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Optimization of preparation conditions for calcium pectinate with response surface methodology and its application for cell encapsulation

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ABSTRACT

The most notable and unique property of pectin is its ability to form gels, which is the foundation of its many functions and applications. To obtain the desired pectin hydrogel beads for tissue engineering or biological applications, the combined effect of the key factors of gel properties of calcium pectinate (CP) beads were investigated by response surface methodology (RSM). The results derived from RSM indicated that the model equation of average size and mechanical stability were significant and could be used to describe the process under a wide range of preparation conditions. The optimum condition for preparing variables were gained graphically. Moreover, the degree of methyl-esterification (DE) of pectin and pectin - calcium concentrates showed significant and combined effort on sphericity factor (SF). The mechanical stability of CP beads was significantly affected by the quadratic contributions of the pectin concentration, the interaction effects between pectin and calcium concentration, and the linear contribution of pectin concentration. The viability and proliferation of cells encapsulated in optimal CP beads demonstrated that the optimal formula results in better gel properties and are more suitable to cell encapsulation.

1. Introduction

Hydrogel beads are hydrophilic, three dimensional cross linked polymer networks, and present good biocompatible, porous network structure with a high water content that mimics the extracellular matrix environment in vivo, which are widely used in tissue engineering [1]. Polymer materials, especially natural polysaccharides, are potential materials for hydrogel beads. Pectin, a major component of the cell walls and soft tissues of all terrestrial plants, has a long history in the food and drug industries as a stabilizer and thickener [2,3]. The most notable and unique property of pectin is its ability to form gels, which is the foundation of its biological applications [3,5–7].

It is well recognized that the properties of hydrogel beads, such as their structure, pore size, and permeability, influence the ability and proliferation of the encapsulated cells [8,9]. To obtain the desired hydrogel beads for tissue engineering or biological application, it is necessary to optimize the properties of hydrogel beads. Pectin, one of the natural polysaccharides, is characterized by its structural and molecular weight (Mw), heterogeneity, and polydispersity. Thus, it can be seen that the gelation properties of pectin are affected by many factors. The structure of pectin generally comprises homogalacturonan (HG), and type-one and type-two rhamnogalacturonan (RG I and RG II) [10,11]. The percentage of galacturonic acid (Gal A) residues that are methylesterified is defined as the degree of methyl-esterification (DE), which is considered as a key parameter that determines pectin functionality [12]. High methoxyl (HM; DE > 50%) pectin can only form gels in the presence of sugars in an acidic environment, whereas the presence of divalent ions, such as calcium, induces low methoxyl (LM; DE < 50%) pectin to form hydrogels [13]. In addition, Capel and colleagues found that LM pectin with small molar masses could not form gel beads. Moreover, the molar mass of pectin has a positive influence on gel strength. The higher molecular weight or pectin concentration, the stronger the gel strength [2,14,15]. The ionic bridges between pectin carboxyl groups and calcium ions lead to the arrangement of molecular chains and gel formation [16,17]. Hence, the calcium content, pectin concentration, and other extrinsic factors, also have important influences on the gel properties [12,18]. Our previously published results indicated that the gelling

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properties, such as gel formation, morphology, and particle distribution were significantly influenced by DE and pectin concentration. Furthermore, DE (below 40%), Mw, and calcium concentration had negative effects on the sphericity factor (SF) of the calcium pectinate (CP) beads [19]. Although the present work involves the optimization of different parameters that affect the properties of CP beads, the most common practice of determination is via single variable optimization, however the combined influence of the interaction among the factors remained unidentified [20,21]. Therefore, investigation of the combined effects of the processing variables on the properties of CP beads is necessary, and until now, little research on the subject has been conducted.

The optimization of the process and investigation of the combined effects can be chaotic and time-consuming, while many factors and interactions influence the desired response. Response surface methodology (RSM) is a statistical and mathematical technique that has proved effective. RSM is more precise and less time-consuming than conventional methods. It can obtain ideal results with less experiment trials that would be needed to evaluate multiple parameters and their interactions [22,23]. It is widely used for developing, modeling, and optimizing processes.

Therefore, based on our previously published results [19], the aim of this study is to optimize the preparation conditions of CP gel beads with the Box-Behnken RSM design [24]. Furthermore, the model is built with RSM according to the responses to particle size, swelling degree, and mechanical stability, all of which could provide more reliable predictive data for CP bead preparation. The aim of this study is to obtain the optimized CP beads with these desired traits, which are more suitable for cell encapsulation.

2. Materials and method

2.1. Materials

Pectin (DE 65%, P_{65}) was obtained from Yantai Andre Pectin Co. Ltd. (Shandong, China). The C3A cell line was a kind gift from Professor Lijian Hui, Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China). The fetal bovine serum (FBS) was purchased from PAN-Biotech GmbH (Aidenbach Bavaria, Germany). The Minimum Essential Medium was procured from Gibco, Life Technologies Corporation (Grand Island, USA). The Trypsin-EDTA was supplied by Biosharp (Anhui, China). Cell counting kit-8 (CCK8) was acquired from Dojindo Laboratories (Kumamoto, Japan). All other chemicals were obtained from Aladdin (Shanghai, China).

The LM pectins were prepared by alkaline de-esterification. The values corresponding to the DE and molecular parameters were obtained through the titrimetric method and gel permeation chromatography (GPC) [19].

2.2. Preparation of CP

The pectin solutions were prepared by dissolution of 0.9% NaCl solution to obtain a pectin concentration of 0.5%–5%. Then, the pectin solutions were dropped into the 0.1 M $CaCl_2$ gelling bath using a needle. Following 30 min of gelling, the wet CP were collected.

2.3. Characterization of CP

2.3.1. Average size and sphericity factor (SF) of CP beads

Images of wet CP were captured by a digital camera (Canon EOS M2; Japan). Diameters of the CP beads were measured and processed with Image J software. Average size and SF were selected as the quantification criteria of the CP beads. The SFs were calculated using the following equation, Eq. (1) [21]:

$$SF = (d_{max} - d_{min})/(d_{max} + d_{min}) \quad (1)$$

where d_{max} and d_{min} are the maximum and minimum diameters of the CP beads, respectively.

2.3.2. Swelling degree (Sw) of CP beads

The CP beads were dried in a drying oven at 37 °C for 24 h and then examined for average size (D_0). Each dried sample was incubated with 10 ml phosphate buffered saline (PBS) solution (pH 7.4) at 37 °C under gentle shaking. Samples were removed from the incubation solution after 7 days, rinsed with distilled water, removed from the surface water with filter paper, and average size determined (D_t). The swelling degree was calculated using Eq. (2) [4]:

$$Sw(w/w) = (D_t - D_0)/D_0 \times 100\%. \quad (2)$$

2.3.3. Mechanical stability of CP beads

The mechanical stability of the CP beads were determined using the assay of bead agitation method [25]. The breakage rates of CP beads was obtained by Eq. (3):

$$Ir\ (\%) = (N_0 - N_r)/N_0 \times 100\% \quad (3)$$

where:

N_0 represents the total number of CP beads, and N_r is the number of ruptured beads.

2.4. Experimental design

RSM, coupled with Box-Behnhen Design (BBD), were employed to measure the individual and interactive effects of the process variable. The experiment design and data analysis used design-expert 8 (Stat-Ease, Inc., Minneapolis, USA). DE (X_1), pectin concentration (X_2) and calcium concentration (X_3) were selected as independent variables, while the average size (Y_1), swelling degree (Y_2), and mechanism stability (Y_3) were chosen as dependent variables. The results were entered into an empirical second order polynomial model. Statistical analysis of the data were considered significant at $p < 0.05$ [26]. All experiments were carried out at random to minimize the effect of unexplained variability in observed responses caused by systematic errors.

2.5. Cell culture

C3A cells were cultured under standard condition (5% CO_2 at 37 °C) in MEM containing 10% FBS. The media were renewed every two days. The cells were sub-cultured until they reached 70–80% confluence.

2.6. CP beads for cell encapsulation

The C3A cells (10^6 cells/ml pectin solution) were suspended in sterilized pectin solution, and extruded into 250 ml 100 mM $CaCl_2$ using a 10 ml syringe with a #23 needle. After 30 min, the CP beads were collected and cultured in MEM with 10% FBS in a 5% CO_2 atmosphere at 37 °C with 95% humidity. The medium was changed every second day.

2.6.1. Live/dead staining analysis

As with the previously method [27], the CP beads with C3A cells were collected and incubated with live/dead staining solution composed of 2 μM calcein AM and 4 μM ethidium homodimer−1 at 37 °C for 90 min. After washing with 0.9% (*w*/*v*) NaCl solution, samples were scanned using confocal laser scanning microscopy(FV1000MPE, Olympus, Japan).

2.6.2. Cell viability analysis

Cell viability was investigated by CCK8 assay [27]. Equal amounts of CP beads encapsulated C3A cells were rinsed with PBS three times and incubated with fresh MEM containing 10% CCK8 (*v*/*v*). After 2 h, the