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Production of Chemically Modified Chitosan Microspheres by a Spraying and Coagulation Method

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Chitosan microspheres can be used in several applications, as biomaterials, in biotechnology processes and as adsorbents. The control of particle size in microsphere production is important to make these applications viable. This study focuses on the production and morphological characterization of chitosan microspheres. Microspheres were prepared by a spraying and coagulation process. Particles underwent chemical modifications with glutaraldehyde, epichlorohydrin or acetic anhydride treatments. The microspheres presented a fairly good sphericity (0.74) but an irregular micro-surface morphology. The mean particle size (MPS) ranged from 140 to 281 μm and the mean standard deviation (MSD) from 119 to 238 μm. The conditions were modelled to provide desired MPS and MSD by using the response surface methodology (RSM).

Keywords: *chitosan, microspheres*

1. Introduction

Chitosan is a biodegradable, hydrophilic, non-toxic and biocompatible polysaccharide that presents a remarkable economic interest due to its functional versatility, with potential applications in medical and pharmaceutical fields[1-6]. Yamaguchi observed chitosan composites and their application in nerve regeneration[7]. Mi studied chitosan microspheres as a coating material for the controlled release of vaccines[8]. Furthermore, small chitosan microspheres (<10 μm) prepared by a spray drying process, have been developed for the specific release of drug agents[9]. Large chitosan microspheres (>50 μm) prepared by the emulsion method have been used in delivery systems[10] or as adsorbents to remove acid pollutants or heavy metals. Recently, a number of articles have been published describing the preparation of microspheres by spray drying and emulsion process methods. Microspheres obtained from these methods present a relatively narrow distribution of particles. Most chitosan particle applications are greatly influenced by their size distribution[11].

In the present study, chitosan porous microspheres were prepared by a spraying and coagulation method. This new method is rapid, reproducible, easy-to-scale-up and economic and can represent an alternative to previously known techniques. It also presents the advantage of not using high temperatures (as in the spray drying process) and there is no need to use other solvents in the emulsification steps. The sphericity, internal morphology and internal cross-section of particles were also characterized after performing chemical modifications that may improve their chemical and mechanical stability.

Chitosan is usually produced by deacetylation of chitin to different degrees. It is formed by β-(1 → 4)-linked 2-amino-2-deoxy-D-glucopyranose (GlcN, D-unit) and 2-acetamido-2-deoxy-D-glucopyranose (GlcAc, A-unit) units[12]. The presence of free amino groups in chitosan is responsible for its polycationic nature in acidic solutions.

An important advantage of chitosan is the possibility of agents that perform chemical modifications on its structure by binding to amino and hydroxyl groups[13]. Crosslinking with epichlorohydrin maintains the cationic amino function and improves the mechanical properties of the material[14]. On the other hand, Muzzarelli studied the crosslinking mechanism of chitosan with the bifunctional glutaraldehyde agent. This crosslinking reaction occurs between primary amino groups and aldehyde groups, resulting in the formation of schiff bases[15].

As another way to modify chitosan, acetylation modifies the structure and functionality of chitosan, improving its chemical resistance, producing an IR spectrum similar to that of chitin[16]. These structures are shown in Figure 1. In the present methods, the three of modifying chitosan were applied on microspheres after their production in order to investigate the induced changes in their morphology and properties.

2. Materials and Methods

2.1. Materials

Chitosan (from Sigma), with a minimum of 85% of deacetylation (extracted from crab shells) was used. Glutaraldehyde (25%, $v.v^{-1}$) aqueous solution was provided by Nuclear. Epichlorohydrin was provided by Merck. Acetic anhydride and methanol were purchased from Vetec and Synth, respectively. The nozzle (Figure 2) was purchased from Spraying Systems of Brazil. Tubing was provided by Masterflex.

2.2. Preparation of chitosan microspheres

Chitosan was dissolved in a (3%, $v.v^{-1}$) acetic acid solution in Milli-Q water. The scheme for the spraying and coagulation process is shown in Figure 3. The solution fed into the nozzle with a peristaltic pump was sprayed using compressed nitrogen. The atomization occurred by the force of the compressed nitrogen, which breaks up the chitosan solution into small droplets. The sprayed particles were kept in contact with NaOH coagulating solution for 12 hours. The particles were then collected and washed with abundant Milli-Q water. Several operating parameters can affect the preparation of the produced microspheres and their characteristics: inlet solution

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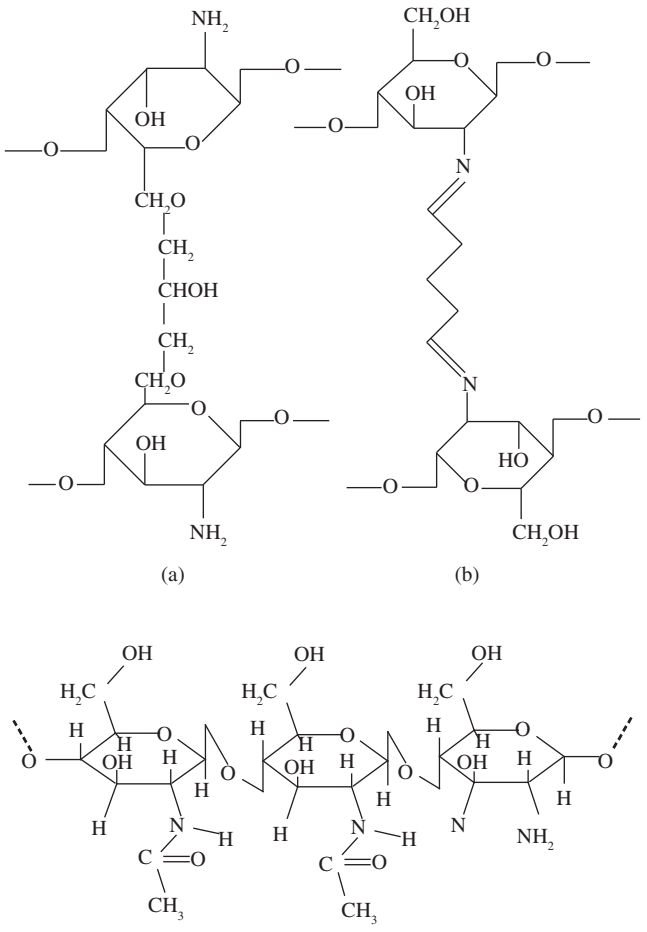


Figure 1. Chemical structures of chitosan modified with: a) epychlorohydrin; b) glutaraldehyde; and c) acetic anhydride.

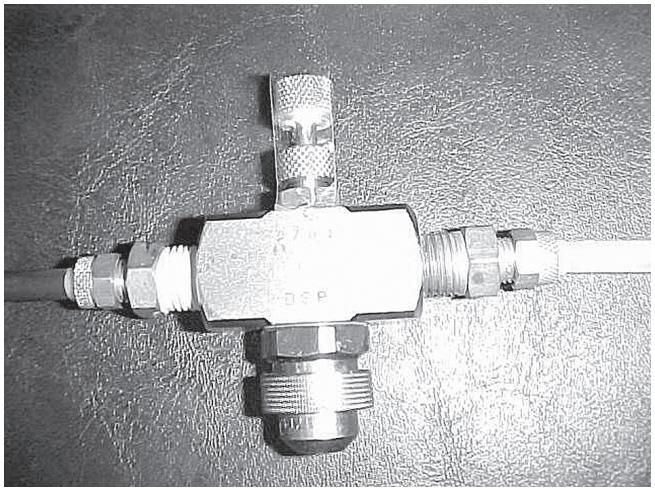


Figure 2. Nozzle used in microsphere obtention.

temperature, chitosan viscosity (i.e. chitosan concentration whilst biopolymer molecular weight distribution was kept constant), nitrogen pressure and chitosan solution flow rate. The following parameters were kept constant for microsphere production (set by preliminar analyses): inlet solution temperature at 25 °C; chitosan concentration

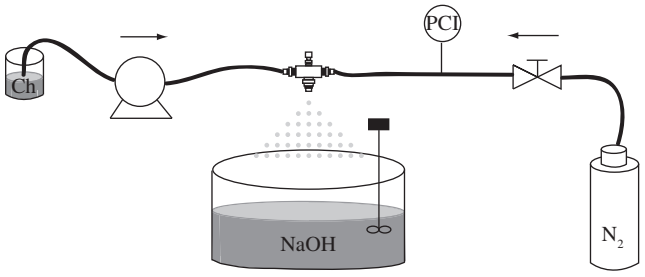


Figure 3. Spraying and coagulation process scheme.

at 0.025 g.mL^{-1} and nozzle aperture at 1 mm. Nitrogen pressure and chitosan solution flow rate were chosen as parameters to be studied by response surface methodology.

2.3. Preliminar set-ups

Some parameters, despite being important in the process, had their values fixed for experimental design. The temperature of the chitosan solution had little influence on the results. Temperatures of 15 and 35 °C (room temperature 25 °C ± 10 °C) were tested, not producing significant changes in microsphere size and standard deviation results. These findings are in accordance with studies performed with chitosan solution at the concentration of 0.025 g.mL^{-1} and molecular mass of 65 kDa[17], which showed that variations in the range of ±10 °C did not cause important changes in the viscosity and, hence, in the shear conditions observed during the process studied[18]. Chitosan solution presents non-newtonian fluid behavior, with polymeric chains that are highly auto-interactive.

With regard to the concentration of chitosan solution, a change in solution viscosity could be observed when its concentration changed between 0.03 and 0.02 g.mL^{-1}. Due to the practical limitations of the peristaltic pump (Cole Parmer model 302FM-B), the concentration of 0.025 g.mL^{-1} was set in the design of experiments. The nozzle opening was set at 1 mm. The height in which the nozzle was positioned, relatively to the surface of the NaOH solution, only had influence on the microspheres morphology. Higher distances produced a less distorted microsphere shape (allowing enough time for a viscous material droplet to acquire spherical shape before reaching NaOH coagulant solution). The height was then set at 30 cm (Tables 1 and 2).

2.4. Crosslinking of chitosan microspheres

Microspheres were heterogeneously crosslinked in (0.75%, w.w^{-1}) aqueous glutaraldehyde solution (5 g of wet chitosan microspheres in 50 mL of glutaraldehyde solution), without agitation, at room temperature for 2 hours. The particles were then rinsed with deionized water to remove the unreacted glutaraldehyde residues.

For the epichlorohydrin crosslinking process, 5 g of wet natural chitosan microspheres were immersed in 0.01 M epichlorohydrin solution, which was prepared in 0.067 M NaOH solution, at 40 °C under continuous agitation for 2 hours. The particles were then rinsed with deionized water to remove the unreacted epichlorohydrin.

Other microspheres were modified in a (0.6%, v.v^{-1}) methanol acetic anhydride solution, at room temperature under continuous agitation for 3 minutes. Afterwards, the micropaticles were rinsed with methanol to remove the unreacted acetic anhydride. The deacetylation values of microspheres were found to be about 70%, as calculated by potentiometric titration[19]. All microspheres were stored in Milli-Q water at 7 °C.

2.5. Response surface

Response surface methodology (RSM) with two variables, nitrogen pressure (NP) and chitosan solution flow rate (CSFR), at 3 nominal levels (–1, 0, 1) was used to study the response pattern for mean microspheres size and for mean standard deviation. Three replicates at the center of the design were used to estimate the error, assuming system homoscedasticity. The coordinates are given by a 2^n factorial design to provide the estimation of the model curvature through the non-linear relationship[20]. Table 3 depicts the correspondent values for experimental levels of each factor.

2.6. Characterization of chitosan microspheres

Microspheres had their mean particle size (MPS) and mean standard deviation (MSD) measured using a Malvern MasterSizer (model S-1000) equipment. Experiments were performed in triplicate and average values were reported. Microsphere sphericity was calculated by taking the ratio between the larger internal diameter and smaller external diameter of circumferences of microspheres, as observed by SEM[21]. Ten images were used to determine an average sphericity value.

The morphology of microspheres was observed using a Jeol scanning electron microscope. The microspheres were prepared after freeze-drying and sputter-coating with a gold layer (SCD 050 -Baltec, Liechenstein). The internal morphology of crosslinked microspheres was observed by imersing them in a polyacryamide resin, which hardened allowing the particles to be freeze-fractured, making the observation of the internal structures and distribution of pores possible.

3. Results and Discussion

3.1. Size results

Table 4 depicts the values equation that correlates to the final particle size with variables (chitosan solution flow rate (CSFR) and nitrogen pressure (NP)) in an appropriate regression model fit by a multiple regression program. The response surfaces are shown in Figures 4a and 4b. The effects of these variables on the responses are shown in Table 5. A value of $p < 0.05$ was considered significant for both first order factors and for their combined effect. The NP quadratic term was not considered significant. The values found for

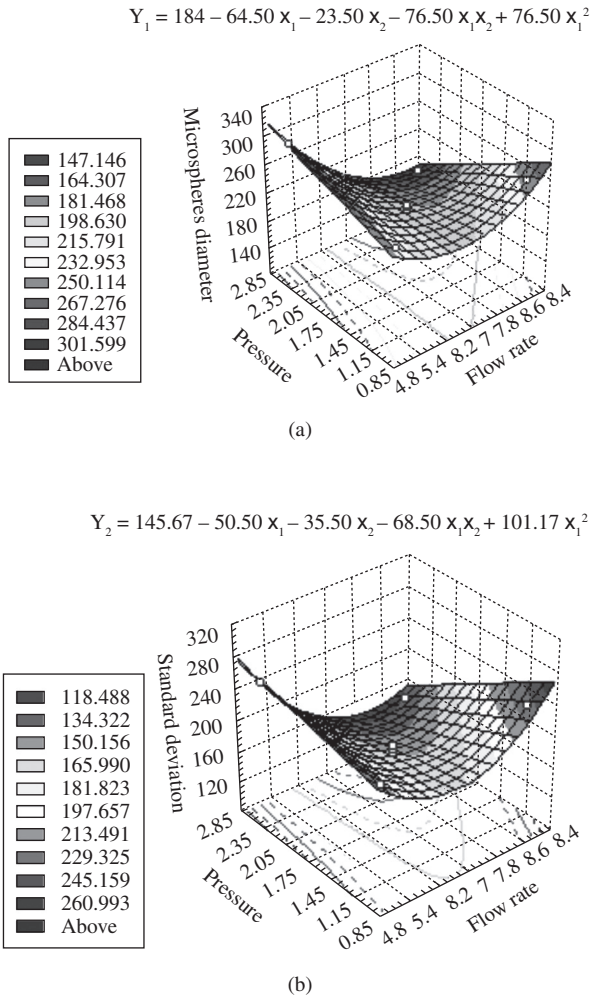


Figure 4. a) Response surface, showing the effects of chitosan solution flow rate (CSFR) (mL/min) and nitrogen pressure (NP) (kgf.cm^{-2}) on microsphere diameter (μm); and b) Response surface, showing the effects of chitosan solution flow rate (CSFR) (mL/min) and nitrogen pressure (NP) (kgf.cm^{-2}) on standard deviation (μm).

Table 1. Influence of parameters.

Parameters	Values	Effect (microns)
Concentration of chitosan solution	0.02 g.mL^{-1}	–20
	0.03 g.mL^{-1}	ND
Inlet temperature	15 °C	10
	35 °C	–5

Table 2. Values of constant parameters used in the study.

Parameters	Values
Concentration of chitosan solution	0.025 g.mL^{-1}
Inlet temperature	25 °C
Nozzle	1 mm
Height (nozzle-NaoH solution)	30 cm

Table 3. Factors used in the design of experiments and their levels.

Levels	–1	0	1
Chitosan solution flow rate (mL/min)	5	7	9
Nitrogen pressure (kgf.cm^{-2})	1	1.75	2.5

Table 4. Treatment schemes for a two-factor design of experiments and responses: mean particle size (MPS) and mean standard deviation (MSD).

Experiment number	CSFR	NP	MPS (μm)	MSD (μm)
1	–1	–1	228	205
2	1	–1	240	223
3	–1	1	281	238
4	1	1	140	119
5	0	0	179	140
6	0	0	186	145
7	0	0	187	152

mean particle size and mean standard deviation of microsphere sizes were between 140 to 281 μm and 119 to 238 μm, respectively.

Results indicate that increased values of CSFR and NP decrease the values of mean particle size and mean standard deviation of microspheres. There are, however, limits in the values to be assigned to these variables. These limits are intrinsic to the system and are related to the equipment and physical constraints of connections. Values of higher than 2.5 kgf.cm^{-2} to NP cause the reflux of the chitosan solution line. Values of higher than 10 mL/min CSFR cause the dilatation of the silicone line through which the chitosan solution flows (see Figure 3). Conversely, decreasing CSFR and NP values produce high mean particle size and standard deviations values. The R^2 correlation coefficient was 0.99 and statistical parameter (F) values suggest that the model fits the experimental data well. The suitability of model equations for predicting the optimum response values was tested. The experimental values were found to be in agreement with the predicted ones (Figures 5a and b). The values of the mean particle size and its standard deviation of particles, obtained by the present method, are higher when compared to the values found for spray dried[22] particles, but the necessary investment in equipment is approximately 50 times lower.

3.2. Optimized production

Specific characteristics of particle size and standard deviations are necessary for each microparticle application. When used as a biomaterial or adsorbent, small particle size and standard deviation are required. Particle size can influence the efficiency of drug delivery systems or the manner by which an adsorption column works.

In our case, the microspheres are to be used as adsorbents. A 140 μm mean particle size was then set as an acceptable target value. This was reached by using the initial conditions of 9 mL/min for CSFR and 2.5 kgf.cm^{-2} for NP. By observing the response surfaces, it is possible to visualize the operation point in the minimum areas of the surfaces. The microsphere size distribution in optimum conditions is shown in Figure 6. The corresponding optimized conditions are presented in Table 6.

Table 5. Effects of chitosan solution flow rate (CSFR) and nitrogen pressure (NP) parameters on mean particle size (MPS) and mean standard deviation (MSD).

Factors	Effect	on
	MPS (microns)	MSD (microns)
NP	(-)23.50	(-)35.50
CSFR	(-)64.50	(-)50.50
NP.CSFR	(-)76.50	(-)68.50
CSFR^2*	76.50	101.17

* NP and CSFR, NP.CSFR, CSFR^2 are the linear, cross and quadratic terms respectively.

Table 6. The optimized conditions for chitosan microparticle production.

Parameters	Values
Concentration of chitosan solution	0.025 g.mL^{-1}
Inlet temperature	25 °C
Nozzle	1 mm
Height (nozzle-NaoH solution)	30 cm
Nitrogen pressure NP	2.5 kgf.cm^{-2}
Chitosan solution flow rate CSFR	9 mL/min

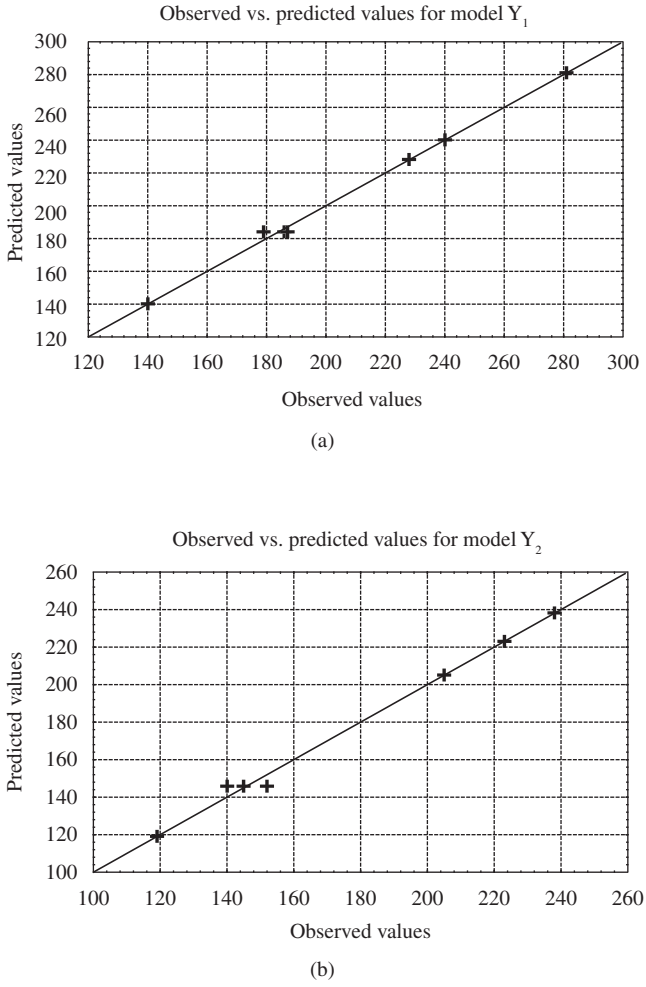


Figure 5. a) Predicted and observed values for model Y_1 = microsphere diameter (μm); and b) Predicted and observed values for model Y_2 = standard deviation (μm).

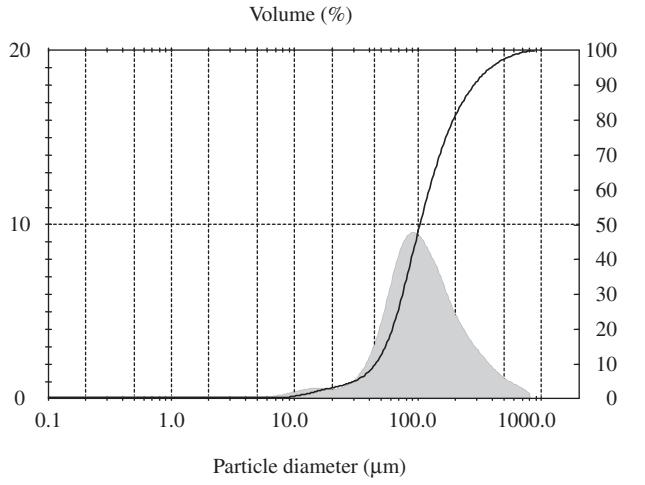
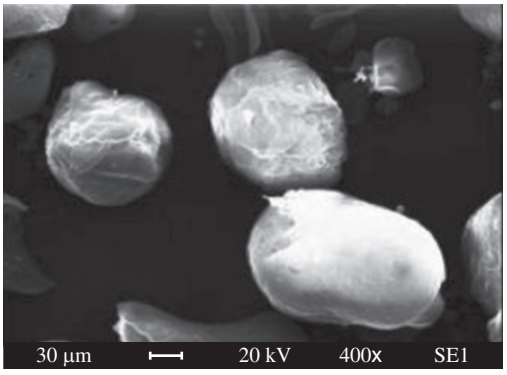


Figure 6. Chitosan microsphere size distribution, produced in optimum conditions for nitrogen pressure, 2.5 kgf.cm^{-2}, and for chitosan solution flow rate, 9 mL/min.

3.3. Morphology

The morphology of chitosan microspheres, prepared by the spraying and coagulation method, was examined by scanning electron microscopy (SEM). The sphericity of the microspheres was calculated as 0.74 ± 0.14. Similar morphology was observed for microspheres modified with epichlorohydrin and glutaraldehyde: good sphericity and a slightly wrinkled surface (Figures 7a and 7b), respectively. Improved sphericity was achieved for glutaraldehyde and epichlorohydrin – crosslinked microspheres, because the other structures tend to collapse. However, it is know that longer spray distances could improve the quality of spray dried particles[23]. For acetylated chitosan, microspheres presented a collapsed surface, as shown in Figure 7c. The deformation of the acetylated structure may be explained due to the differences of hydrophobicity between internal and external parts of formed microspheres. The heterogeneous acetylation (the reaction occurs from outside to inside) may have intensified the deformation, when compared to the homogeneous acetylation.

Chitosan microsphere cross-sections with epichlorohydrin and glutaraldehyde presented pores with irregular shapes and sizes (Figures 8a and 8b), respectively. In acetylated chitosan microspheres, the fracture surface revealed collapsed internal structures (Figure 8c). This finding indicates that the acetylation degree obtained was not



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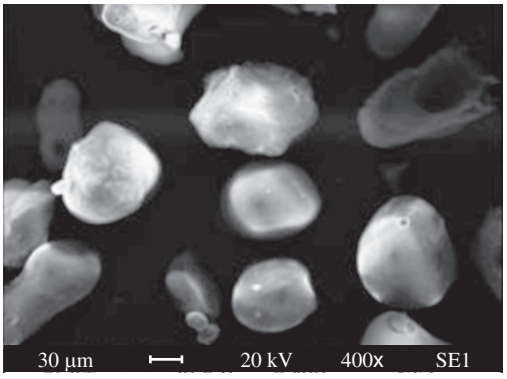

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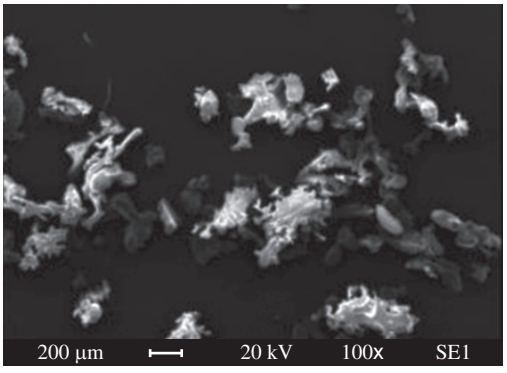
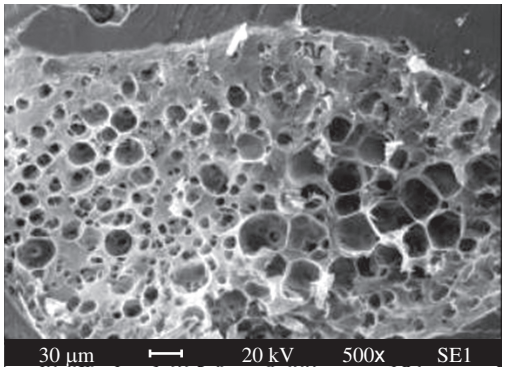


(c)

Figure 7. a) Scanning electron micrograph of chitosan microspheres, modified with epychlorohydrin; b) Scanning electron micrograph of chitosan microspheres, modified with glutaraldehyde; and c) Scanning electron micrograph of chitosan microspheres, modified with acetic anhydride.



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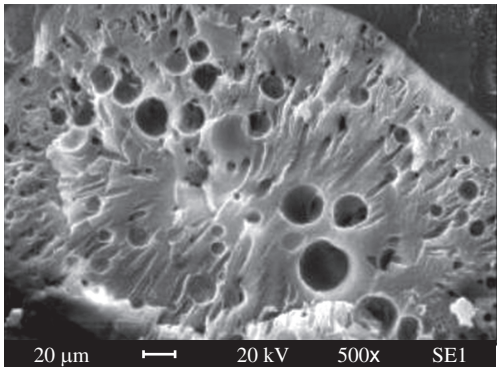

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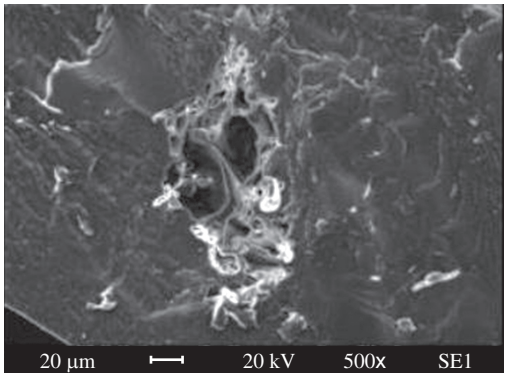

(c)

Figure 8. a) Scanning electron micrograph of epychlorohydrin-treated chitosan microsphere fracture; b) Scanning electron micrograph of glutaraldehyde-treated chitosan microsphere fracture; and c) Scanning electron micrograph of chitosan microspheres cross section morphology modified with acetic anhydride.

sufficient to maintain the microsphere structure intact during the SEM sample-preparation process.

Chitosan microspheres crosslinked with epychlorohydrin presented better resistance to handling than natural chitosan microspheres. The method presented of chitosan microsphere production proved to be very versatile and demostrated the possibility of associating the material with further chemical modifications, making this technique a very useful and easy route for achieving chitosan porous microparticles suitable for many applications. The chemical differences between the two obtained structures indicate that all interactions between the polymeric matrix and the other species will have an important effect on the final performance and specificity of the adsortion processes.

4. Conclusions

The spray and coagulation process, under different operating conditions, can produce chitosan microspheres of different sizes with a relatively narrow particle distribution. The microspheres produced by this method and the optimization results indicate a possibility of controlling particle size and, consequently, standard deviation, according to the application aimed. Chemical modifications to these particles allow the production of microspheres with different mechanical resistance and final morphology.

Acknowledgments

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