International Journal of Biological Macromolecules 88 (2016) 288–295

Contents lists available at ScienceDirect

International Journal of Biological Macromolecules

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

Fabrication of porous gelatin-chitosan microcarriers and modeling of process parameters via the RSM method

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ARTICLE INFO

Article history:
Received 17 January 2016
Received in revised form 27 March 2016
Accepted 28 March 2016
Available online 29 March 2016

Keywords:
Response surface methodology
Gelatin/chitosan
Mesenchymal stem cells
Microcarriers
Cell expansion

ABSTRACT

Porous gelatin-chitosan microcarriers (MCs) with the size of 350 ± 50 μm were fabricated with blends of different gelatin/chitosan (G/C) weight ratio using an electrospraying technique. Response surface methodology (RSM) was used to study the quantitative influence of process parameters, including blend ratio, voltage, and syringe pump flow rate, on MCs diameter and density. In the following, MCs of the same diameter and different G/C weight ratio (1, 2, and 3) were fabricated and their porosity and biocompatibility were investigated via SEM images and MTT assay, respectively. The results showed that mesenchymal stem cells (MSCs) could attach, proliferate, and spread on fabricated porous MCs during 7 days of culturing especially on those prepared with a G/C weight ratio of 1. Such porous gelatin-chitosan MCs with a G/C weight ratio of 1 may be considered as a promising candidate for injectable carriers supporting attachment and proliferation of MSCs.

1. Introduction

Mesenchymal stem cells (MSCs) can be easily isolated from patients and have the capacity to differentiate into many cell types. Therefore, they have potential clinical utility in the treatment of a multitude of diseases [1]. One of the main weak points of MSCs hindering the development of clinical therapies is their sparseness and the fact that they can only be isolated in very small quantities. The microcarrier (MC)-based stirred suspension culture is an appropriate approach to expand stem cell populations and to overcome this limitation [1–3]. MCs which are small spherical particles can be maintained in a suspended state within a stirred bioreactor and provide a large overall surface area for cells to attach and proliferate [4–7]. Different types of materials including dextran, polystyrene, gelatin, collagen, chitosan, and alginate are commonly used for fabrication of porous MCs [7–9].

Chitosan is a linear polysaccharide, which has attracted much attention as a biomaterial due to its unique characteristics such as low cost, large-scale availability, anti-microbial activity, and biocompatibility [10]. However, one of the most exceptional features of chitosan is its excellent ability to be molded into porous structures, which is a very important issue for use in cell transplantation and tissue regeneration [11,12].

Several methods have been used to modify physical and/or chemical properties of chitosan. These modifications generally included improvement of the pore size, mechanical strength, chemical stability, hydrophilicity, and biocompatibility of chitosan [13–15]. Due to the lack of cell binding sites, the use of chitosan as a substrate for cell culturing is limited. Blending with a natural protein such as gelatin is one of the current approaches for overcoming this limitation [16–19]. Gelatin is a biocompatible protein derived from collagen, possesses low antigenicity, and promotes cell adhesion and migration [16–18]. MCs made from a blend of chitosan and gelatin has been previously reported to be effective for hepatocyte cultures [8]. The cross-linked gelatin-chitosan MCs are very stable and maintain their strength even in acidic and basic solutions [13,20].

The gelatin/chitosan (G/C) weight ratio is one of the most important parameters influencing the MC fabrication process. Furthermore, the fabrication conditions, including applied voltage and syringe pump flow rate, also have significant influences on the final properties of the fabricated MCs. The final properties, including density, diameter and porosity, are all important factors for their application as expansion and delivery scaffolds for cells. Therefore, it is necessary to study these parameters and investigate the relationships between them to be able to fabricate MCs with the most desirable properties.

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http://dx.doi.org/10.1016/j.ijbiomac.2016.03.061
0141-8130/

Response surface methodology (RSM) is a combination of mathematical and statistical methods applied for experiential modeling and analysis of several input data, this data potentially influences the results or quality characteristics of the process [21,22]. To the best of our knowledge, there is no previous report in the literature on modeling of gelatin-chitosan MCs fabrication, and the current study is the first attempt to find a quantitative relationship between electrospraying process parameters and physical characteristics of manufactured MCs. In this study, RSM based on the Box-Behnken design was applied to investigate the effects of three electrospraying parameters (G/C weight ratio, applied voltage, and syringe pump flow rate) on MCs characteristics including diameter and density. Results showed that thereis a quadratic relationship between selected operational parameters and the MCs diameter and density. Furthermore, the prepared gelatin-chitosan MCs showed proper potential for use as a carrier for attachment and proliferation of MSCs as well as injectable tissue engineering scaffolds.

2. Experimental

2.1. Materials

Low molecular weight chitosan (from shrimp shells, M_w = 50 kD and DD = 75–85%), gelatin (from porcine, type A), 3-(4,5-dimethylthiazol-2-yl)-2,5diphenyltetrazolium bromide (MTT) powder, and sodium tripolyphosphate (TPP, 85%) were purchased from Sigma-Aldrich. Sodium hydroxide (NaOH), glacial acetic acid, and 2-propanol were obtained from Merck. Dulbecco's modified eagle's medium (DMEM, high glucose), fetal bovine serum (FBS), trypsin/EDTA, trypan blue stain (0.4%), saline buffer phosphate (PBS), and Roswell Park Memorial Institute (RPMI 1640, without phenol red) were provided from Bio-Idea group.

2.2. Preparation of porous gelatin-chitosan MCs

Gelatin powder was dissolved in deionized water at 40 °C to obtain 8 wt.% solution. Various amounts of chitosan powder were also dissolved in acetic acid solution (2% v/v) for 4 h at ambient temperature to obtain 2.66, 4, and 8 wt.% solutions. Chitosan solutions were added to gelatin with volume ratio of 1:1 to get gelation-chitosan solutions with various G/C weight ratios (1, 2, and 3). The obtained solutions were stirred at 40 °C for 4 h to become homogenous. The homogeneous gelation-chitosan solution was dropped into a crosslinker solution containing 2% w/v TPP through 16-gauge needle driven by a syringe pump under a high voltage electrostatic field to produce spherical shaped microcarriers (MCs). The produced cross-linked MCs were washed several times with distillated water to remove un-reacted TPP, kept at −20 °C for 24 h to freeze absorbed water, and then made porous by freeze drying at temperature and pressure of −40 °C and 0.6 mbar, respectively for 24 h.

2.3. Experimental design and data analysis

Response surface methodology (RSM) was used to design experiments and analyze the effects of considered parameters. G/C weight ratio, voltage, and syringe pump flow rate were considered as the important parameters affecting the MCs diameter and density. A Box–Behnken design of an experiment by each of these parameters at three levels, i.e., G/C weight ratio (1, 2, and 3), voltage (7, 9, and 11 kV), and syringe pump flow rate (300, 500, and 700 μl/min) were used to determine the optimal condition for preparation of MCs. The Box–Behnken experimental design has been established using Design Expert 7.0 software. Seventeen runs were performed to optimize the process parameters and experiments were carried out according to the actual experimental design matrix. Three levels were attributed to each factor, coded as −1 (low), 0 (intermediate), and +1 (high). The range and levels used in the experiments are listed in Table 1. The mathematical relationship between the three variables and the response can be expressed by the second-order polynomial equation:

Table 1
Independent factors and their coded level in Box-Behnken design.

Variables	Real values of coded levels		
	−1	0	+1
G/C weight ratio, A	1	2	3
Voltage (kV), B	7	9	11
Flow rate (μl/min), C	300	500	700

$$Y = \beta_0 + \beta_1 A + \beta_2 B + \beta_3 C + \beta_{11} A^2 + \beta_{22} B^2 + \beta_{33} C^2 + \beta_{12} AB + \beta_{13} AC + \beta_{23} BC \quad (1)$$

where Y is the response; β_0 is a constant; β1, β2, β3 are linear coefficients; β11, β22, β33 are quadratic coefficients, and β12, β22, β23 are cross-product coefficient. A, B, and C are the independent variables.

2.4. Dynamic cell culturing

Human umbilical cord (UC) was obtained from the term placental samples. Isolation of the Wharton's Jelly (WJ) MSCs was carried as previously described by Sabapathy et al. [23] WJ-derived UC-MSCs were cultured in DMEM supplemented with 10% FBS. Cells were maintained in a humidified CO_2 incubator at 37 °C until they reached confluence and were fed by fresh medium every two days. Passage 6 cells was used in this study.

The prepared porous MCs were sterilized using ethanol (70%) and washed three times with PBS solution then incubated with DMEM at 37 °C and 5% CO_2. 20 mg of the sterilized MCs were immersed into 1 ml DMEM and added to 48 well plate. The cell suspension with concentration of 1×10^5 cell/ml was also added to the plate. The cell seeded MCs were incubated for 3 h at 37 °C and 5% CO_2. The plate was then removed and the culture medium was replaced with a fresh one. The plate was put onto a mini-rocker at 15 rpm to facilitate the expansion of MSCs and incubated again at 37 °C and 5% CO_2 for specified period of time. The culture medium was replaced daily with a fresh one.

2.5. Characterization

2.5.1. Porosity and density of MCs

The porosity (Φ) was calculated using MC apparent volume (V_{MC}) and the volume of MC pores (V_p) according to the procedure previously described by Hsieh et al. [24]. We assumed that the single MC has spherical geometry, therefore V_{MC} was calculated according to one single sphere volume ($1/6\pi d^3$). The diameter of MC (d) was calculated using a hemocytometer. The pores volume (V_p) was assumed as the volume of absorbed ethanol by MCs. The MCs with dry weight of (W_d) was immersed into ethanol and kept in the vacuum desiccator for 15 min to let the ethanol to diffuse completely into the pores. The MCs were then taken out and weighted again (W_e) after removing surface ethanol with blotting paper. The pores volume, porosity, and density of MC were calculated according to the following equations:

$$V_p = \frac{W_e - W_d}{\rho_s} \quad (2)$$

$$\phi(\%) = \frac{V_p}{V_{MC}} \times 100(\%) \quad (3)$$

$$\rho_{MC} = \frac{W_d}{V_{MC}} \quad (4)$$

where, ρ_s and ρ_{MC} are ethanol and MC density, respectively.