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Modeling and optimization of gelatin-chitosan micro-carriers preparation for soft tissue engineering: Using Response Surface Methodology

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ABSTRACT

Electrospray ionization is a wide spread technique for producing polymeric microcarriers (MCs) by applying electrostatic force and ionic cross-linker, simultaneously. In this study, fabrication process of gelatin-chitosan MCs and its optimization using the Response Surface Methodology (RSM) is reported. Gelatin/chitosan (G/C) blend ratio, applied voltage and feeding flow rate, their individual and interaction effects on the diameter and mechanical strength of the MCs were investigated. The obtained models for diameter and mechanical strength of MCs have a quadratic relationship with G/C blend ratio, applied voltage and feeding flow rate. Using the desirability curve, optimized G/C blend ratios that are introduced, include the desirable quantities for MCs diameter and mechanical strength. MCs of the same desirable diameter (350 μm) and different G/C blend ratio (1, 2, and 3) were fabricated and their elasticity was investigated via Atomic Force Microscopy (AFM). The biocompatibility of the MCs was evaluated using MTT assay. The results showed that human Umbilical Cord Mesenchymal Stem Cells (hUCMSCs) could attach and proliferate on fabricated MCs during 7 days of culturing especially on those prepared with G/C blend ratios of 1 and 2. Such gelatin-chitosan MCs may be considered as a promising candidate for injectable tissue engineering scaffolds, supporting attachment and proliferation of hUCMSCs.

1. Introduction

Application of common scaffolds in soft tissue engineering is restricted due to problems such as vascularization limitation [1]. Additionally, prefabricated scaffolds require invasive surgery for implantation. The healing of the defect is difficult and may pose further problems on the patient [2,3]. Injectable biomaterials, such as collagen, gelatin, alginate, hyaluronic acid, and chitosan gels have therefore emerged as candidates for soft tissue replacement [4,5]. Injectable materials are advantageous because they require a minimally invasive procedure, are mechanically similar to soft tissue, and have the capability of conforming to irregular shapes; however, many cells poorly perform when suspended within a gel [5,6].

Using injectable hydrogel microcarriers (MCs) has the potential to revolutionize tissue engineering technology. Tissue-engineered injectable composite was developed for the first time by Burg and coworkers, wherein the cells were seeded onto the gelatin/chitosan MCs [7].

In vitro and in vivo behavior of mammalian stem cells is strongly related to the surface features, and the interactions between the cell-matrix. Biopolymer MCs inherent properties such as biocompatibility, biodegradability and bio-adhesivity, are critical to obtain the suitable injectable scaffolds for tissue engineering [8]. To achieve this purpose, a variety of natural materials such as chitosan, hyaluronic acid, alginate and gelatin have been studied to provide optimal conditions for stem cell culture [9]. Sun et al. have shown that biopolymer MCs' diameter range between 100 and 400 μm with proper mechanical properties is required for cells attachment and expansion [10]. Hajiabbas et al. reported that the blend of chitosan and gelatin biopolymers achieves suitable results for soft tissue engineering application [11]. Chitosan-gelatin linking represents a suitable composite biopolymer for tissue engineering with improved mechanical properties and cell biding sites, that are related to the chitosan and gelatin, respectively [12].

There are several methods to prepare biopolymeric MCs such as emulsification-solvent evaporation, emulsification–coacervation, phase separation–coacervation, ionotropic gelation, electrostatic spray and spray drying [13]. Along these methods, emulsification–coacervation has attracted considerable interest in the last decade due to mild operation conditions and material activity preservation [14]. However because of the high size fluctuation and low biocompatibility of the fabricated MCs [12,14], other methods are being considered. Among these methods is electrospraying, which combines spraying a solution using a syringe pump under a high voltage electrostatic field with ionotropic gelation methodology [15]. To achieve a standardized

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diameter range for MCs, experimental parameters such as flow rate of syringe pump and voltage of electrostatic field (provided using a high voltage power supply) should be manipulated.

In order to optimize the diameter and mechanical strength of MCs, variation of mentioned experimental parameters and blend ratio of the polymers should be obtained using an experimental design technique such as Response Surface Methodology (RSM) [16]. RSM is a statistical approach applied for building and analyzing models between several input and response variables. RSM is a well-organized modeling method which uses polynomials in place of local rough calculation to the true input or output relationship. In RSM the aim is to optimize the response (output variables: diameter and mechanical strength of MCs) which is dependent on several experimental variables (input variables: biopolymers blend ratio, feeding flow rate of the syringe pump and voltage of power supply). Using RSM is beneficial to reduce the number of experimental runs, cost and time [17].

Although, a number of polymeric MCs have been produced using electrospray technology [15,18–21]; however, to date, no studies on gelatin-chitosan MCs optimization prepared by electrospraying are available in the literature. In this study, electrospray ionization in the presence of tri-polyphosphate (TPP) as the stabilizer was applied to fabricate gelatin-chitosan MCs for soft tissue engineering applications. The design of experiments using RSM methodology was employed to optimize and predict the diameter and mechanical strength of the prepared MCs with varying gelatin/chitosan (G/C) blend ratios, feeding flow rate of the syringe pump and voltage of power supply.

Furthermore, to ascertain the value of the fabricated optimized MCs for tissue engineering applications, the scaffold properties such as elasticity and the ability to support the attachment and proliferation of hUCMSCs were evaluated.

2. Materials and methods

2.1. Materials

Medium molecular weight chitosan from shrimp shells (low viscosity), gelatin (type A, from porcine skin), sodium tri-polyphosphate (TPP), and 3-(4,5-dimethylthiazol-2-yl)-2, 5 diphenyltetrazolium bromide (MTT) solution were supplied by Sigma-Aldrich. 2-propanol were purchased from Merck (Darmstadt, Germany). 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. Solution preparation

Chitosan and gelatin powders were sanitized first prior to solution preparation. Specified amount of chitosan and gelatin were wetted with 70% ethanol and let to dry thoroughly. The polymer weight should be balanced before and after sanitization procedure. Gelatin powder was dissolved in deionized water at 40 °C to obtain 8 wt% solution. Various amounts of chitosan powder was also dissolved in acetic acid solution (2% v/v) for an overnight 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) as shown in Table 1. The obtained solutions were stirred at 40 °C for 4 h to become homogenous It is worth mentioning that for the solution with highest amount of chitosan (8 wt%), the gelatin solution was added to the chitosan solution followed by blending together for 8 h at 40 °C to get a homogeneous solution.

Table 1
MCs' preparation data.

Sample	MCs with various blend ratio		
	G1C1	G2C1	G3C1
Chitosan solution in 2% acetic acid (w/v)	4%	2%	1.3%
Gelatin solution in de-ionized water (w/v)	4%	4%	4%

2.3. MCs fabrication

Gelatin-chitosan MCs were prepared by a protocol based on the methods previously introduced [12,15] using the electrospraying technique. Briefly, chitosan and gelatin solution were separately prepared and then were blended with specified ratio (Table 1). The homogeneous gelation-chitosan solution was dropped into a coagulation solution containing 5% w/v TPP through 16-gauge needle driven by a syringe pump under a high voltage electrostatic field to produce spherical shaped MCs. After 45 min stirring, 1 N NaOH was added to TPP solution with TPP/NaOH volume ratio of 2/1 for 15 min. The needle electrically connected to the positive electrode of a high voltage electrostatic generator, the negative electrode of which electrically connected in to an annular stainless steel plate fixed under the coagulation solution. The optimized potential between the needle and the stainless steel plate and the pumping rate was obtained between 7 and 11 kV and 300 to 700 μL/min, respectively. The collected MCs were washed several times with distillated water to remove the un-reacted TPP.

2.4. Physical and mechanical characterization of MCs

The mean diameter and morphology of MCs were determined by using the inverted microscopy. In order to investigate the mechanical strength of MCs, 100 random MCs were put into 40 mL water and they were stirred at 400 rpm stirring speed for 30 min. Then fragmented MCs were separated and the residual (unfragmented) MCs were renumbered. The mechanical strength was estimated by noting the percent of residue MCs' [22]. Elasticity was analyzed using atomic force microscopy (AFM) apparatus. The basic AFM technique for quantitative study of mechanical characteristics of micro- and nanostructures is the force spectroscopy (namely, force-curve analysis). The force value versus distance between the probe and each point of MC surface can be plotted. The force curve represents a basis for estimation of local Young's modulus of the sample [23]. In order to compare the MCs' groups, five random MCs were selected from each group and ten independent tests were done for each MC. During these tests, the set point was selected as 14 nN for quadratic pyramid tip and cantilever forced each point by descending velocity of 1 μm/s. The slope of force-displacement curve indicates elasticity of the sample according to the Hertz model equation [24]. Indentation depth of MCs resulting from descending cantilever represented force-displacement curve.

2.5. Experimental design and data analysis

Response Surface Methodology (RSM) was used to design experiments and analyze the effects of considered parameters. The biopolymers blend ratio, syringe pump flow rate and voltage of high voltage device were considered as studied parameters while the MCs' diameters and mechanical strength were assumed as responses. A Box–Behnken design of experiments 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) was used to determine the optimal condition for preparation of MCs. Box–Behnken experimental design has been established using Design Expert software (7.0 trial version).

A total of 20 trials, including 5 replicates at the center point for the estimation of error sum of squares, were performed based on the quadratic model generated by RSM software. 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 2.