After a certain time for assembling, the gelation of PAM was triggered by heating with a ceramic heating flake to 50 °C. The disorganized magnetic hydrogel was fabricated as the same procedure but in absence of magnetic field. Then the obtained magnetic PAM hydrogel was fixed in an aluminum mould and sliced with a low profile microtome blade. The final size of sliced magnetic hydrogel for 3D cell culture was $0.2 \times 1.0 \times 1.0$ cm^3. Before the biological experiments, the magnetic hydrogel was dialyzed for 2 days.

2.3 Morphology characterization of sliced magnetic hydrogel

Optical microscope images of sliced magnetic hydrogel and fluorescence microscope images were taken with a BX63 Olympus microscope. The optical microscope images were transferred into binary images with ImageJ and the section areas of assemblies and interval between magnetic nanoparticle assemblies was measured based on these binary images with ImageJ. Atomic force microscopy (AFM) images were taken with a Bruker Dimension FastScan Atomic force microscope. The sample was firstly replicated with polydimethylsiloxane (PDMS) then scanned with AFM.

2.4 Cell culture

Sliced magnetic hydrogel first was rinsed with PBS twice, then sterilized by immersed in ethanol solution (75%) for 24 hour and in PBS for 24 hour. Then the slice magnetic hydrogel was incubated with cell culture medium for 12 hour before cell culturing. GFP-293A, MCF-7 and SK-OV-3 cells (1×10^6 cells/mL, 15μL) in single cell suspension were grown on sliced magnetic hydrogel placed in 24 well cell-culture plates. Following initial plating of cells, they were allowed to adhere to the hydrogel before addition of complete growth medium to 3 mL. All cultures were maintained in an incubator at 37 °C in an atmosphere of 5% CO_2. Live/dead cells were stained with Fluorescein diacetate (FDA)/Propidium Iodide (PI) dye. Cell viability of tumor cells (SK-OV-3) cultured on cell culture plates and sliced magnetic hydrogel were characterized with cell counting kit-8 (CCK-8).

2.5 Time lapse-microscopy

Time-lapse images of cells seeded on sliced magnetic hydrogel were acquired every 20 min at 100 × magnification for 1 day using an X-living cell workstation (Olympus).

2.6 Laser confocal fluorescence microscopy

Laser confocal fluorescence images were acquired at 100 × magnification using a SP8

(Leica) laser confocal fluorescence microscopy.

2.7 Drug cytotoxicity analysis

To establish dose response of cells on monolayer and sliced magnetic hydrogel, cells were treated with 2, 25 and 100 μg/mL doxorubicin in standard medium after a given culture period. Cytotoxicity was evaluated following a 24-hour incubation on both monolayer and sliced magnetic hydrogel.

3. RESULTS AND DISCUSSION

3.1 Fabrication and characterization of sliced magnetic hydrogel

In our experiments, owing to good biocompatibility and magnetism, Fe_3O_4 nanoparticles coated by polyglucose sorbitol carboxymethyether (Fe_3O_4@PSC) were used as the building block (Supporting Information Figure s1)[22]. The average hydrodynamic diameter of Fe_3O_4@PSC nanoparticles was 201 nm and the magnetic force on this nanomaterial can effectively overwhelm the thermal perturbation based on our calculation[18]. PAM hydrogel was chosen as host material because it is biocompatible after dialysis and the mechanical property of hydrogel is suitable as mimic for ECM[14]. By combining static magnetic field-assisted assembly of magnetic nanoparticles and gelation together, we fabricated this magnetic hydrogel with anisotropic properties. Since the assemblies were encapsulated inside the hydrogel, magnetic hydrogels were sliced in order to expose assemblies on the interface.

Sample (1.0 cm × 1.0 cm × 1.0 cm) was fixed in a mould to avoid break caused by deformation of anisotropic hydrogel when slicing and sliced with a low profile microtome blade. The final size of sliced magnetic hydrogel for 3D cell culture was 0.2 cm × 1.0 cm × 1.0 cm.

A series of sliced magnetic with different concentration of Fe_3O_4 nanoparticles (0.05 mg/mL, 0.3 mg/mL and 1.8 mg/mL, respectively) were fabricated and labeled as SMH-1, SMH-2 and SMH-3. The increase of concentration of Fe_3O_4 nanoparticles could be easily distinguished with naked eyes (Figure 2a) and the optical microscope images of side view of sliced magnetic hydrogel accorded with this tendency (Supporting Information Figure s2). The section morphologies of sliced magnetic hydrogel with Fe_3O_4 nanoparticles as building block are characterized with optical microscope (Figure 2b). Previous studies showed that assembly process could promote the homogeneity of the distribution of nanomaterials inside the hydrogel[23]. The optical microscope images of sliced magnetic hydrogels were analyzed with image processing software. Specifically, sectional areas of and intervals between assemblies were measured and percentage of assemblies in the section area of sliced magnetic hydrogel was calculated. Results demonstrated that structure properties of assemblies array (assemblies' number and sectional areas) on the surface were of no significant difference between each sample with different slicing depth and showed a good reproducibility (Supporting Information Figure s3). As in Figure 3, the area of one assembly, which largely distributed around 4-12 μm^2, is growing slightly when the concentration of nanoparticles increased. According to Figure 3a, the peak value emerges at area of 4 μm^2, 8 μm^2 and 12 μm^2 in SMH-1, SMH-2, and SMH-3, respectively. At lower nanoparticle concentration (SMH-1 and SMH-2), the ratio of assemblies in area ranging from 4 to 8 μm^2 is near to 90% while at higher concentration more than 60% of assemblies are in range of 8-12 μm^2. Furthermore, as showed in Figure 3b, the concentration impacts the interval between assemblies significantly. The distribution of interval of SMH-1 is quite wide, from 10 to 40 μm. With the concentration increasing, the distribution become narrower and the interval between assemblies of SMH-3 is only ranging from 5-10 μm, which showed that with the increasing of concentration the degree of homogeneity of assemblies also improved.

3.2 Sliced magnetic hydrogel applied as 3D cell culture matrix

Previous studies showed that cell-cell interaction played an important role in cell behaviors and functions[23]. Strong cell-matrix interaction inhibited the formation of cell-cell interaction when cells were cultured on 2D plates. Based on this, researchers fabricated hydrogels and electro spinning scaffolds with limited cell-adhesive sites to promote the cell-cell interaction and several cancer cell lines were able to form multicellular spheroids when cultured on these matrices[25,26]. Sliced magnetic hydrogel composed by cell-adhesive nanoparticle assemblies and anti cell-adhesive hydrogel also capable of providing a matrix with cell adhesive properties more possibly accord with the ECM than the traditional monolayer culture plate. Since cells can not adhere on the hydrophilic surface of the PAM hydrogel, the cell adhesive function of magnetic hydrogel are provided by the magnetic nanomaterials. However, we found that very few cells could adhere on the surface of disorganized sliced magnetic hydrogel which was fabricated in absence of magnetic field (Supporting Information Figure s4). This might because that the array consisted of randomly distributed magnetic nanoparticles were too small to support cells to form focal adhesion[27]. Compared to the unassembled magnetic nanoparticles, the sectional area of magnetic colloidal assemblies were large enough for single cell to form focal adhesion. Moreover, magnetic nanoparticles of the colloidal assemblies were less likely to be encapsulated inside the hydrogel after slicing. Since the magnetic hydrogels were sliced before being applied as cell culture matrix, the roughness of sliced surface was characterized by atomic force microscope in area of 400 μm^2 (20 μm × 20 μm) which is close to the area of one single cell after moulding with polydimethylsiloxane (PDMS) (Supporting Information Figure s5). Generally, the height of the peaks and valleys were ranging from tens to hundreds of nanometers, while specifically the surface roughness (Ra) is 422 ± 169 nm, which quite close to a polished surface[27]. Research showed that surface roughness ranged from 1 μm to tens of micron would affect the behavior of cells[29]. The roughness of magnetic hydrogel caused by slicing was out of this range (both larger and smaller than) and had little impacts on cells. Consequently, we believe the sliced surface of magnetic hydrogel could be regarded as smooth surface to cells[30].

To evaluate the performance of sliced magnetic hydrogel as 3D cell culture platform, human embryonic kidney 293A (stably transfected with enhanced green fluorescent protein encoding gene (EGFP)) was chosen because 293A cells tended to form multicellular spheroid in traditional non-adhesive 3D culture model. A GFP-293A single cell suspension (15 μL of 1×10^6 cells/mL) was pipetted onto the surface of the composite material placed in a 24 well plate. After 1 hour incubation in CO_2 incubator, 3 mL culture medium was added. According to the optical microscope images, the proportion of cell adhesive sites areas on the surface of sliced magnetic hydrogel were rose with increasing concentration of magnetic nanoparticles. Sliced magnetic hydrogels with concentration of Fe_3O_4 nanoparticles higher than 0.3 mg/mL were light-tight and morphology of cells cultured on these composite materials could hardly be observed with inverted microscope. So the cell cultured on sliced magnetic hydrogel with concentration of Fe_3O_4 nanoparticles higher than 0.4 mg/mL was observed after the composite material was overturned. After preliminary experiments we found that GFP-293A cells cultured on all of three samples with different density of cell-adhesion sites could form multicellular spheroids after 6 days (Supporting Information Figure s6). The number and size of the multicellular spheroids formed on SMH-1, SMH-2 and SMH-3 were counted and measured (Supporting Information Figure s7). Researches showed that the size and size distribution of multicellular spheroids were cell-line dependent and the numbers of multicellular spheroids formed increased with the density of adhering cells caused by partially exposed Fe_3O_4 nanoparticles [12, 31]. Results showed that the size of multicellular spheroids formed on different samples were of no significant difference. The numbers of multicellular spheroids formed on sliced magnetic hydrogel per sample slightly increased when the concentration of magnetic nanoparticles increased. Based on Figure 3a, the cell adhesive area of sliced magnetic hydrogel increased from approximately 0.9% to 17% when the concentration of magnetic nanoparticles rose from 0.05 mg/mL to 1.8 mg/mL and the density of adhesive site was still not large enough to prevent cells from forming multicellular spheroids[17]. However, farther increasing the density of magnetic nanoparticles will induce the aggregate and precipitate of nanoparticles when mixed with the polymer monomer solution. This makes it not possible to fabricate magnetic hydrogel containing higher concentration of magnetic nanoparticles.

Considering the suitable concentration of magnetic nanoparticle was demanded for alternating magnetic field induced hyperthermia of magnetic hydrogel[32], we chose SMH-2 for further evaluation. Results showed that GFP-293A cells overlaid as single-cell suspension on SMH-2 formed 3D cell multicellular spheroids one day after plating (Figure 4a) and the average diameter and total numbers of multicellular spheroids clearly increased in 6 days post plating (Figure 4b), which was not observed with the GFP-293A cells cultured in 24-well plates (Figure 4c). 3D reconstruction images obtained with Laser confocal fluorescence microscope showed that multicellular spheroids were not formed inside the sliced magnetic hydrogel but on the surface (Supporting Information, S1 mov.). These multicellular spheroids won't drop off when underwent a gentle shaking. To further investigate the formation process of multicellular spheroids on the surface of sliced magnetic hydrogel, an Olympus X- living cell workstation was used to acquire the time-lapse images of seeded cells (cell seeding density was doubled to accelerate the spheroid formation) every 20 min at 100 × magnification for 1 day (Supporting Information, S2 mov.). Results demonstrated that GFP-293A cells initially formed small aggregates *via* proliferation and migration, and then these aggregates further merged into irregular spheroids and finally became mature with inerratic geometry (Figure 5). The co-localization of magnetic nanoparticle assemblies and multicellular spheroids were demonstrated with optical microscopy images at low exposure. Results showed that GFP-293A cells started to aggregate 2 hours after seeding (Supporting Information Figure s8). Intervals among assemblies were smaller than the size of single cell and were able to be stepped over easily by cells. After two days, multicellular spheroids had formed and localized on top of the nanoparticle assemblies. Since the diameter of multicellular spheroids ranged in hundreds of micrometers, the optical microscope could not focus on the surface of sliced magnetic hydrogel and multicellular spheroids at the same time (Supporting Information Figure s9). After culturing for 6 days, with the growing of those spheroids, they became opaque therefore the nanoparticle assemblies underneath became invisible (Supporting Information Figure s10). However, the assemblies around the multicellular spheroids proved that those areas underneath spheroid certainly had assemblies exposed, owing to the homo-distribution of assemblies that area with no assemblies surrounded by area with assemblies does not possibly exist. The spontaneous formation process of multicellular spheroids, cell morphology and behaviors were similar with cells culture on staple commercial materials (such as Matrigel) [33], but costs of sliced magnetic hydrogel were much lower and almost have no batch-to-batch discrepancy. Furthermore, the spheroids formation efficiency of cells cultured on sliced magnetic hydrogels was also no worse than those developed techniques[34].

Most fully-fledged techniques used to generate multicellular spheroids (such as hanging drops) nowadays were designed based on the "non-adhesion" strategy. Findings in these models demonstrated the spheroid formation capability among different cell lines were obviously different. Researchers found that many tumor cell lines could only form loose aggregates, an architecture that loses tight cell-cell conjunction thereby significantly different from the *in vivo* tumor features[34]. We chose breast cancer cell line MCF-7 and ovarian cancer cell line SK-OV-3 as models to study the multicellular spheroid formation capability on the sliced magnetic hydrogel. Results showed that after 3 days culture, MCF-7 cells initially formed a large amount of small cell aggregates (Figure 6a). These cell aggregates continued to grow and merge to form multicellular spheroids after 6 days culture (Figure 6b). Results of live/dead staining with Fluorescein diacetate (FDA) and Propidium Iodide (PI) demonstrated that most dead cells were located in the center of partial multicellular spheroids after 9 days culture (Figure 6c-d), which should be induced by the diffusion limitation of nutrition and metabolic wastes. SK-OV-3 cells cultured on sliced magnetic hydrogel presented similar results (Figure 5e-f), although dead cells (red) were observed after cultured for 12 days (Figure 6g-h). The proliferation of tumor cells in monolayer was comparably faster than those cultured in 3D[10]. Results showed that proliferation rate of SK-OV-3 cultured on sliced magnetic hydrogel was clearly higher than those cultured in monolayer (Supporting Information Figure s11), which was close to the growth rate in vivo[35]. However, when cultured in hanging drop system SK-OV-3 cells could only form cell aggregates with no penetration resistance (Supporting Information Figure s12). The drug resistance of SK-OV-3 cells cultured on the sliced magnetic hydrogel and in the culture plates was preliminarily evaluated with doxorubicin as model drug. Results demonstrated that the cells cultured on sliced magnetic hydrogel presented stronger drug resistance than those cultured on plates when the concentration of doxorubicin increased to 100 μg/ml after culturing for 9 days, which was due to the compact structures of multicellular spheroids (Supporting Information Figure s13). Previous studies showed that SK-OV-3 cells could form multicellular spheroids and showed drug resistance when cultured inside RGD-modified, cell-secreted metalloproteinases (MMP) sensitive hydrogel[10]. Furthermore, researches demonstrated that adding extracellular adhesion molecules (fibronectin, laminin or reconstituted basement membrane) into the hanging drop culture system could induce SK-OV-3 cells to form multicellular spheroids[36]. Combined with our findings, we speculate that the matrix with appropriate cell-adhesion properties were necessary for the formation of multicellular spheroid[37]. Further studies on the cytobiology mechanism of spheroid formation could be carried on by applying this composite material as platform.

4. CONCLUSION

In summary, a novel sliced magnetic hydrogel with certain regularity and reproducibility was prepared and applied as 3D cell culture platform. By combining anti cell-adhesive hydrogel and cell-adhesive magnetic colloidal assemblies together, this composite material provided a matrix with cell-adhesive microarray on the surface which could enhance the cell- cell interaction. Cells from different cell lines could spontaneously form multicellular spheroids when cultured on this functionalized interface, and on this platform cells which could only form cell aggregates in traditional 3D cell culture system were able to form multicellular spheroids, which promote the applicability of this matrix. This low-cost magnetic hydrogel will have a great potential in *in vitro* evaluation of hyperthermia and chemotherapy or cytobiology researches.

■ ASSOCIATED CONTENT

* S Supporting Information

The morphology and magnetic properties of Fe_3O_4@PSC MNPs, the optical microscope images of side view of sliced magnetic hydrogel and sectional view of sliced magnetic hydrogel with different slicing depth, AFM characterization of sliced magnetic hydrogel, Statistical diagram of numbers and size of multicellular spheroids formed on SMH-1, SMH-2 and SMH-3,morphology of GFP-293A cells cultured on sliced disorganized magnetic hydrogel, SMH-1 and SMH-3, morphology of SK-OV-3 cells cultured in hanging drop system, 3D reconstruction images obtained with Laser confocal fluorescence microscope and time lapsed video of GFP-293A cells cultured on SMH-2.

■ AUTHOR INFORMATION

Corresponding Authors

*E-mail: zhangtianzhu@seu.edu.cn(T.Z.) Fax: +86 25 83272460.

*E-mail: guning@seu.edu.cn(N.G.) Fax: +86 25 83272460.

Author Contributions

† These two authors contributed equally in this manuscript. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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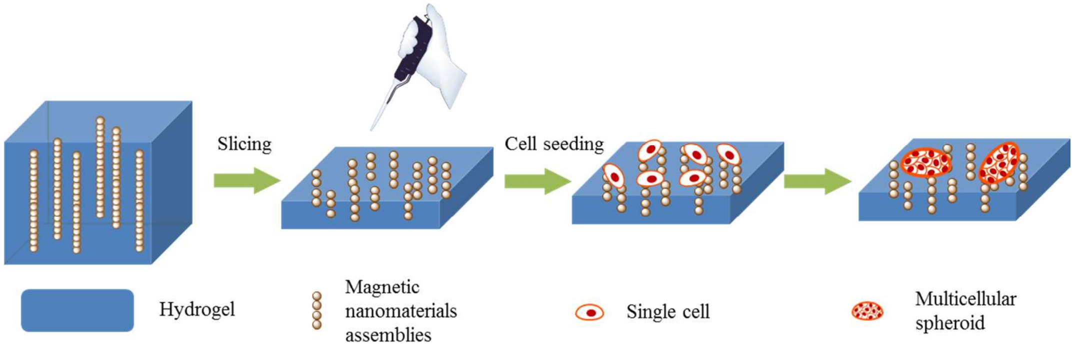


Figure 1. Schematic show of sliced magnetic hydrogel applied as 3D cell culture matrix.

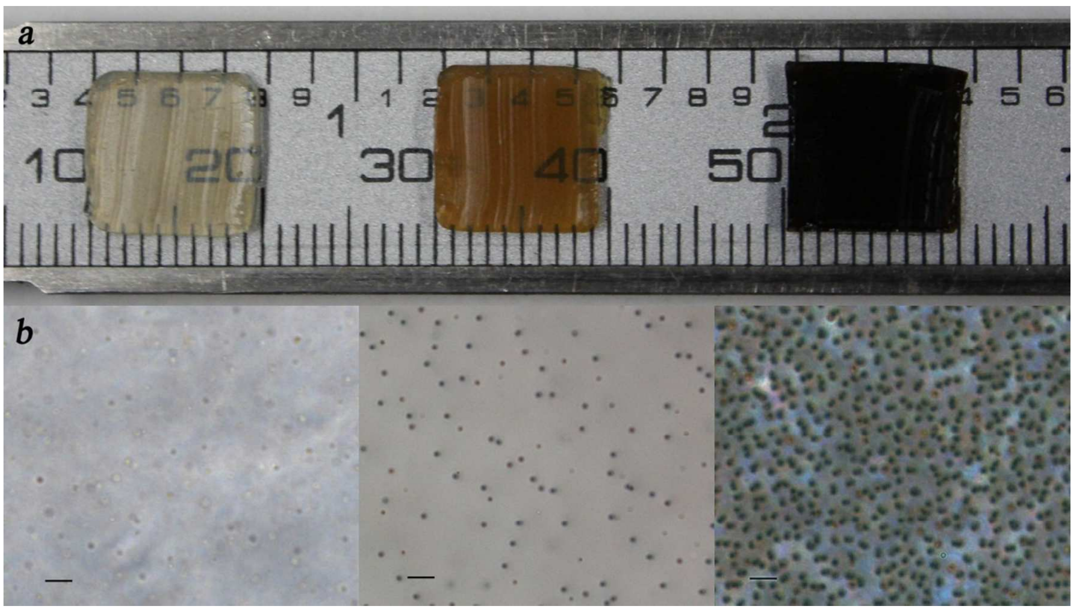


Figure 2. Photographs (a) and optical microscope images (b) of sliced magnetic hydrogel samples SMH-1, SMH-2 and SMH-3 (from the left to the right). Scale bar in (b): 10 μm.

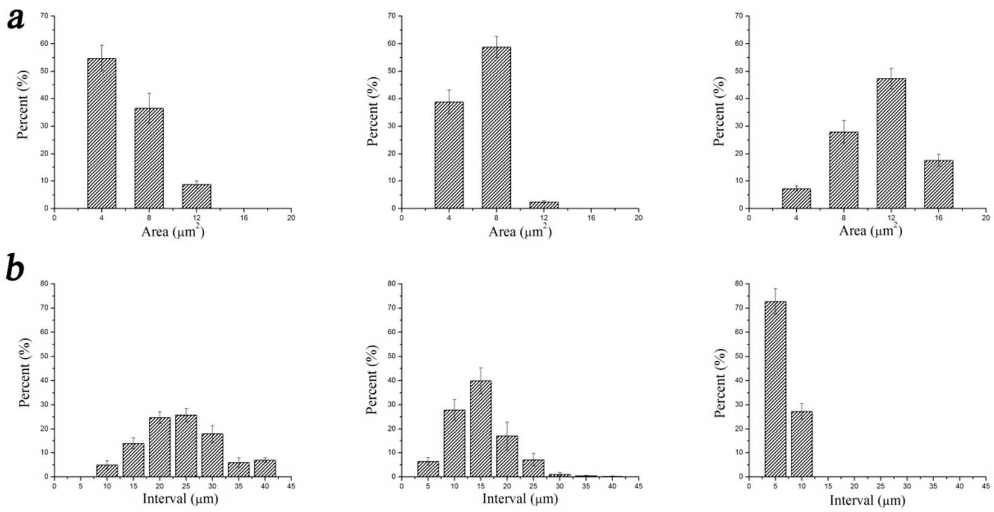


Figure 3. Statistical diagrams of assembly sectional area (a) and intervals between magnetic nanoparticle assemblies (b) of sliced magnetic hydrogel samples SMH-1, SMH-2 and SMH-3 (from the left to the right).

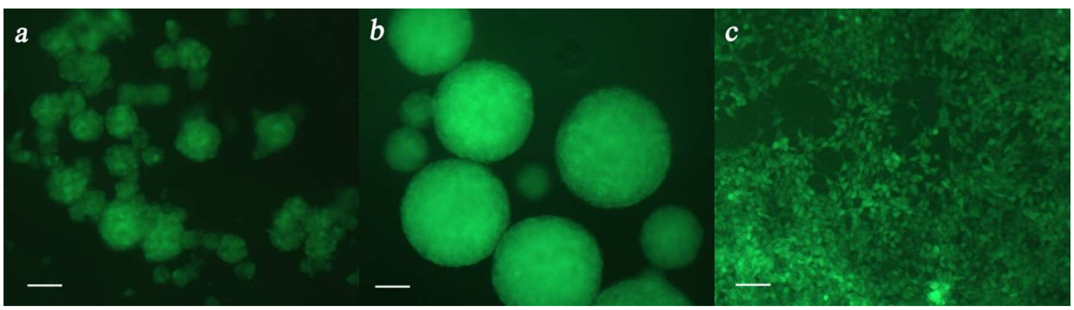


Figure 4. Florescent microscope image of GFA-293A cells cultured on sliced magnetic hydrogel sample SMH-2 for 1 day (a) and 6 days (b) and on 24-well plate for 6 days (c). Scale bar: 100 μm.

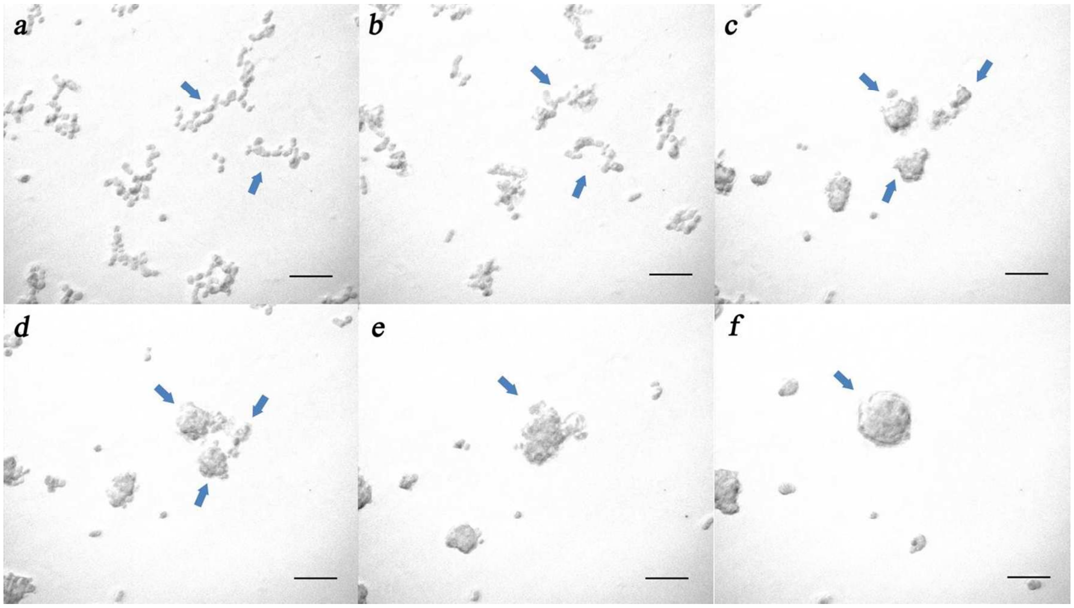


Figure 5. (a-f) time lapse microscope images of GFP-293A cells cultured on sliced magnetic hydrogel. Blue arrows point the spontaneous formation process of multicellular spheroids via migration, proliferation and mergence. Scale bar: 100 μm.

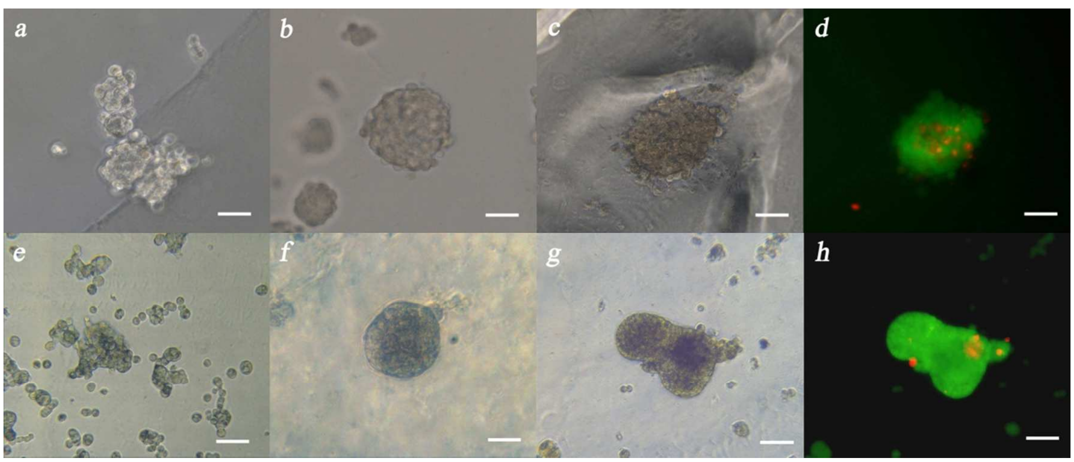
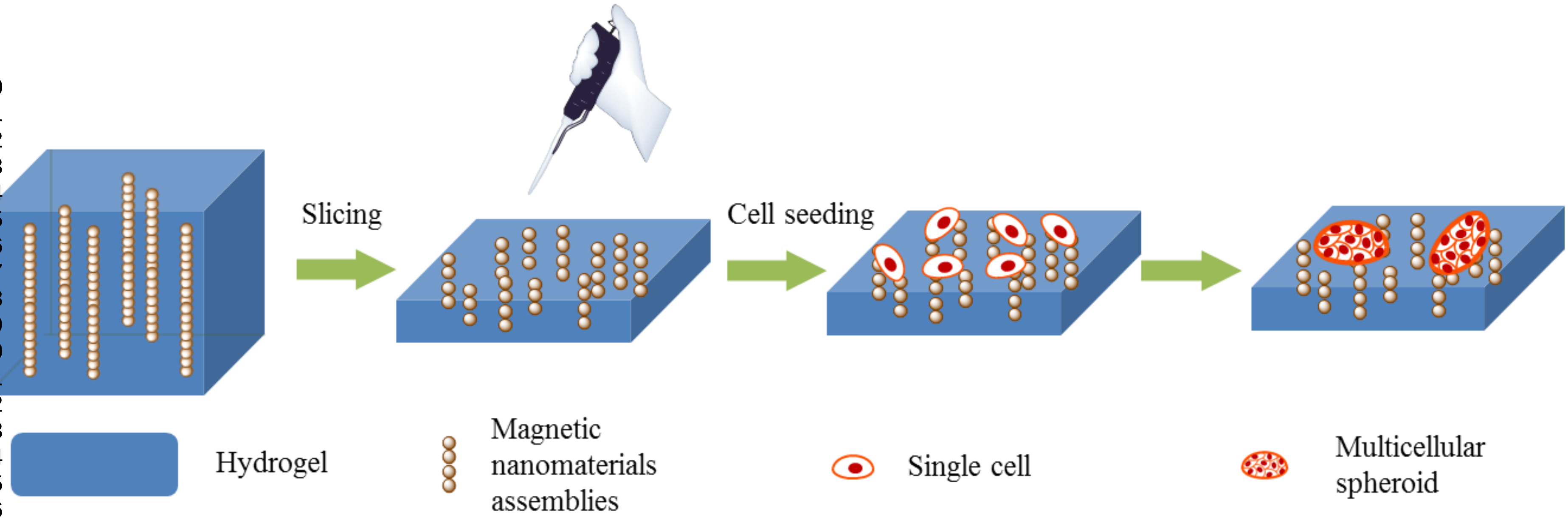


Figure 6. Optical microscope images of MCF-7 cells and SK-OV-3 cells cultured on sliced magnetic hydrogel for 3 (a & e), 6 (b & f), 9 (c) and 12 (g) days. Live/dead cells were stained with PDA (green)/PI (red) (d & h). Scale bar: 50 μm.


Slicing
Cell seeding
Hydrogel
Magnetic nanomaterials assemblies
Single cell
Multicellular spheroid