Polyacrylamide/GelMA hydrogel templates for MCF-7 cancer cell spheroids fabrication

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ABSTRACT

Fabrication of cellular spheroids is critical for creating functional tissue units and investigating mechanism of tumorigenesis, development, intercellular and cell–matrix interactions in vitro. Herein, we developed a novel, simple and facile method for cell spheroid fabrication by using polyacrylamide/gelatin methacrylate (PA/GelMA) hydrogel composites. Arrays of MCF-7 breast cancer cell spheroids can be easily formed by tuning the GelMA composition. The shape and size of cell spheroids can be also well controlled by regulating cell seeding density and culturing time. All these results suggested that this simple and facile platform can serve as a useful tool to generate 3D cell spheroids and can be integrated within high-throughput screening platforms, which will be of great help in engineering functional tissue models and regenerative medicines.

1. INTRODUCTION

Multiple cell types such as pancreatic cells, embryonic stem cells (ESCs) and cancerous cells exist as three-dimensional (3D) spheroids *in vivo*, which commonly scale from tens to hundreds of microns [1-4]. Cell spheroids with enhanced cell-cell interactions can better maintain cell activities than monolayer cells such as production of metabolic enzymes (*e.g.,* matrix metalloproteinase) to remodel local microenvironment, especially for tumor invasion [5, 6]. Homogenous shape and size of embryoid body have also been demonstrated to be crucial for regulating the differentiation of ESCs during development [7, 8]. Fabrication of cellular spheroids is therefore critical for generating functional tissue units and investigating mechanism of tumorigenesis, development and intercellular and cell–matrix interactions.

A large set of tools has been developed for fabricating cellular spheroids *in vitro*, including those based on hanging drop, cell-nonadhesive substrata and magnetic levitation [9-12]. Important challenges that these approaches face include limited control of spheroid shape and size, difficult massive production and limited cell types. Microwells have recently emerged as a useful tool to generate cell spheroids with well-controlled size, cell composition and geometry [13-16]. Microwells of defined size and aspect ratio can be fabricated by micromolding on cell-nonadhesive inert materials like poly (ethylene glycol) diacrylate or agarose [17-20]. Suspended cells are reorganized in microwells through gravity and hydrodynamic force, and subsequently assembled into spheroids according to microwell geometry. Although microwells hold several advantages including good controllability of cellular spheroid size and high-throughput

capacity, several limitations including non-uniform cell seeding and complexity of microwell fabrication still existed. Here, we developed a novel, simple and facile method to generate cell spheroids using polyacrylamide/gelatin methacrylate (PA/GelMA) hydrogel templates. Breast cancer cells (MCF-7) were seeded onto hydrogel templates and formed into high-throughput spheroid arrays due to the distributed arginine-glycine-aspartic acid (RGD) cell-adhesive binding sites on hydrogel surface. The shape and size of cell spheroids can be well controlled by regulating cell seeding density and culturing time. Given its tunable chemical modification features, this hydrogel template holds great potential in the formation and analysis of cell aggregates and can be integrated within high-throughput screening platforms.

2. Materials and Methods

2.1 Hydrogel template fabrication

Gelatin methacrylate was firstly synthesized following the protocol as described previously [21]. A mixture contained GelMA solution, acrylamide monomers, crosslinker N,N methylene-bis-acrylamide, ammonium persulphate and N,N,N´,N´-tetramethylethylenediamine (TEMED) was prepared. The ratio of acrylamide%/bis-acrylamide% was 6%/0.45% here. The 0.5% w/v photoinitiator 2-hydoxy-2-methylpropiophenone was then added to the prepolymer solution. Hydrogel mixture was then exposed to 365 nm UV light for crosslinking. The exposure time was 60 seconds for each simple. Two mass ratios of GelMA (5% w/v and 10% w/v)

were used here. Hydrogel template was put in PBS to swell for 24 hours and then removed to 6-well plate before cell seeding.

2.2 **Scanning electron microscopy (SEM)**

For sample preparation, the hydrogel templates were serially transitioned from dH_2O into absolute ethanol with 30 min incubations in 20, 40, 60, 80, and 100% ethanol solutions. Samples were then placed into the freeze-drying chamber of a freeze-dryer (VFD-2000, Boyikang, Beijing, China) at -70°C for 3 hours. The porous structure of hydrogel templates was sputter coated with platinum (JFC-1600, JEOL), and the specimens were examined using a JEOL JSM-6700F SEM. Hydrogel templates seeded with cells were fixed in 4% paraformaldehyde and prepared for SEM as described above. The pore size of hydrogel scaffolds was analyzed using Image-Pro Plus (IPP; version 6.0, Media Cybernetics).

2.2 **Mechanical testing**

Hydrogel samples were cut into bone shape using molding method. Hydrogel samples were then tested at a rate of 10% strain/min on a Bose 3200 mechanical tester. The data were analyzed using Origin 8.0 and Young's modulus was determined as the slope of the linear region corresponding to 20% strain.

2.3 **Cell seeding**

MCF-7 breast cancer cells were cultured in RPMI 1640 medium (HyClone, South Logan, UT, USA) and supplemented with 10% fetal bovine serum (Gibco Industries, Inc.,

Big Cabin, OK, USA) in a humidifified 5% CO_2 incubator at 37 °C. The cells were digested with trypsin (EDTA 1×, Mediatech, Inc., Manassas, VA, USA) and centrifuged, and the liquid supernatant was removed. The prepared cellular suspension was then gently mixed with a pre-filtered gelatin solution at a final cell density of 1×10^4 cell mL^{-1} to 1×10^6 cells mL^{-1}. The cell suspension was then seeded onto hydrogel templates and incubated at 37 °C.

2.4 **Immunofluorescence**

To measure cell spheroid morphology, cells seeded on hydrogel templates were stained using a live/dead assay (Molecular Probes) following manufacturer's instructions. Briefly, each hydrogel template was incubated in a solution of 2 μg mL^{-1} calcein AM and 5 μg mL^{-1} propidium iodide at 37 °C for 30 min. Confocal microscopy (LSM 700, Carl Zeiss) was performed to identify cells that were live (stained green by calcein AM) and dead (stained red by propidium iodide). F-actin stress fibers and nuclei of cells were stained by fluorescein rhodamine conjugated phalloidin (Acti-stain rhodamine phalloidin, Cytoskeleton, Inc.) and 4', 6-diamidino-2-Phenylindole (DAPI; InvitrogenTM, Life Technologies, Inc.), respectively. The size of cell spheroids were analyzed using ImagePro Plus.

2.4 **Statistical analysis**

All data collected were presented as the mean ± standard deviation (s.d.) of 5 samples.

3. Results and Discussion

We have synthesized hydrogel templates by mixing two types of crosslinked polymer: covalently crosslinked polyacrylamide (PA) and photocrosslinkable gelatin methacrylate (GelMA) (Fig. 1). PA gels coated with collagen are typically used to tune substrate stiffness independent of ligand density for 2D cell culture due to their good biocompatibility and tunable mechanical properties [22]. As the hydrolysis product of collagen, GelMA contains lots of arginine-glycine-aspartic acid (RGD) sequences that promote cell adhesion (Fig. 1(*b-c*)). The RGD sequences introduced by GelMA can provide cell adhesive binding sites on hydrogel templates. To characterize the microstructure of synthesized hydrogel templates with varying compositions, we quantified the pore size of four kinds of hydrogel templates (PA, 5% w/v GelMA, PA/5% w/v GelMA and PA/10% w/v GelMA hydrogel). Analysis of SEM micrographs suggested that the pore size of PA hydrogel (130±8 μm) is smaller than 5% w/v GelMA hydrogel (80±5 μm). The pore size of PA/GelMA hydrogel composites decreased with increasing GelMA monomer fraction (Fig. 2(*a-b*)).

To verify the influence of introduced GelMA on hydrogel mechanical properties, we stretched PA, PA/5% w/v GelMA and PA/10% w/v GelMA hydrogel samples, respectively. The stress/strain curves revealed that Young's modulus of hydrogels increased by introducing GelMA polymer, indicating that GelMA monomers can enhance the mechanical properties of hydrogel composite as a result of the double network formation after crosslinking reaction [23] (Fig. 2(*c*)). We then assessed the MCF-7 cancer

cell spheroid formation on four kinds of hydrogel templates (PA, 5% w/v GelMA, PA/5% w/v GelMA and PA/10% w/v GelMA hydrogel). Live/dead staining results showed that few cells attached on PA hydrogel surface due to its non-adherent property (Fig. 3(*a*)), whereas cells adhered and spread on 5% GelMA hydrogel surface as a result of its RGD composition. Surprisingly, cell spheroid well formed on both PA/5% w/v GelMA and PA/10% w/v GelMA hydrogel surface (Fig. 3(*c*)). We hypothesized that a few cells firstly attached on RGD binding sites introduced by GelMA and non-adherent cells subsequently assembled, adhered to attached cells and proliferated to form cell spheroids. We therefore used the PA/10% w/v GelMA hydrogel template to investigate cell spheroid morphology due to the larger amount of formed cell spheroids than PA/5% w/v GelMA groups. To verify the effect of culturing time on spheroid size, we analyzed the SEM micrographs of formed cell spheroid at 1, 3, 7 and 10 days of culture, respectively (Fig. 3(*b and f*)). The results indicated that spheroid diameter increased with culturing time, which suggested that cell mitosis and proliferation occurred. The diameter of cell spheroid can achieve 115 ± 12 μm after culture for 10 days and the immunofluorescent images showed tight junction between individual cells in cell spheroid (Fig. 3(*d*)). This indicated that such hydrogel templates hold potential to generate large and maturated cell spheroids which can be later utilized as a 3D tissue model *in vitro*. Next, we studied the influence of cell seeding density on the cell spheroid morphology. The quantification of spheroid diameter indicated that larger cell seeding density enhanced the cell spheroid formation and maturation. Further investigation should mainly focus on visualization of cell spheroid growth, maturation,

morphological changes and even gene expression in real time on such hydrogel template without destroying 3D cellular structures.

4. **Conclusions**

In summary, we have developed a novel, simple and facile method for cell spheroid fabrication by using polyacrylamide/gelatin methacrylate (PA/GelMA) hydrogel composites. Arrays of Breast cancer cell spheroids can be easily formed by tuning the GelMA composition. The shape and size of cell spheroids can be also well controlled by regulating cell seeding density and culturing time. All these results suggested that this simple and facile platform can be acted as a useful tool to generate 3D cell spheroids and can be integrated within high-throughput screening devices, which will be of great help in engineering functional tissue models and regenerative medicines.

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Figure Captions List

Figure 1 Schematics of PA/GelMA hydrogel template synthesis. (a) In a polyacrylamide gel, the polymer chains form covalent crosslinks through N,N methylene-bis-acrylamide (yellow circles). (b) Reaction of gelatin and methacrylic anhydride for grafting of methacryloyl substitution groups. The modification occurs at primary amine and hydroxyl groups. The RGD domains are presented as green segments along the GelMA polymer chains, and their chemical structure is depicted within the inset. To create a hydrogel network, the methacrylated gelatin was crosslinked using UV light in the presence of a photoinitiator. (c) In a PA/GelMA hybrid gel, the two types of polymer network are intertwined and formed double networks. (d) Schematics of cell spheroid fabrication on PA/GelMA hydrogel template.

Figure 2 Characterization of PA/GelMA hydrogel composite. (a) SEM micrographs of hydrogels with varying compositions. (b) Quantification of pore size of different kinds of hydrogels. (c) Stress/strain curves of three kinds of hydrogels (PA, PA/5% w/v GelMA and PA/10% w/v GelMA). Scale bar: 100 um (up) and 50um (bottom)

Figure 3 Characterization of cell spheroids generated on PA/GelMA hydrogel templates. (a) Live/Dead staining of MCF-7 breast cancer cells on PA, 5% w/v GelMA, PA/5% w/v GelMA and PA/10% w/v GelMA hydrogel samples, respectively. Cell spheroids were well formed on both PA/5% w/v GelMA and PA/10% w/v GelMA hydrogel groups. (b) SEM micrographs of single cell spheroid on PA/10% w/v GelMA hydrogel templates at 1, 3, 7 day of culturing, respectively. (c) Diameter distribution of cell spheroids on PA/5% w/v

GelMA and PA/10% w/v GelMA hydrogel templates. (d) Immunofluorescent staining of cell spheroids formed on PA/10% w/v GelMA hydrogel templates after 10 days of culturing. (red: F-actin (phalloidin); blue: nuclei (DAPI)). (e,f) The influence of cell seeding density (e) and culturing time (f) on the diameter of cell spheroid formed on PA/10% w/v GelMA hydrogel templates. Scale bar: (a) 50 μm (b,d) 20 μm.