Optimization and Characteristics of Preparing Chitosan Microspheres Using Response Surface Methodology

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ABSTRACT: In this study, response surface methodology (RSM) was employed to optimize preparation of chitosan microspheres (CMs). Firstly, Plackett–Burman design (PBD) was applied to screen out the factors, which influence preparation of CMs significantly. The results showed that the concentration of chitosan and acetic acid as well as the volume of toluene were the key factors. Then, steepest ascent experiment and Box–Behnken design were introduced to optimize the levels of the key factors. As a result, the appropriate conditions of preparing CMs were chosen as follows: 2% (w/v) chitosan, 1.7% (v/v) acetic acid, 7 mL span-80, 488 mL toluene, 1100 rpm, 60 min (emulsification time), 10 mL formaldehyde, and 60 min (crosslinking time). Also, the morphology, size particle, and FTIR spectra of CMs were studied by scanning electron microscopy, small angle laser light scattering, and FTIR spectrometer. Results showed that CMs had quite smooth surface spherical and sharp size distribution, which indicated that the CMs were successfully prepared by W/O emulsification crosslinking method using RSM. © 2012 Wiley Periodicals, Inc. J. Appl. Polym. Sci. 000: 000–000, 2012

INTRODUCTION

Chitosan, composed of β-(1.4)-2-acetamido-2-deoxyglucopyranose and 2-amino-2-deoxyglucopyranose units, is a biodegradable high molecular weight cationic polysaccharide.[1–5] Chitosan is attracting a lot of concerns recently because of its useful features such as biocompatibility, low toxicity, and biodegradability.[6,7] Chitosan and its derivatives show important functional properties in many areas. Some researchers had reported chitosan microspheres (CMs) or nanoparticles had an advantage on drug carriers,[8,9] immune enhancing,[10] antibacterial,[11] embolization agents,[12] etc.

Various methods of preparing CMs have been reported, such as water/oil (W/O) emulsification crosslinking technique,[13] solvent evaporation,[14] spray-drying,[15,16] ionic gelation,[17] and so on. However, CMs attained in these ways are mostly of irregular shape and heterogeneity particle size distribution, which may limit their pharmaceutical application as drug carriers. It has been found that the shape and particle size distribution of CMs could be affected by many factors such as proportion of W/O phase, stirring speed, emulsification time, and so on. But still now, the researches on optimization of preparing CMs mainly focus on single-factor analysis, and multivariate analysis[18] had little been reported. In our article, response surface methods

(RSM) have been applied in order to attain the CMs with smooth spherical surface and homogeneous size distribution. When compared with the previous research, the optimization of preparing CMs was systematically studied by multivariate analysis using RSM.

RSM[19] has been used to optimize conditions of preparing CMs. Thus, the basic goal of RSM was to locate the optimal settings. A sequential experimentation could be used to solve the procedure of optimization. Typically, a factorial or fractional factorial designs [Plackett–Burman design (PBD)][20,21] were carried out to screen out the significant factors from lots of impact factors. After the response got from regression model, extrapolation and experiment along the path of steepest ascent could maximize the response.[22] To approach the optimum region, steepest ascent experiments were used for choosing the proper levels of factors. Finally, response surface design [Box–Behnken design (BBD)][21,23] was selected to model curvature and locate the optimum response.

The purposes of this study was to obtain CMs with smooth spherical surface and homogeneous size distribution by W/O emulsification crosslinking method using RSM and optimize the experiment conditions according to experimental needs. The characterization of CMs such as morphology, size particle, and FTIR spectra were also studied.

Table I. Matrix of PBD and Results of Evaluation of Factors Affecting CMs (Y) Production

Run order	Variables: code levels (real value)								Response Y (CMs/g)
	A	B	C	D	E	F	G	H	
1	1(2)	−1(1)	1(7)	−1(300)	−1(700)	−1(30)	1(10)	1(60)	5.19
2	1(2)	1(3)	−1(3)	1(400)	−1(700)	−1(30)	−1(5)	1(60)	5.54
3	−1(1)	1(3)	1(7)	−1(300)	1(1100)	−1(30)	−1(5)	−1(30)	2.23
4	1(2)	−1(1)	1(7)	1(400)	−1(700)	1(60)	−1(5)	−1(30)	6.45
5	1(2)	1(3)	−1(3)	1(400)	1(1100)	−1(30)	1(10)	−1(30)	5.97
6	1(2)	1(3)	1(7)	−1(300)	1(1100)	1(60)	−1(5)	1(60)	5.06
7	−1(1)	1(3)	1(7)	1(400)	−1(700)	1(60)	1(10)	−1(30)	3.6
8	−1(1)	−1(1)	1(7)	1(400)	1(1100)	−1(30)	1(10)	1(60)	4.58
9	−1(1)	−1(1)	−1(3)	1(400)	1(1100)	1(60)	−1(5)	1(60)	3.94
10	1(2)	−1(1)	−1(3)	−1(300)	1(1100)	1(60)	1(10)	−1(30)	5.46
11	−1(1)	1(3)	−1(3)	−1(300)	−1(700)	1(60)	1(10)	1(60)	2.76
12	−1(1)	−1(1)	−1(3)	−1(300)	−1(700)	−1(30)	−1(5)	−1(30)	2.58

MATERIALS AND METHODS

Materials

Chitosan with molecular weight (MW) 136 kDa and deacetylation degree 83.7–85% was obtained from Biochemical Medicine Plant of Qingdao (Qingdao, China). Tween-80, span-80, acetic acid, toluene, ethanol, and formaldehyde solution (37%) were supplied by Sigma Chemical of China.

Chitosan Microspheres Preparation

CMs were prepared by the W/O emulsion technique.[24] The process of preparing as follows: 2 g of chitosan powders (A) were fully dissolved in 100 mL 2% (v/v) acetic acid solution (B), and then 100 mL chitosan solution was added into 400 mL toluene (D) with 7 mL span-80 (C) and 2 mL tween-80. The mixture were then mechanically stirred (E) at 1100 rpm by IKA RW 20 stirrer (IKA, Germany) for 60 min (F) to form an emulsion. And then 10 mL formaldehyde solution (37%) (G) was added into the system with continuous stirring for 60 min (H). The mixture was rinsed by ethanol (30%), distilled water until the CMs turn to clear and transparent. The CMs with different particle sizes were collected and then successively dehydrated in series grade ethanol (30, 50, 80, 95, and 100%) and ether, respectively. Finally, the microspheres were dried and stored in sealed bags.

Formation and size distribution of CMs were affected by at least eight impact factors,[1] including concentration of chitosan (A), concentration of acetic acid (B), volume of span-80 (C), volume of toluene (D), stirring speed (E), emulsification time (F), volume of formaldehyde solution (G), and crosslinking time (H). To investigate the influence of eight variables and maximize weight of CMs, the RSM, the PBD followed by (BBD) had been used with the software Minitab, version15.1.30 (Minitab, PA). The wet weight of CMs (g) was chosen as the evaluation objectives (response Y).

EXPERIMENTAL DESIGN

Plackett–Burman Design

Plackett–Burman's factorial design method[20] was employed in this study as the first step for screening the significant factors, which affect CMs production. The Plackette–Burman experimental design was an eight-factor-two-level factorial design based on the first-order polynomial model:

$$Y = \beta_0 + \sum \beta_i X_i \qquad (1)$$

where Y is the response (wet weight of CMs), β_0 is the model intercept, β_i is the linear coefficient, and X_i is the level of the independent variables. This model did not consider the interaction effects among variables and it was used to screen out and evaluate importance of variables that influence the response Y. On the basis of PBD, each factor was examined in two levels: −1 for low level and +1 for high level, and the (−) value and (+) values of the eight variables were shown in Tables I and II. Twelve runs were carried out in PBD and the wet weights of CMs were taken as responses. The result of PBD was exhibited in Table II. Through the regression analysis, the factors significant at 95% level ($P < 0.05$) were considered to have significant effect on the wet weight of CMs and thus used for further optimization by steepest ascent experiment.

Steepest Ascent Experiment

The method of steepest ascent[25] was for choosing the proper levels of key factors. The first step was to obtain the optimum response settings, such as A (concentration of chitosan), B (concentration of acetic acid), and D (volume of toluene), and then to explore the region around the current operating conditions to decide what direction needs to be taken to move toward the optimum region. It needed to move from the current operating conditions to the optimum region in the most efficient way by using the minimum number of experiments. This was done using the method of steepest ascent. Table III showed the experimental design and corresponding response of the steepest ascent path. The steepest ascent experiments were performed along the steepest ascent path until the response did not further increase any more. This point would be near the optimal range and used as the center point of BBD. The response Y (wet weight of CMs) of row three and four (7.98 and7.84) arrived at the optimum domain as shown in Table III. Accordingly, the

Table II. Estimated Effects and Coefficients for CMs (Y) Production

Term	Factors	Low level(-1)	High level($+1$)	Effect	Coef	SE Coef	T	P-value
Constant					4.4574	0.07420	60.07	0.000
A	Concentration of chitosan (g/100 mL)	1	2	2.3231	1.1615	0.07420	15.65	0.001[a]
B	Concentration of acetic acid (mL/100 mL)	1	3	−0.4991	−0.2495	0.07420	−3.36	0.044[a]
C	Volume of Span-80 (mL)	3	7	0.1531	0.0766	0.07420	1.03	0.378
D	Volume of toluene (mL)	300	400	1.1478	0.5739	0.07420	7.73	0.004[a]
E	Stirring speed (rpm)	700	1100	0.1742	0.0871	0.07420	1.17	0.325
F	Emulsification time (min)	30	60	0.2095	0.1048	0.07420	1.41	0.253
G	Volume of formaldehyde (mL)	5	10	0.3064	0.1532	0.07420	2.06	0.131
H	Crosslinking time (min)	30	60	0.1207	0.0603	0.07420	0.81	0.476

S = 0.269485, PRESS = 3.48587, R-Sq = 99.00%, R-Sq (pred) = 83.95%, R-Sq (adj) = 96.32%.
[a]Statistically significant at 95% of confidence level ($P < 0.05$).

values of factors, corresponding to the max response Y (7.98), were chosen for the center point.

Box–Behnken Design and Response Surface Methodology
Three key factors, affecting the CMs production, were further studied by RSM. A three-factor-three-level BBD[23] was applied to determine the optimization condition matrix for CMs production. The factorial model, all linear and interaction terms were considered and analyzed using the BBD. For statistical calculations, the relation between the coded values and real values were described as follows:

$$X_i = \frac{x_i - x_0}{\Delta x} \quad (2)$$

where X_i is a coded value of the variable (-1, 0, $+1$), x_i is the real value of variable, x_0 is the real value of the x_i at the centre point, and Δx is the step change value. In this study, a set of 15 runs were carried out in BBD and the independent variables were studied at three different levels: low (-1), center (0), and high ($+1$). The three levels of the significant variables and the results of experimental design were shown in Tables IV–VI and Figure 1. The relationships and interrelationships of the variables were determined by fitting the second-order polynomial equation to data obtained.

$$Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum \beta_{ij} X_i X_j. \quad (3)$$

where Y is the predicted response (the wet weight of CMs), X_i and X_j were the level of independent variable, β_0 is the constant, β_i, β_{ii}, and β_{ij} were the linear, quadratic, and interaction coefficients, respectively.

Morphology Observation and Size Distribution of CMs
The surface feature of dried CMs was observed by optical microscopy (OM) (CKX-31, Olympus) and scanning electron microscopy (SEM) (KYKY-2800B, Scientific Instrument, Chinese Academy of Sciences, China). CMs were fixed onto an aluminum stub using two-sided carbon tape and then coated with gold in an argon atmosphere by an ion sputter current.

Table IV. Matrix of BBD and Results of Evaluation of Factors Affecting CMs (Y) Production

	Variables: code levels (real value)			
Run order	A Concentration of chitosan (g/100 mL)	B Concentration of acetic acid (mL/100 mL)	D Volume of toluene (mL)	Response Y (CMs/g)
1	−1(1)	−1(0.5)	0(400)	4.86
2	1(3)	−1(0.5)	0(400)	6.72
3	−1(1)	1(2.5)	0(400)	4.98
4	1(3)	1(2.5)	0(400)	6.16
5	−1(1)	0(1.5)	−1(300)	4.52
6	1(3)	0(1.5)	−1(300)	6.83
7	−1(1)	0(1.5)	1(500)	5.16
8	1(3)	0(1.5)	1(500)	7.17
9	0(2)	−1(0.5)	−1(300)	7.43
10	0(2)	1(2.5)	−1(300)	7.08
11	0(2)	−1(0.5)	1(500)	8.03
12	0(2)	1(2.5)	1(500)	7.57
13	0(2)	0(1.5)	0(400)	7.95
14	0(2)	0(1.5)	0(400)	8.16
15	0(2)	0(1.5)	0(400)	8.09

Table III. Experiment Design and Results of Steepest Ascent Path

	Factors			
Run order	A Concentration of chitosan (g/100 mL)	B Concentration of acetic acid (mL/100 mL)	D Volume of toluene (mL)	Response Y (CMs/g)
1	1	0.5	300	4.47
2	1.5	1	350	5.94
3	2	1.5	400	7.98
4	2.5	2	450	7.84
5	3	2.5	500	6.72

Table V. Analysis of BBD Estimated Regression Coefficients for CMs (Y) Production

Term	Coef	SE Coef	T	P-value
Constant	8.06667	0.12814	62.953	0.000
A	0.92000	0.07847	11.724	0.000[a]
B	−0.15625	0.07847	−1.991	0.103
D	0.25875	0.07847	3.298	0.022[a]
A*A	−1.99708	0.11550	−17.290	0.000[a]
B*B	−0.38958	0.11550	−3.373	0.020[a]
D*D	−0.14958	0.11550	−1.295	0.252
A*B	−0.17000	0.11097	−1.532	0.186
A*D	−0.07500	0.11097	−0.676	0.529
B*D	−0.02750	0.11097	−0.248	0.814

S = 0.221942. PRESS = 3.62625. R-Sq = 98.92%. R-Sq (pred) = 84.11%. R-Sq (adj) = 96.98%.
[a]Statistically significant at 95% of confidence level ($P<0.05$).

The average diameter and particle distribution were measured by small angle laser light scattering (Mastersizer 2000, Malvern Instruments, UK).

FTIR Spectra of CMs
The infrared spectra of CMs were recorded in KBr pellets on a Nicolet FTIR 5700 spectrophotometer (Madison, WI) at room temperature (25°C) by the method of transmission. Samples of CMs were dried and triturated before used.

Statistical Analytical Methods
All experiments and measurements were performed in triplicates. A probability level of $P < 0.05$ was considered statistically significant. The related data analyzed by software Minitab, version15.1.30 (Minitab, PA).

RESULTS AND DISCUSSION

Screening the Factors Effecting the CMs Production by PBD
PBD was used to efficiently screen out the key variables affecting the CMs production. The CMs yields varied markedly from 2.23 to 6.45 g under different levels of factors, as shown in Table I. From the regression analysis of PBD, the confidence level of the factors above 95% ($P < 0.05$) were selected as significant effect. Table II showed that the confidence level of variables A

($P = 0.001$), B ($P = 0.044$), and D ($P = 0.004$) were above 95% ($P < 0.05$) and they were considered to be the most significant factors. The rest five variables(C, E, F, G, and H) had insignificant effects for response and were not included in the next optimization experiment because their confidence levels were below 95% ($P > 0.05$). Meanwhile, the effects of C, E, F, G, and H were positive effects for CMs (Y) production. On the basis of PBD, the variables with insignificant effect were chosen in trials at their (-1) level for negative contribution and ($+1$) level for the positive contribution. Accordingly, the factors: C, E, F, G, and H were set at 7 mL ($+$), 1100 rpm ($+$), 60 min ($+$), 10 mL ($+$), and 60 min ($+$), respectively. To investigate the optimum levels of significant factors for CMs production in trial, the steepest ascent experiment should be performed to reach of the optimum domain of the maximum response.

Determining the Levels of Significant Factors Affecting the CMs Production by Steepest Ascent Experiment
The steepest ascent method was used to determine the most effective factors in improving CMs production. On the basis of eq. (1) and Table II, the direction of steepest ascent should increase concentration of chitosan (A) and volume of toluene (D), decrease concentration of acetic acid (B) in order to approach the optimal experimental region of maximum response. Five sets of experiment design of the steepest ascent and corresponding results were shown in Table III. The yield of CMs was the highest in Run 3 when A, B, and D was selected to be 2%, 1.5%, and 400 mL, which suggested that it was near the region of maximum response (CMs). Accordingly, these levels of the three factors in Run3 were set as the center point of BBD (Table IV).

The Results of BBD
The BBD was utilized to study the interactions among the three significant factors and also determine their optimal levels. The design matrix of the variables and experimental results showed in the Table IV. The BBD comprised of 15 experimental runs including 3 runs under the same conditions. By applying multiple regression analysis on the experimental data; the following second-order polynomial equation was obtained:

$$Y = 8.06667 + 0.92X_1 - 0.15625X_2 + 0.25875X_3 - 1.99708X_1^2$$
$$- 0.38958X_2^2 - 0.14958X_3^2 - 0.17X_1X_2 - 0.075X_1X_3 - 0.0275X_2X_3$$
$$(4)$$

Table VI. Analysis of Variance for CMs (Y) Production

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Regression	9	22.5779	22.5779	2.50865	50.93	0[a]
Linear	3	7.5021	7.5021	2.50071	50.77	0[a]
Square	3	14.9346	14.9346	4.97821	101.06	0[a]
Interaction	3	0.1411	0.1411	0.04704	0.95	0.481
Residual error	5	0.2463	0.2463	0.04926		
Lack-of-fit	3	0.2234	0.2234	0.07448	6.51	0.136
Pure error	2	0.0229	0.0229	0.01143		
Total	14	22.8242				

[a]Statistically significant at 95% of confidence level ($P < 0.05$).
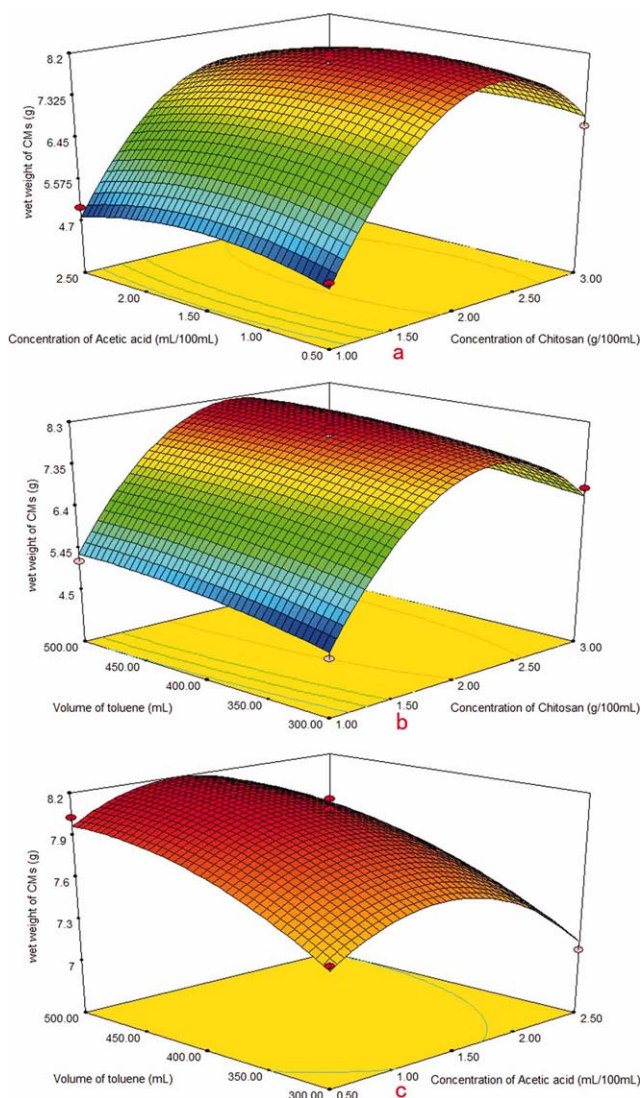
Figure 1. The response surface plot and the corresponding contour plot showing the effects of concentration of chitosan, concentration of acetic acid, and volume of toluene on the wet weight of CMs (g). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

where Y is the predicted wet weight of CMs, X_1, X_2, and X_3 are the coded values of A, B, and D, respectively.

The significance of the regression coefficients was given by ANOVA. The regression coefficients and corresponding P-values for the model were presented in Tables V and VI. The confidence level of A ($P = 0.000$), D ($P = 0.022$), A^2 ($P = 0.000$), and B^2 ($P = 0.020$) were above 95% ($P < 0.05$), which suggested that they had significant effects on response Y (CMs) (Table V). Linear terms of A, D, and quadratic terms of A^2 and B^2 had significant effect on response Y (CMs), interactive terms among the three were not significant (Table VI). The coefficient of determination (R^2) was 98.92%, which indicated that 98.92% of the variability in the response could be explained by the model. The R^2 (pred) of 84.11% was in reasonable agreement with R^2 (adj) of 96.98%. The F-value of "Lack-of-Fit" was 6.51 (>0.05), which implied that the Lack-of-Fit was not significant relative to the pure error. The model was found to be adequate for prediction within the range of variables used. Comparing the predicted values with the experiment values, the results indicated that these data were in reasonably close agreement.

Optimization of the Response by RSM
Response surface plot which provided a method to predict the CMs for different values of the test variables was helpful in identification of the type of interactions between test variables. The 3D response surface from the calculated response surface in the CMs was plotted to further explain the results of statistical analyses. Figure 1 (a–c) presented the effect of two variables, while the third variable was held at zero level. As can be seen from Figure 1, the wet weight of CMs rapidly increased as increasing concentration of chitosan (A) and acetic acid (B) to optimum conditions, and then did not produce a corresponding increase with further increase. Meanwhile, the wet weight of CMs lowly increased with volume of toluene (D) increasing.

The optimum levels of the variables were calculated from the data obtained using the RSM. The optimal values of X_1(A), X_2(B), and X_3(D) in the coded units were found to be $X_1 = 0.01647$, $X_2 = 0.235$, and $X_3 = 0.88240$. Correspondingly, referring to eq. (2), the real value of the model was obtained, which were chitosan (A) of 2.0%, acetic acid (B) of 1.7% and toluene (D) of 488.2 mL. The maximum predicted value of the wet weight of CMs was estimated as 7.926 g. To confirm the predicted results of the model, the repeated experiments under
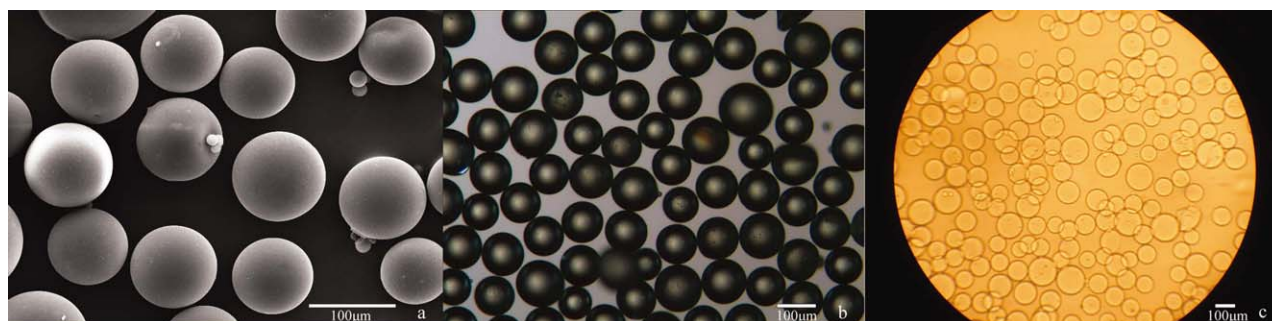
Figure 2. Morphology of the smooth-surface microspheres. (a) SEM micrograph of CMs ($\times 350$); (b) microscopic photograph of dried CMs ($\times 100\times$); and (c) Microscopic photograph of wet CMs ($\times 40\times$). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]
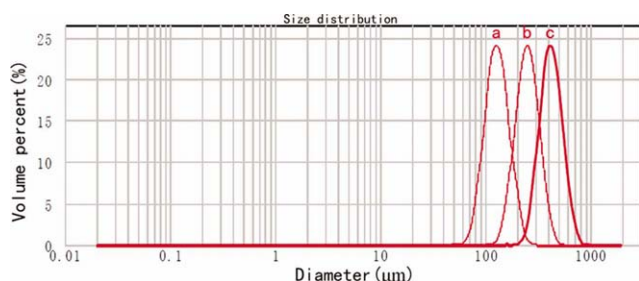
Figure 3. Particle diameter distribution of different size CMs (a) 132 μm; (b) 259 μm; and (c) 429 μm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

optimal conditions were carried out and an average value of 8.03 ± 0.26 ($N = 3$) was obtained. This result was obviously in close agreement with the model prediction. The excellent correlation between predicted and measured values verifies the model validation.

Characteristics of CMs

CMs with uniform size and smooth surface were prepared by W/O emulsification crosslinking technique. The CMs were spherical shape with smooth surface as shown in Figure 2. The SEM micrographs, the optical micrograph of dry and wet CMs were shown in Figure 2(a–c). The size and morphology of CMs were affected by kinds of factors, such as proportion of W/O phase, stirring speed, emulsification time, formaldehyde solution, and crosslinking time. Chitosan solution should achieve a proper viscosity during preparing CMs, the higher or lower concentration will affect the formation of CMs. Chitosan as a cationic polysaccharide only dissolved in diluted acid and degraded in concentrated acid.[4] The rate of W/O phase also was one of the significant factors affecting morphology, size, and distribution of CMs.[13] For instance, the particle size of CMs decreased with increasing rate of O/W and no more obviously decreasing was observed when the rate of O/W was beyond 488 : 100. Moreover, the other factors, such as span-80, stirring speed, emulsification time, formaldehyde solution, and crosslinking time, were also positive effects for the size of CMs in experimental design. As a result, these positive effects factors were chosen for the high levels (+1) in trial. Anyway, proper levels of

the factors could be chosen for preparing CMs according to the experiment requirements.

The dried CMs were measured by Mastersizer 2000 and the particle size distribution was 132 μm [Figure 3(a)]. Correspondingly, CMs with different particle size could be prepared via altering the variables in another experiment and the particle sizes were 259 and 429 μm [Figure 3(b, c)]. The results of morphology and size distribution of CMs indicated that the CMs were successfully prepared by W/O emulsification crosslinking technique in the appropriate conditions.

The FTIR spectra of Chitosan and CMs were shown in Figure 4. The absorption peaks at 1655 and 1600 cm^{-1} (shoulder) are assigned to the NH$_2$ groups in chitosan formed during the deacetylation, mixed with the amide I and amide II modes of the residual —NHCOCH$_3$ groups in Chitosan. When compared with the FTIR spectrum of chitosan, CMs showed significant reduction of the NH$_2$ stretching region (3400 cm^{-1}) and NH$_2$ bending region (1655 cm^{-1}), indicating the consumption of the NH$_2$ groups during their reaction with the crosslinker formaldehyde. In fact, the 1655 cm^{-1} peak in chitosan has moved to 1643 cm^{-1} in CMs, which corresponds to crosslinking points formed between NH$_2$ groups and formaldehyde. Furthermore, there was no significant difference in other absorption peaks between chitosan and CMs. In addition, there were no characteristic absorption peaks of benzene (1600, 1585, 1500, and 1450 cm^{-1})[26] in FTIR spectra; it was shown that toluene had been removed in experiment.

CONCLUSIONS

PBD, steepest ascent experiment, and BBD had been proved to be effective for optimizing the process conditions of CMs. Experimental results indicated that the concentration of chitosan, concentration of acetic acid, and volume of toluene had significant effects on CMs. Meanwhile, the other factors, such as span-80, stirring speed, formaldehyde solution, emulsification time, and crosslinking time, had no significant effects on CMs. However, based on PBD, these factors with insignificant effect were chosen in trials at their (+1) level for the positive contribution. Accordingly, the appropriate conditions were chosen in trial as followed: 2% (w/v) chitosan dissolved in 1.7% (v/v)
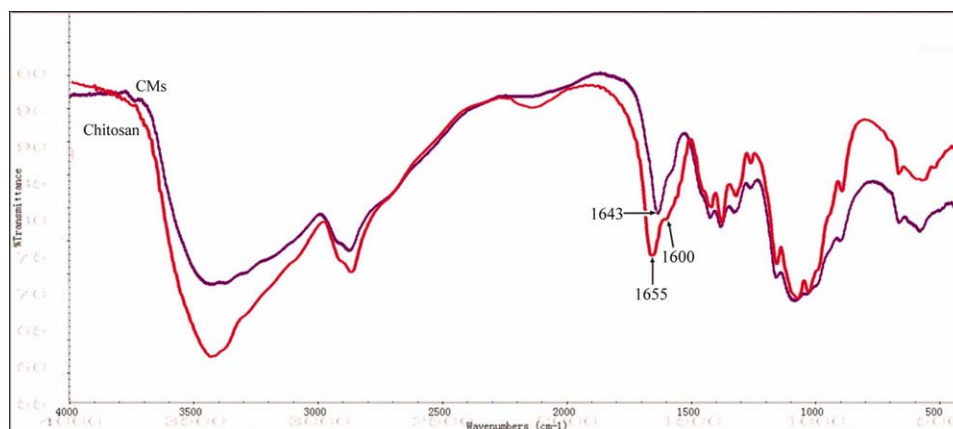
Figure 4. FTIR spectra of chitosan and CMs. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

acetic acid, and then 100 mL chitosan solution were added into 488 mL toluene with 7 mL span-80 and 2 mL tween-80, the mixture were mechanically stirred at 1100 rpm for 60 min, 10 mL formaldehyde solution was added to the system under continuous stirring for 60 min. The maximum predicted value of wet CMs can be achieved at 7.93 g and a mean value of 8.03 ± 0.26 g ($N = 3$) was obtained in the experiment under optimal conditions.

In optimal conditions, the smooth-spherical CMs in the mean sizes of 132 μm can be successfully prepared by W/O emulsification crosslinking methods. These were obviously in close agreement with the model prediction. The further experiment will be investigated in biocompatibility of CMs.

ACKNOWLEDGMENTS

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