High Quality Multicellular Tumor Spheroid Induction Platform Based on Anisotropic Magnetic Hydrogel

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ABSTRACT: In recent years, multicellular spheroid (MCS) culture has been extensively studied both in fundamental research and application fields since it inherits much more characteristics from in vivo solid tumor than conventional two-dimensional (2D) cell culture. However, anticell adhesive MCS culture systems such as hanging drop allow certain cell lines only to form loose, irregular aggregates rather than MCS with physiological barriers and pathophysiological gradients, which failed to mimic in vivo solid tumor in these aspects. To address this issue, we improved our previously established anisotropic magnetic hydrogel platform, enabling it to generate multicellular spheroids with higher efficiency. The qualities of multicellular tumor spheroids (MCTSs) obtained on our platform and from classic 3D culture systems were compared in terms of morphology, biological molecule expression profiles, and drug resistance. In this novel platform, mature MCTSs with necrotic cores could be observed in 1 week. And results of molecular biological assays with real time-PCR and western-blot confirmed that MCTSs obtained from our platform performed higher cell pluripotency than those obtained from the hanging drop system. Moreover, a lower cell apoptosis ratio and better viability of cancer cells were observed on our platform both under culturing and drug treatment. In conclusion, higher quality of MCTSs obtained from this anisotropic magnetic hydrogel than classic hanging drop system validate its potential to be an in vitro platform of inducing tumor MCTS formation and drug efficacy evaluation.

KEYWORDS: anisotropic magnetic hydrogel, multicellular tumor spheroids, 3D cell culture, microenvironment, cell−matrix interaction

1. INTRODUCTION

Multicellular tumor spheroid (MCTS) is one of the reliable models which maintains the functional phenotype of human tumor cells, sharing many cell biological similarities with avascular tumors. Its unique potential and flexibility in drug screening and pathophysiological analysis has been gradually recognized by biologists and bioengineers; therefore, its prospects are increasing. But simple and reproducible means of MCTS generation are prerequisites and fundamental for subsequent applications, while few researchers paid attentions to the equivalence and generation efficiency of MCTS obtained with such methods.

Preventing cells from adhering to the culture substratum is the common strategy to obtain MCTS. However, research demonstrated that tumor cells from certain cell lines could only form irregular aggregates with no pathophysiological gradients in some systems based on this strategy, and these aggregates would easily be disintegrated by mild perturbation. This is owing to the lacking of sufficient cell−cell interaction, which not only failed to give cell aggregates with avascular tumor mimicking gradients but also unable to provide aggregates with physiological barriers. This barrier has been recognized as protection of MCTS, losing which will lead to abnormally higher therapeutic reagent penetration than human tumor and might cause erroneous drug efficacy prediction in preclinical trials. Results obtained from several three-dimensional (3D) cell culture substrates were quite the opposite. Cells from different tumor or normal cell lines could spontaneously form MCTS when cultured on these matrices, including those which could not form MCTS in nonadhesive systems. These differences might be caused by the in vitro microenvironment provided by the cell culturing system. Although recent research has demonstrated that the microenvironment greatly affected the fate of tumor cells, most classic 3D cell culturing...
systems only provided microenvironments which were either limited aqueous space or inactive interface (e.g., hanging drop system) rather than a biologically relevant one. We believe that a substrate which could allow cells to form 3D organizes via in vivo mimicking behaviors might be a better alternative.

Among biomaterials investigated by researchers for mimicking the extracellular matrix (ECM), hydrogel has become popular for its similarity with the nature of most soft tissues and flexibility in combining with other functionalized organic or inorganic materials.\(^{14,16}\) While it is often challenging to explore the influence of biological, chemical, and physical properties separately due to the inherent characteristics of naturally derived matrices, synthetic hydrogels are more and more attractive due to its ease of tailoring. Moreover, diverse functions brought by the composition of hydrogel and nanomaterials have drawn the attention of many researchers to use it as cell culture matrices.\(^{16,17}\)

In previous studies, we fabricated anisotropic magnetic hydrogels (AMHs) based on the composition of antitcell adhesive polyacrylamide hydrogel and cell adhesive magnetic nanoparticle.\(^{6,19}\) MCTS could spontaneously form on this multifunctional composite with low cell adhesive interface. However, the difference between MCTS spontaneously formed on this platform and those generated in the nonadhesion systems has not been examined yet. In this study, we improved the MCTS culturing platform based on anisotropic magnetic hydrogels and attempted to elucidate this issue by preparing hanging drop system as the contradistinctive platform.\(^{20}\) The cytobiological and molecular biological characteristics of MCTS obtained via di
cytobiological and molecular biological characteristics of MCTS separately due to the inherent characteristics of naturally derived matrices, synthetic hydrogels are more and more attractive due to its ease of tailoring. Moreover, diverse functions brought by the composition of hydrogel and nanomaterials have drawn the attention of many researchers to use it as cell culture matrices.\(^{16,17}\)

**2. MATERIALS AND METHODS**

**2.1. Materials.** Polyglucose sorbitol carboxymethyl ether encapsu-
lated Fe\(_3\)O\(_4\) magnetic nanoparticles (Fe\(_3\)O\(_4\)@PSC MNPs) were provided by Jiangsu Key Laboratory for Biomaterials and Devices. Characterization of Fe\(_3\)O\(_4\)@PSC MNPs was carried out by vibrating sample magnetometer (VSM; Lakeshore 7407, USA) to measure the magnetic properties, Fourier transform infrared spectroscopy (FTIR) spectrophotometer (Nicolet5700, USA) to identify the molecular structure, and transmission electron microscope (TEM) (JEOLJEM-2100, Japan) to exam morphology. Strong magnetic properties of Fe\(_3\)O\(_4\)@PSC MNPs and the presence of Fe\(_3\)O\(_4\) were validated by hysteresis loops (saturation magnetization of MNPs is 69.2 emu/g) shown in Figure S1A and the absorption peak in 582–640 cm\(^{-1}\) bands (generated by stretching vibration of Fe–O bonds)\(^{21}\) indicated by arrow 1 in the FTIR spectrum shown in Figure S1B, respectively. Human colon cancer cell line HT-29 and ovarian cancer cell line SKOV-3 were purchased from NanjingKebai Biotechnology Co. Ltd. Unless further stated, all reagents were purchased from Sigma-Aldrich.

**2.2. Fabrication of Anisotropic Magnetic Hydrogel.** The method of fabricating anisotropic magnetic hydrogel has been previously described. Briefly, Fe\(_3\)O\(_4\)@PSC MNPs solution (Figure S1C), acrylamide monomer (87 mg), \(N,N'\)-methylen-bis-acrylamide (9 mg), and ammonium persulfate (2.4 mg) were mixed in 1 mL water first and then the cross-linking agent tetraethylenepetamine (0.2 \(\mu\)L) was added. After ultrasonic vibration for 10 min, the mixture solution was poured into a poly(tetrafluoroethylene) (PTFE) module and subsequently exposed to a magnetostatic field. Later on, the reaction was triggered by heating to 50 °C with a ceramic heating

flake.\(^{17}\) Then the gelatinized PAM hydrogel was sliced by a customized module and the final size was 0.1 cm \(\times\) 1.0 cm \(\times\) 1.0 cm (Figure S2). The magnetic hydrogel was dialyzed in ultrapure water for 2 days and sterilized with ethylene oxide before cell seeding.

**2.3. Cell Seeding.** Before cell seeding, the hydrogels were first washed with PBS (PH = 7.4) twice and then conditioned in the culture medium for 24 h. The cells were cultured in DMEM supplement with 10% fetal bovine serum and 1% penicillin/streptomycin (Gibco) at 37 °C in a humidified incubator in the presence of 5% CO\(_2\). When confluence reached about 90%, cells were trypsinized and resuspended as concentration of \(5 \times 10^3\) cell/mL. In order to allow cells only adhere to surface of hydrogel rather than everywhere in well plate, 20 \(\mu\)L cell suspension was added on top of each AMH placed in 12 well plate (10 000 cells/well), followed by adding 2 mL complete medium after 12 h of seeding when initial adhesion between cells and AHM has formed. Culture medium was changed every 2 days. The same batch of cells was seeded directly into 12 well cell culture plates in same concentration as 2D control. A 20 \(\mu\)L portion of cell suspensions were dropped onto the lid of the cell culture dish and then turned over and cultured in the same condition. A 10 \(\mu\)L portion of cell culture medium was added into the drop every 2 days. Both monolayer cells and multicellular spheroids were viewed using an inverted microscope (LEICA DMC2900) on a bright field and then photographed at 400× magnification using LAS Vision 4.4 software.

**2.4. Cell Viability Assay.** Cell proliferation was analyzed using cell counting kit-8 (CCK-8, NanjingKebai Biotechnology Co. Ltd.). Anisotropic magnetic hydrogel was cut to fit 96 well plate and 5000/20 \(\mu\)L cells were seeded onto each well. The assay was performed by adding 10 \(\mu\)L of CCK-8 solution to each well. Hanging drop system was performed in the same cell concentration and 10 \(\mu\)L of CCK-8 solution was added into each drop. Cells cultured on 2D plates were set as control. Two hours after treatment, cells in hanging drop system were collected into a 96 well plate, and the color reaction in all three groups were determined at the absorbance of 450 nm by using a microplate reader (BioTek ELX808). Cell apoptosis was detected by Flow Cytometry. The specific experimental procedure was performed as previously described.\(^{22}\)

**2.5. Quantitative RT-PCR.** RNA was extracted from the HT-29 and SKOV-3 cells using TRIzol (Invitrogen). Quantitative RT-PCR was used to detect the levels of CD44, ALDH1A3, CD133, POU5F1, NANOG, and SOX2 which were performed using Fermentas reverse transcription reagents and SYBR Green PCR Master Mix (Applied Biosystems) according to the manufacturer’s protocols. GAPDH was used for normalization. Primers are shown in Table S1. Analysis was performed using the \(2^{-\Delta\Delta CT}\) method. Each experiment was run in triplicate (ABI7300).

**2.6. Western Blot Analysis.** Western blot was performed as previously described.\(^{23}\) Immunoblot was performed using appropriate primary antibodies: CD44, ALDH1A3, CD133, POU5F1, NANOG and SOX2 and GAPDH (ImageQuant LAS 4000 mini).

**2.7. Laser Confocal Fluorescence Microscopy.** After 7 days culture, live/dead cells were stained with fluorescein diacetate (FDA)/propidium iodide (PI) dye. The image was observed and captured with a laser scanning confocal microscope (ZEISS LSM T-PMT). The excitation wavelength for FDA and PI was 488 and 535 nm and emission wavelength was 530 and 617 nm, respectively.

**2.8. Drug Resistance Assay.** The solutions of doxorubicin were added into culture medium of AMH, HD, and 2D control group on day 10 with three different final concentration (1, 10, 100 \(\mu\)g/mL). After 2 days of doxorubicin treatment, the viability of cells in all three groups was determined by a CCK-8 assay.

**2.9. Statistical Analysis.** Each type of experiments was performed at least three times independently with multiple samples at each time in order to ensure the reproducibility, and data were showed as mean ± standard deviation. Evaluation of statistic differences among the experimental groups were carried out by analysis of variance and Student’s t test, and p values < 0.05 were considered to be statistically significant.
3. RESULTS AND DISCUSSION

Anisotropic magnetic hydrogels (AMHs) were prepared by combining magnetostatic field induced magnetic nanoparticles assembly and hydrogel gelation and were sliced and applied as 3D cell culture matrices. The surface of this composite material consisted of cell adhesive magnetic nanoparticle assemblies and anticell adhesive hydrogel. We compared the differences among cells cultured on our AMH platform and those in classical hanging drop (HD) platform, which schematically illustrated in Figure 1. Colon cancer cell line HT-29 and ovarian cancer cell line SKOV-3 were selected as models (Figure 2A and D). After 12 h of culturing, HT-29 cultured on AMH tended to centralize into aggregates while cells in the HD system were observed to concentrate to the center of the droplet. Cells on AMH would not detach when underwent a gentle shaking. After 4 days of culture, cell aggregates on AMH continued to grow and fuse to form multicellular spheroids and most of them are still attached to the surface of composite material (Figure 2C). However, HT-29 cells in the hanging drop kept concentrating with the progressing of culture and formed loose aggregate, whose geometric configuration changed with a gentle shaking (Figure 2B). Similar divergences on different platforms can also be observed in SKOV-3 cells (Figure 2E and F). Meanwhile MCSs prepared by HD and AMH method do not have significant differences (Figure S3) while MCTSs prepared by HD is slightly larger than those prepared by AMH. We infer the difference in size was caused by the compaction of MCSs during maturation. It is worth noting that according to our previous studies, the transparency of materials would be reduced when higher concentration of nanoparticles were assembling, which limited the acquirement of higher density of cell adhesion sites. Here we enhanced the optical transparency of materials via regulating the cutting model, which allowed the use of higher concentrations of nanoparticles, thereby further improving the yield of MCTSs (Figure S4). Based on these, the formation efficiency of MCTSs was improved and mature multicellular spheroids could be observed after culturing for 7 days. Images obtained by laser scanning confocal fluorescence microscope indicated that a necrotic core was formed inside the MCTSs (Figure 3), which was caused by suppression of nutrition and metabolite infiltration. This result demonstrated the potentiality of AMH as a promising platform to induce the mimic of avascular tumor formation.

According to the above results, we noticed that while cells could form MCTSs on AMH, only loose aggregates form in HD and both HT-29 and SKOV-3 showed the same trends. These results not only indicated the cell line dependent MCTS production, in other words, the limited adaption of HD platform but also indicated to us that the existing of matrix as well as interaction between cells and matrices plays an important role in forming multicellular spheroids. Therefore, to further investigate the impact that different culture systems had on cells, a series of biological detections were performed.

First of all, three typical cancer stem markers CD44, CD133, and ALDH1 and three typical stem cell markers POU5F1, NANOG, and SOX2 were selected as molecular biological indicators. Interestingly, the expression of CD133 (∼13-fold)
of the HD group is significantly lower than that of the AMH group (~15-fold) (p < 0.05) although it is much higher than the 2D group. Similar results were found when the SKOV-3 cell line was used, in which AMH groups are ~20-fold higher than 2D group but HD group is only ~17-fold higher than 2D groups. Moreover, to further examine the stemlike properties of cell aggregates, the expression of CD44, ALDH1A3, POU5F1, NANOG, and SOX2 were analyzed. It was found that for SKOV-3 cells, the expression of POU5F1 and SOX2 were highly improved in AMH (~1.9-fold compared with the 2D group) than in HD (~1.5-fold compared with 2D group). A similar situation can be seen in the expression of CD44. As for the expression of NANOG, significant increase can be seen in AMH (~9.5–13-fold) than in HD (~7–10-fold) for both cell lines (Figure 4). To validate the results, western-blot was performed to observe the relative amount of relevant proteins that cell aggregates on different substrates expressed. The results are showed in Figure 5.

These results demonstrated a strong correlation between high-level expression of stemness associated markers and presence of matrices along with MCTS formation. In contrast, HD, which formed loose cell aggregates but lacked compaction, only showed a much lower enhance in cell stemness than AMH group when compared with 2D culture. These all might suggest the mechanism underlying the correlation between MCTS formation and presence of matrices, which inherently link to the increase of stemness of cancer cell. To our knowledge stemness or tumor associated stemness, which reflected by these markers, are directly or indirectly related to the malignancy of tumor and drug resistance.

Figure 3. Optical microscope image (A) and laser confocal microscope image (B) of multicellular spheroid of HT29 cells formed on anisotropic magnetic hydrogel. White arrows indicate the necrotic core inside the multicellular spheroid. Scale bar: 50 μm.

Figure 4. Expression for markers associated with stemness analyzed by qRT-PCR. In order, the mRNA expression of CD44, ALDH1A3, CD133, POU5F1, NANOG, and SOX2 (stemness marker genes) at 4 days were shown. The normalized value was then expressed as the relative ratio in the 2D group (control, CON). Each bar represents the means of three determinations ± SD *p < 0.05, **p < 0.01, ***p < 0.001 among the indicated groups.
risks of tumor reoccurrence, meanwhile a strong link between tumorigenicity and pluripotency, which is reflected by POU5F1, NANOG, and SOX2, have also been found.

In classic anticell adhesion ways to obtain MCTS, seeded cells lost adhesion sites, thus quite a number of cells apoptosis during the early stages. We recognize that distinguishing the drug-induced apoptosis from anoikis, which is of high ratio in HD system, is challenging if we were going to evaluate the drug induced apoptosis. Meanwhile we infer that in non-adhesive state, proliferation of cancer cell will also be inhibited, thereby compromise the predictive capacity in application as drug screen platform. However, in our composite material, the cell adhesive magnetic nanoparticle assemblies served as adhesion sites might somehow reduce the influence bring by anoikis. In order to verify our conjecture, the apoptosis of HT-29 and SKOV-3 cells in 2D, AMH, and HD were detected using flow cytometry after 24 h of seeding. Figure 6 showed cells on AMH performs significant lower ratio of apoptosis than in HD. Furthermore, cell counting kit-8 (CCK-8) was performed to characterize cell viability on different materials and AMH was testified to be more efficient to promote cell proliferation (Figure S5).

At the meantime, we also investigate MCTS that prepared on surface of noncell adhesive hydrogel. On this platform, tumor cells acquire solid surface rather than liquid–air interface support from matrices, however cell adhesion was missing. Figure S6 shows the expression for markers associated with stemness in agarose hydrogel (AGA) and AMH which analyzed by qRT-PCR. Results demonstrate a high promotion of stemness by both platforms when compared with 2D control but relatively divergent when compared with each other. It seems that AHM promotes the enhancement of stemness slightly more than AGA since when we compare AMH with AGA, 4 out of 12 indicators, namely CD44 and NANOG from both cell lines are significant higher while only 3 of 12 indicators, namely ALDH1A3 from both cell lines and POU5F1 from SKOV-3, are significant lower, while the remaining 5 out of 12 are of no significant differences. Although divergent results were found in expression of cell stemness indicators between AMH and nonadhesive AGA, a significantly higher ratio of apoptosis (Figure S7) was exhibited when spheroids were prepared from agarose hydrogel than from AMH. These results suggest not only the essential role a mechanical support provided by a solid surface plays in promoting the stemness of MCTS, but also the necessitation of adhesion sites provided by nanoparticle assemblies that reduce the apoptosis ratio.

Additionally, doxorubicin in three different concentrations was used as drug model to perform drug resistance assay. Results demonstrated that after 7 days culture, compared with HD and cell culture plates, cells seeded on AMH that formed compact spheroids presented significantly higher viability ($P < 0.05$) than HD when doxorubicin reached to 10 μg/mL, (Figure 7) both with HT-29 and SKOV-3 cell line. Even higher cell viability was observed when doxorubicin reached to 100 μg/mL, which demonstrated its superior drug resistance compared with HD. This evidence suggests that our new
polymer material may offer a simple and valuable biomaterial platform for rapid generation of tumor 3D spheroids in vitro as well as for further applications in cancer stem cell research and cancer drug screening.36,37

4. CONCLUSION

In conclusion, this novel anisotropic composite material showed great potential as MCTSs culturing matrices in vitro. Better applicability in multicellular spheroids formation and higher viability of cells culturing on this substrate made it a better mimic of the in vivo microenvironment, which also makes it a better platform for drug screening. Not only so, experiments exploring the biological effect of magnetic therapy or thermal therapy might be feasible using AMHs as platforms because of the magnetic nanoparticle assemblies.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsami.6b15918.

TEM image of Fe₃O₄@PSC MNPs, the pictures and optical microscope images of AMHs in different thickness, optical microscope image of sliced anisotropic magnetic hydrogel with different thicknesses, the cell proliferation assay performed with CCK-8 in AMH, HD, and 2D culture plates for HT-29 and SKOV-3 cell lines, and the primer sequences used for qRT-PCR analyses (PDF)

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This work was supported by grants from the State Key Research and Development Project, China (2016YFA0201704/2016YFA0201700), the Priority Academic Program Development of Jiangsu Higher Education Institutions (PAPD, 2014-37), the National Natural Science Foundation of China (61127002, 61420106012), and the National Natural Science Foundation of China (NSFC, 21273002). We were also thankful for the support from Suzhou Key Laboratory of Biomaterials and Technologies & Collaborative Innovation Center, Suzhou Nano Science and Technology, Suzhou, China.

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