Materials Science and Engineering C 63 (2016) 644–649

Contents lists available at ScienceDirect

Materials Science and Engineering C

journal homepage: www.elsevier.com/locate/msec

Development of size-customized hepatocarcinoma spheroids as a potential drug testing platform using a sacrificial gelatin microsphere system

Wenyan Leong [a], Antje Kremer [a,b], Dong-An Wang [a,*]

[a] Division of BioEngineering, School of Chemical & Biomedical Engineering, Nanyang Technological University, 70 Nanyang Drive, N1.3-B2-13, Singapore 637457, Singapore
[b] Department for Tissue Engineering and Regenerative Medicine, University Hospital Würzburg, Röntgenring 11, 97070 Würzburg, Germany

ARTICLE INFO

Article history:
Received 5 September 2015
Received in revised form 29 January 2016
Accepted 14 March 2016
Available online 16 March 2016

Keywords:
Sacrificial microsphere
Cell encapsulation
Tumor spheroid formation
3D drug testing model

ABSTRACT

Sacrificial gelatin microspheres can be developed as a cell delivery vehicle for non-anchorage dependent cells – its incorporation into a macroscopic scaffold system not only allows the cells to be cultured in suspension within cavities left behind by the sacrificial material, it also allows scaffold-free tissue development to be confined within the cavities. In this study, dense and highly viable hepatocarcinoma spheroids were developed by means of encapsulation in sacrificial gelatin microspheres produced via a simple water-in-oil emulsion technique. By initial selection of microsphere size and distribution, spheroid size can be controlled for various applications such as uniform tumor spheroids as a reproducible three-dimensional drug screening and testing platform that better mimics the in vivo nature of tumors (instead of conventional monolayer culture), as this study has suggested as a proof-of-concept with chemotherapy drug Doxorubicin.

1. Introduction

Cancer has become the leading cause of death globally, and its incidence is set to rise as the population grows and ages [1]; 14.1 million new cases were diagnosed in 2012 according to GLOBOCAN [2]. Anticancer drug therapy is a widely studied field which relies heavily on monolayer cell culture and mouse models with human tumor xenografts as preclinical drug screening and testing platforms [3,4]. However, monolayer conditions are not representative of the nature of tumors in vivo, which are 3D in structure [5] and mouse models have not been accurate models of human cancer cell responses [6]. Furthermore, control and regular inspection of tumor development cannot be performed in mouse models. A 3D in vitro tumor model that mimics the in vivo nature of tumors is therefore imperative to provide a more accurate prediction, thereby reducing animal testing as well as the risk of failure at clinical trial phases [7,8].

Self-assembly of 3D spheroids can be achieved by culturing cells in a non-cell adhesive environment e.g. suspension cultures, round bottomed or coated plates [8,9] and molds [10]. However, they may either require specialized and expensive equipment, expertise or intensive labor. 3D polymeric matrices may be used as a convenient scaffolding system, on which cells are seeded onto after fabrication [11,12] or encapsulated within [13], but the presence of material may affect the recapitulation of in vivo phenomena. Furthermore, to our knowledge, regulation of spheroid size has not been reported, although size control may provide a reproducible and convenient method of drug screening and testing.

To overcome these limitations, we propose the use of sacrificial gelatin microsphere as a cell delivery vehicle for formation of size-controlled tumor spheroids in a hydrogel bulk. Temperature-responsive gelatin dissolute quickly at physiological temperature and can therefore be used as a sacrificial material [14,15] when encapsulated into another hydrogel bulk. In this study, a temperature-cured dissolvable gelatin microsphere based cell carrier (tDGMC) was developed as a cell delivery vehicle for hepatocarcinoma cells through a simple and scalable emulsion technique [16] and incorporated into an alginate hydrogel bulk. As gelatin dissolute, similarly-sized cavities are formed and the delivered cells are developed into material-free spheroids akin to suspension culture, but with an additional advantage of size regulation – spheroid size is limited to the cavity size by the surrounding hydrogel. tDGMC can thus serve as a versatile platform for size-customized spheroid formation of non-anchorage dependent cells for various applications such as tissue engineering and drug testing.

2. Materials and methods

All cell culture related reagents were purchased from Invitrogen, and all chemical reagents from Sigma-Aldrich unless otherwise stated.

* Corresponding author.
E-mail addresses: WLeong1@e.ntu.edu.sg (W. Leong), Antje.Kremer@uni-wuerzburg.de (A. Kremer), DAWang@ntu.edu.sg (D.-A. Wang).

http://dx.doi.org/10.1016/j.msec.2016.03.046
0928-4931/

2.1. Cell culture

Human hepatocarcinoma cell line HepG2 was purchased from American Type Culture Collection (ATCC) and cultured in monolayer with Dulbecco's Modified Eagle's Medium (DMEM) containing 10% Fetal Bovine Serum (FBS, 'Gold' Standard, PAA Laboratories) at 37 °C, 5% CO_2. Cells were harvested by trypsin for encapsulation upon reaching 80% confluence.

2.2. Fabrication of tDGMC

A previously established protocol was modified and performed under sterile conditions [16]. 5% w/v gelatin precursor was prepared by dissolution of gelatin type A in PBS and cell culture medium (1:1 ratio) through boiling. The gelatin precursor and filtered soya oil were maintained in a 37 °C water bath prior to use.

1×10^7 HepG2 cells were suspended in 1 mL of 37 °C gelatin precursor and added into a 50 mL beaker containing 15 mL 37 °C soya oil. A water-in-oil emulsion was formed by stirring for 2 min at 350 revolutions per min (rpm) at room temperature. The setup was placed in an iced water bath and stirred for a further 10 min at 300 rpm for sol-gel transition of gelatin. Subsequently, the emulsion was transferred to a 50 mL centrifuge tube and centrifuged at 700 rpm for 3 min. Supernatant was removed and the pellet of HepG2-laden microspheres referred to as HepG2-tDGMC, was washed in 15 mL 4 °C PBS by resuspension and then centrifugation at 700 rpm for 3 min. The washing procedure was repeated.

The procedure was repeated for two other stirring speeds for optimization. The settings are: 500 rpm for 2 min at room temperature, and 350 rpm for 10 min in an iced water bath for the first setting; and 650 rpm for 2 min at room temperature and 350 rpm for 10 min in an iced water bath for the second setting.

For size quantification, microspheres were suspended in PBS and transferred onto petri-dishes. 40 images were taken at random under light microscopy (Carl-Zeiss) for subsequent diameter measurements and size distribution analysis using ImageJ software. At least 300 microspheres were analyzed for each setting.

500 rpm was selected as the initial stirring speed for subsequent experiments.

2.3. Fabrication of 3D constructs

HepG2-tDGMC were firstly sieved using a 40 μm cell strainer (BD Falcon) and mixed in 1.5% alginate solution (dissolved in 0.15 M NaCl, autoclaved and kept at 4 °C) at 0.3 g tDGMC per 1 mL alginate. For gelling, 80 μL suspension was pipetted into each cylindrical silicon mold laid on a gelatin substrate comprising 15% gelatin and 102 mM $CaCl_2$ dissolved in distilled water. The setup was then placed in 4 °C for 4 min. Subsequently, 5 μL 102 mM $CaCl_2$ was carefully added onto the surface of the suspension, and the setup was returned to 4 °C for 4 min for gelling. HepG2-tDGMC constructs were retrieved and cultured in agarose-coated wells containing cell culture medium at 37 °C, 5% CO_2. Upon elevation of temperature to 37 °C, dissolution of gelatin occurred to leave behind suspended cells in the cavities for further development. The process is depicted in Fig. 1B.

For comparison, a similar number of HepG2 cells was encapsulated in a conventional hydrogel bulk setup and named HepG2-alginate, assuming 30% w/v tDGMC in 1 mL alginate translated to 3×10^6 cells in 1 mL alginate. Therefore, 3×10^6 cells were mixed with 1 mL alginate solution and gelled as per above.

2.3.1. Removal of alginate to check size and integrity of spheroids

Sodium citrate (SC) solution comprising 55 mM sodium citrate in 0.15 M NaCl was used to remove alginate [17]. Briefly, each construct was immersed in 3 mL 55 mM SC solution in a centrifuge tube for 10 min prior to centrifugation at 700 rpm for 3 min. The pellet was gently suspended in PBS via pipetting.

2.4. Cell viability assays

Live/dead cell viability assay was performed as per manufacturer's instruction (Invitrogen). Briefly, each construct was incubated in respective cell culture/differentiation medium with the addition of calcein AM and ethidium homodimer-1 for 30 min, prior to observation under fluorescence microscopy.

WST-1 assay (Roche, Switzerland) was used to quantify cell viability. Samples at pre-determined time points were incubated in 10% v/v WST-1 reagent in cell culture medium for 1.5 h in the dark at 37 °C, 5% CO_2. The conditioned medium was collected for absorbance measurement

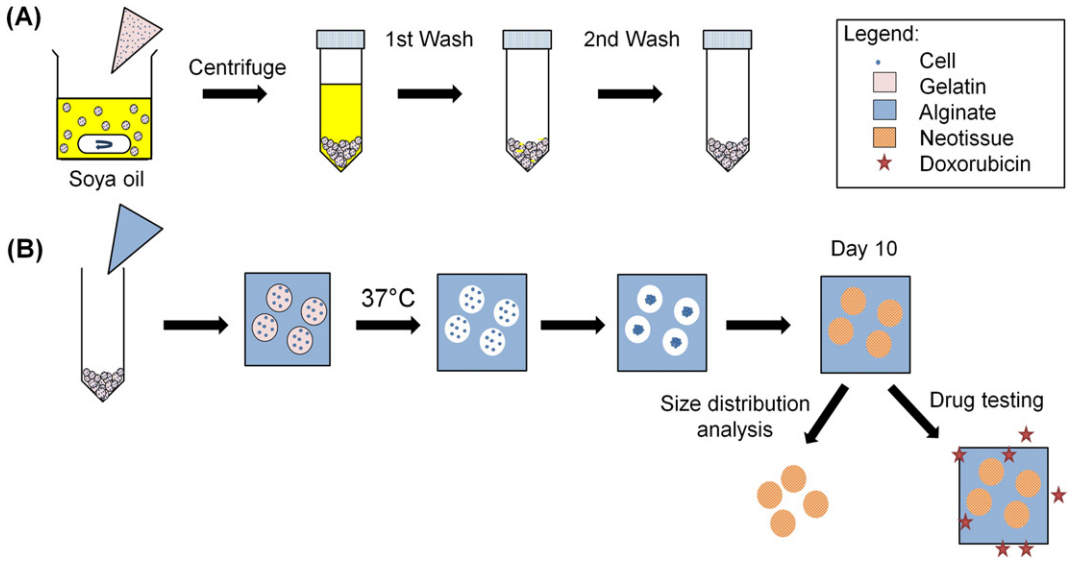

Fig. 1. Schematic diagram of fabrication and developmental process of HepG2-tDGMC constructs. (A) A HepG2-gelatin suspension was added to soya oil to form a water-in-oil emulsion at 37 °C and cooled to 4 °C to set the temperature-responsive gelatin droplets into microspheres (HepG2-tDGMC, or temperature-cured dissolvable gelatin microsphere based cell carrier). Two washes in 4 °C PBS solution was performed to remove oil prior to use; and (B) HepG2-tDGMC were encapsulated in alginate bulk and cultured at 37 °C, during which gelatin quickly dissolute to form cavities containing suspended HepG2 cells. HepG2 cells proliferated to form dense spheroids within 10 days (as optimized in subsequent results), and used to test for size distribution analysis (after alginate removal) and drug testing.