A One-Step Method for Fabricating Chitosan Microspheres

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ABSTRACT: A simple and *in situ* method, by using a high-voltage electrostatic system, for the fabrication of chitosan microspheres (in a form of isolatable microgels) by an extrusion process, exhibiting variable sizes and different membrane structures, was presented. The chitosan microspheres exhibited good sphericity and were in the range of 185.8 ± 13.8 to 380.9 ± 11.5 μm in diameter. There were two significant factors, the pump flow rate and electrostatic field strength, that affected the chitosan microsphere size. The microsphere size decreased when the flow rate was increased from 0.1 to 0.4 mL/h. Also, the microsphere size decreased when the electrostatic field strength was increased from 5.5 to 6.5 kV/cm. However, when the electrostatic field strength was raised to 7 kV/cm and higher, the microsphere size increased. For the latter case, with other parameters fixed, chitosan microsphere size can be controlled by adjusting the electrostatic field strength and predetermined by a simple linear regression equation: Microsphere Diameter (D, in μm) = $-(75.48) + 45.67 \times$ (Electrostatic Field Strength, E, in kV/cm), at [$7 \leq$ (Electrostatic Field Strength) ≤ 10] ($R^2 = 0.956$, $P < 0.001$). Following treatment with various ratios of crosslinking/gelating ($Na_5P_3O_{10}$/NaOH) agents, the prepared chitosan microspheres exhibited distinct membrane structures that yielded various mechanical strengths. In the $Na_5P_3O_{10}$/NaOH ratio of 19, the chitosan microspheres had a distinct two-layer structure. The selection of crosslinking/gelating ratio provided an additional degree of freedom, permitting the simultaneous regulation of mechanical properties and permeability of the microspheres, without extra manipulation, and thus, improved applicability in the biomedical field. When the chitosan microsphere extrusion process was used to encapsulate β-tricalcium phosphate powder for application as bony material, we found that the ultra fine β-tricalcium phosphate powder was trapped inside of the membrane very well. After appropriate collecting procedures, stored microspheres also retained good spherical shape. J Appl Polym Sci 94: 2150–2157, 2004

Key words: polysaccharides; extrusion; microgels; microspheres; crosslinking

INTRODUCTION

Microspheres are defined as particles that range in size from 50 nm to 2 mm in diameter. Microspheres could provide a larger surface area for cell growth and provide easy estimation of diffusion and mass transfer behavior. It could be used as a cell or tissue carrier, bone grafting, or an encapsulated drug delivery system in the biomedical field.[1–5] For example, in application for the treatment of Type I diabetes mellitus, microspheres with a semipermeable membrane encapsulating transplanted live islets tissue could protect them from the host's immune system and be able to induce blood glucose homeostasis. In general, the kinetics of the insulin response is much better with smaller encapsulated islets and the oxygen supply to the encapsulated cells could also be greatly improved by reducing the microsphere's size. In addition, smaller microspheres should also be more resistant to shear and compression forces, further ensuring the performance and/or durability of these tissues.[6–8] To date, there is strong interest in the fundamental and applicative research of microspheres and the preparation methodology of producing even smaller microspheres. Generally, the choice of microsphere manufacturing process depends on the nature of the native starting materials. Frequently used microsphere production methods are suspension–emulsion polymerization from monomeric materials or suspension–emulsion crosslinking from polymeric starting materials.[2,9] In such methods, the prepared microsphere's size distribution is widespread and difficult to regulate from batch to batch. Furthermore, organic solvents or toxic agents, such as glutaraldehyde, are often used to act as a crosslinking agent to strengthen microspheres, thereby impeding the biocompatibility of microspheres and limiting their applications in the biomedical field.

Chitosan, a natural polysaccharide [poly(1,4)-β-D-glucopyranosamine], is obtained by alkaline deacety-

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lation of chitin contents that constitute the shells of crab or shrimp. As a biodegradable material, chitosan has been observed to accelerate rates of wound healing and blood clotting.[10–11] Because of its biocompatibility, chitosan has shown great potential in a number of biomedical applications including drug delivery systems and wound dressing.[12–14] Unique chemical and physical properties, especially its proper viscosity flow characteristics in forms of gel-like aqueous solutions, make the chitosan material widely available to biomaterial researchers through processes of film casting, bead and powder suspension, and other shape forming. Chitosan has been explored extensively to prepare microspheres for a controlled release system. In one of the systems, larger chitosan microspheres are prepared normally by an emulsion method to deliver the anti-inflammatory drug diclofenac sodium and other active materials such as theophylline and aspirin.[15–17] Small chitosan microspheres have also been developed to deliver anticancer agents such as 5-fluorouracil.[18] During recent years, increased interest has been paid to using chitosan microspheres in mucoadhesive drug delivery systems, particularly the nasal delivery of peptide drugs.[19] Spray-drying is the usual method to produce such powders or granules from drug solution or suspensions.[3,20] The microspheres prepared by this method range in size from a few microns to several tens of microns and have a relatively wide range of variation in diameter and sphericity. Although this method can produce smaller microspheres, it requires a dry preparation environment. This prohibits its application where a wet process is unavoidable, such as the microencapsulation of live tissue or cells. Furthermore, due to their uneven membrane thickness, these microspheres could develop fragile spots and thus make it difficult to preserve and evaluate their overall diffusion and mass transport properties. It would be a worthwhile effort to develop a new approach to produce microspheres with a more uniform skin and allow processing in a wet environment.

The main purpose of this study was to identify the factors governing chitosan microspheres production by a high-voltage electrostatic system.[21–23] Besides quantifying the effects on microsphere size, the determination of conditions for producing microspheres of a range of desired sizes was explored. All preparative procedures were conducted in an aqueous environment, and therefore, free from nontoxic solvents; biocompatible crosslinking agents were used to prepare chitosan microspheres with improved mechanical and transport properties. The preliminary results reveal that electrostatic field strength and pump flow rate are two primary factors in determining the microsphere's size. The prepared chitosan microspheres with good sphericity were in the range of 185.8 ± 13.8 to 380.9 ± 11.5 μm in diameter. In addition, changing the crosslinking/gelating ratios could yield a range of distinct microsphere structures and properties, which would lead to versatile applications in the biomedical field. When β-Tricalcium phosphate (β-TCP) was microencapsulated by this process, the β-TCP powder was thoroughly entrapped inside of the chitosan microspheres with an ultrathin skin and still retained good spherical shape.

EXPERIMENTAL

Materials

Chitosan was purchased from TCI (Tokyo, Japan) with a molecular weight of 300,000 and deacetylation degree of 83%. Sodium tripolyphosphate ($Na_5P_3O_{10}$, 5%) and sodium hydroxide (NaOH, 1*N*) were purchased from SHOWA (Tokyo, Japan). Acetic acid was purchased from Sigma (St. Louis, MO). β-TCP was purchased from Merck-Schuchardt (Germany). All chemicals used in this study were of reagent grade. Injecting pump (74900 series, USA) and high-voltage power supply (series 230, USA) were supplied by Cole-Parmer and Bertan, respectively.

Preparation of chitosan microspheres

Chitosan microspheres were produced by extruding chitosan (1.5 w/v % in 0.1*N* acetic acid solution) droplets into a $Na_5P_3O_{10}$/NaOH solution. The droplets were generated with the use of a high-voltage electrostatic system developed in our lab. Figure 1 shows a schematic diagram of the experimental equipment used to prepare chitosan microspheres. The positive electrode of the electrostatic system was connected to the needle assembly, whereas a negative electrode, hollow in the middle, was placed vertically in the midpoint between the needle tip (positive electrode) and the receiving beaker, filled with $Na_5P_3O_{10}$/NaOH solution. The whole apparatus was encased in an insulated acrylic cabinet designed to avoid accidental contact with high-voltage sources during the experiment. This prototype system allowed accurate adjustment of the electrostatic field strength by fine-tuning the distance between the two electrodes. The detailed arrangement of the set design that related to the polymer chemistry and physics, and criterions on parameter selection, will be illustrated and discussed elsewhere. Generally speaking, the electrostatic field strength, the pump flow rate, the needle gauge size, and the distance between the needle tip and the negative electrode all affect the size and other characteristics of the chitosan microspheres. For example, the concentration of chitosan (1.5%) solution could be kept constant and the influence of the viscosity is thus fixed. For each parameter change, such as field strength, electrode gap distance, pump rate, crosslink-

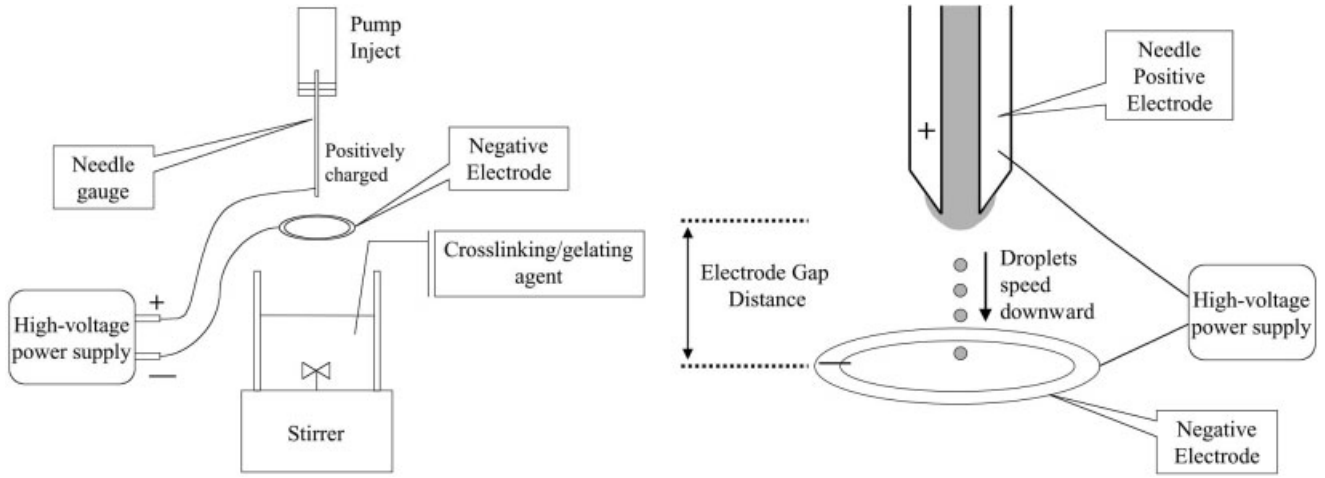


Figure 1 (a) Schematic presentation of chitosan microsphere generator. (b) Close-up view of electrodes.

ing agent ratio, and others, the diameter of the microsphere was determined from the corresponding photomicrograph. Moreover, from time to time, we duplicated reactions of same sets of parameters and found the reproducibility to be excellent.

Evaluation of chitosan microspheres

Samples (from a minute run) containing between 200 and 700 microspheres were produced for each different set of conditions tested. These microspheres were gently stirred and soaked in the $Na_5P_3O_{10}$/NaOH solution for 2.5 h at room temperature before their diameter was determined by an optical microscope (Olympus IX-70, Japan). Size distributions of various makes of microsphere samples were done on a random sampling basis of about 100–150 individual microspheres to minimize potential selection bias. For each sample, the average diameter of long and short axes and the standard deviation was calculated. Scanning electron microscopy (SEM; Hitachi, S-2700, Japan) was used to evaluate the surface morphology of these microspheres.

Mechanical strength of the microspheres

An agitation method was adapted to investigate the mechanical strength of the microspheres. Briefly, these prepared chitosan microspheres (~ 200 microspheres) were immersed in vials each containing 2 mL of phosphate-buffered saline (PBS) solution (pH 7.4). These vials were placed in a shaker at 37°C and vibrated back and forth at a frequency of 50 rpm for varying time periods. This vigorous agitation accelerated the microsphere breakage and therefore shortened the time needed for a fracture test. The vial was removed from the bath and the number of fractured microspheres was counted. The vial was then placed back into the shaker for continued agitation. This measurement was carried out for 8 days and three sets of independent experiments for each sample were performed.

RESULTS AND DISCUSSION

Various chitosan microspheres with a good spherical shape factor were produced. Incidentally, because the needle gauge had only a minor effect on microspheres size, we were able to use a type 25G needle throughout the study. We found that the size and related characteristics of chitosan microspheres are subject to

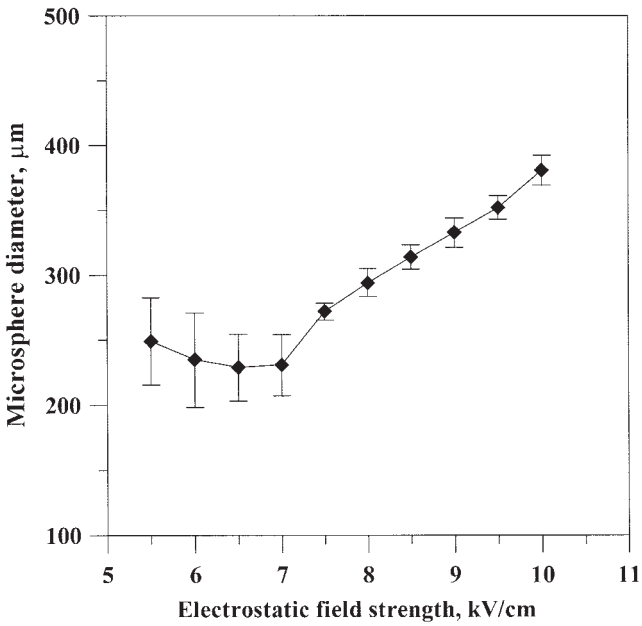


Figure 2 Effect of electrostatic field to microsphere size. Pump rate, 0.2 mL/h; electrode gap distance, 1 cm; needle gauge, 25G.

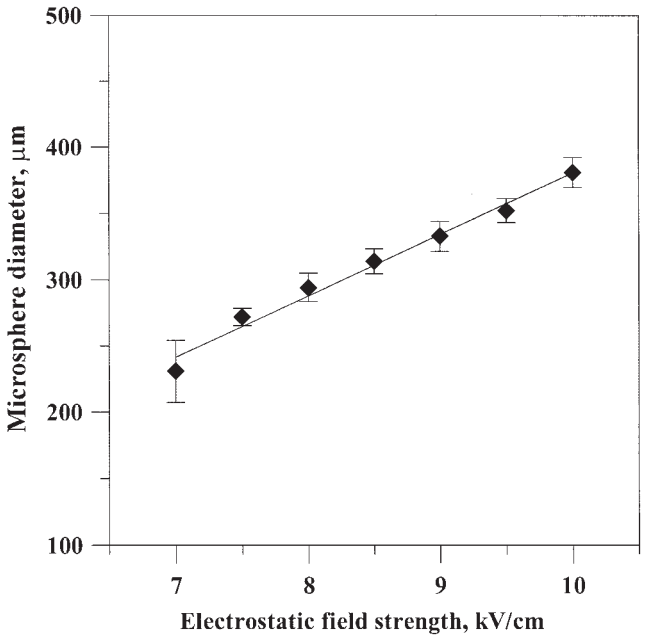


Figure 3 Empirical equation for determination of diameter of microspheres by applied voltage.

variations of electrostatic field strength, pump rate, electrode gap distance, and crosslinking agent concentration:

Effect of electrostatic field on microsphere size

Typically, the pump rate of the chitosan solution through the needle tip and the electrode gap distance were kept constant at 0.2 mL/h and 1 cm apart, respectively, during the experiments. When the voltage applied was increased from 5.5 to 6.5 kV, the microsphere's size decreased (with relatively high deviation). However, when the voltage was increased to 7 kV and higher, the incrementation of microsphere size exhibited a close-to-linear relationship and the deviation in size distribution became smaller (Fig. 2). In the latter case, by using a simple statistical regression analysis on these data sets, an empirical equation can be obtained: Microsphere diameter, D (μm) = $-(75.48) + 45.67 \times$ (Electrostatic Field Strength in kV/cm), [$7 \leq$ (Electrostatic Field Strength, E) ≤ 10] ($R^2 = 0.956$, $P < 0.001$). This proves that the microsphere size could be accurately determined and controlled simply by adjusting the electrostatic field strength (Fig. 3). Under the current apparatus, when the voltage was increased to >10.5 kV, arcing discharges occurred, which consequently destabilized formation and destroyed the microspheres.

Effect of pump flow rate on microsphere size

To study the effect of the chitosan solution pump flow rate on the microsphere size, the applied electrostatic field strength was maintained at 7 kV/cm as the pump flow rates were varied. As shown in Figure 4, there are two distinctive regions for the relationship between flow rates and microsphere size. The microsphere size decreased on the flow rate from 0.1 to 0.4 mL/h. However, at a higher flow rate from 0.4 to 0.8 mL/h, the microspheres obtained increased in size proportional to the increment of flow rate and with a smaller deviation in size.

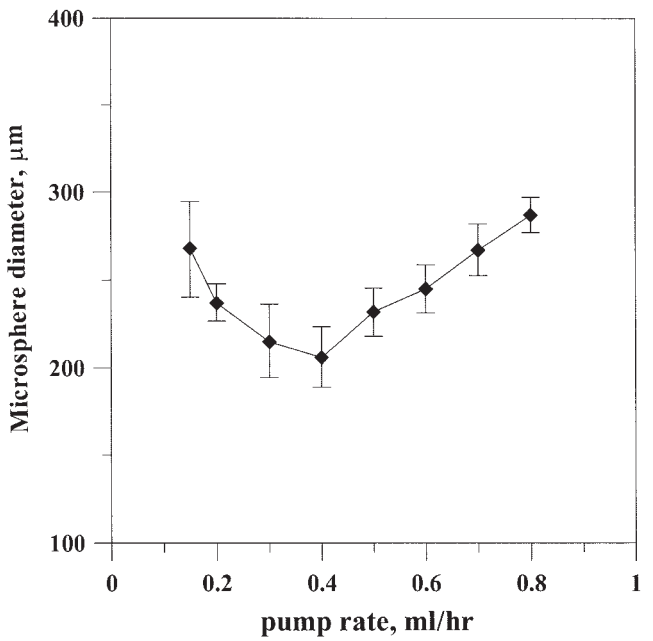


Figure 4 Effect of pump rate to microsphere size. Applied voltage, 7 Kv; electrode gap distance, 1 cm; needle gauge, 25G.

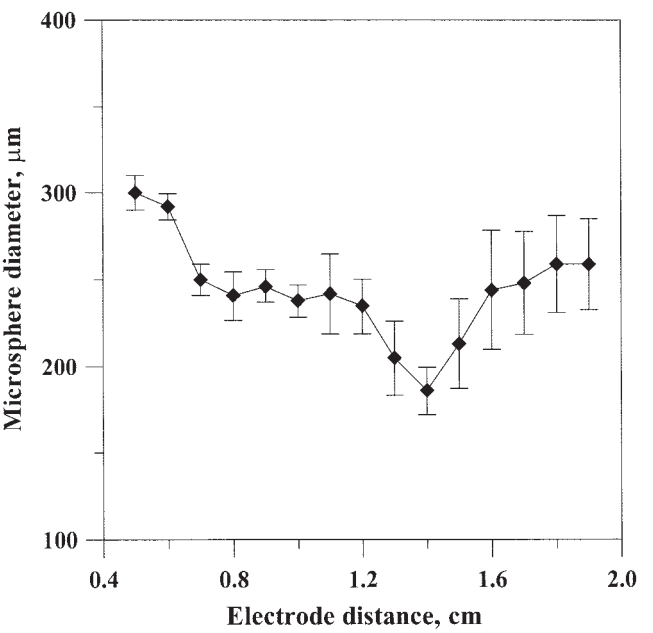


Figure 5 Profile of microsphere size variation in relation to electrode gap distance. Applied voltage, 7 kV; pump rate, 0.2 mL/h; needle gauge, 25G.

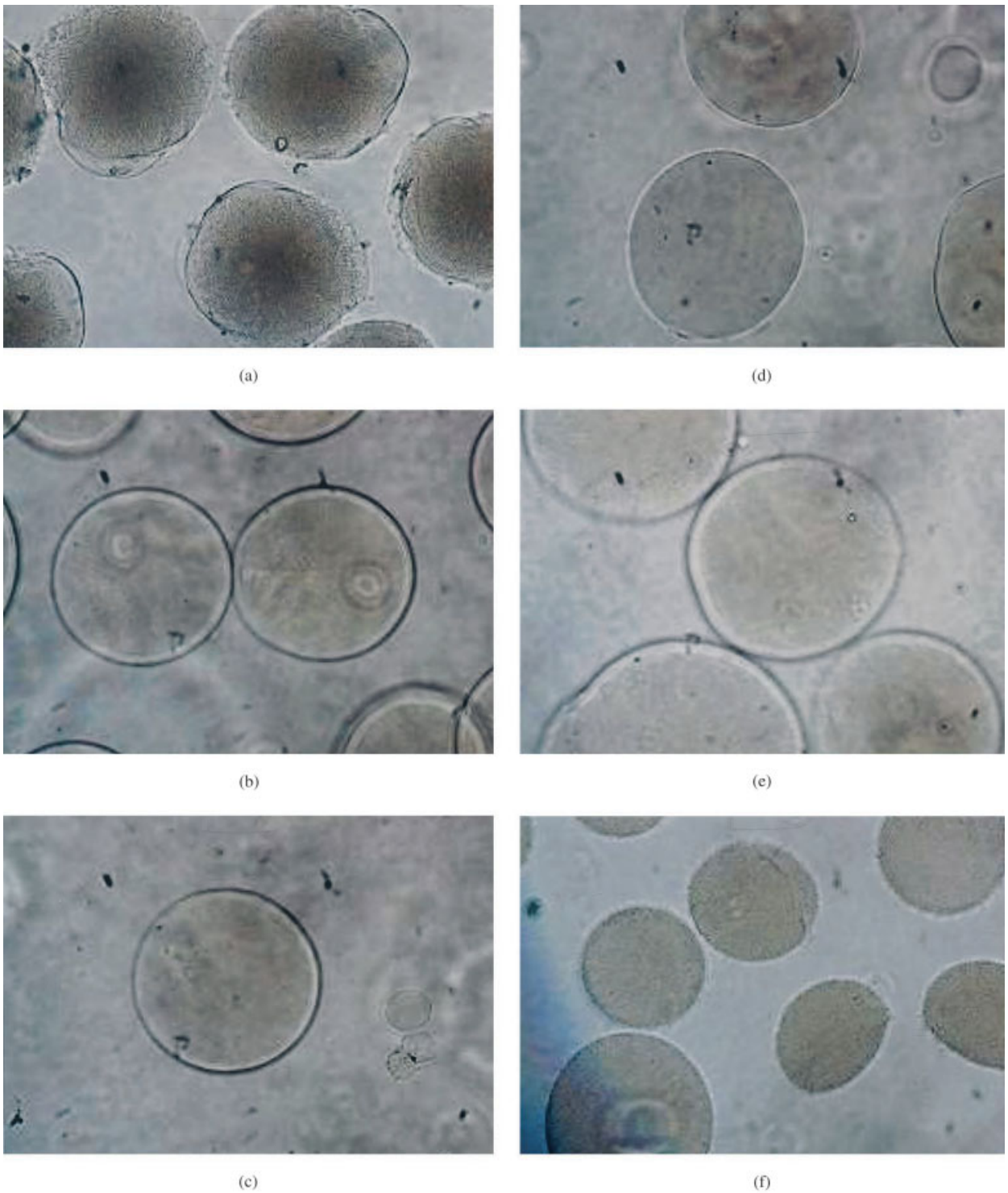


Figure 6 Micrographs of $Na_5P_3O_{10}$/NaOH (v/v) mixture crosslinked microspheres from 1.5% chitosan solution. (a) Pure $Na_5P_3O_{10}$, (b) P/N = 4/1, (c) P/N = 3/2, (d) P/N = 2/3, (e) P/N = 1/4, and (f) pure NaOH. ($Na_5P_3O_{10}$: P; NaOH: N.)

Effect of electrode gap distance on microsphere size

Instead of immersing the negative electrode into the receiving beaker, the electrode was suspended in the air and placed between the positive electrode and the beaker. By usng this construction, we could easily adjust the electrostatic field strength and vary the distance between the two electrodes. Also, the electrostatic field strength could be kept stable in the extrusion process of microspheres even during the addition

of chitosan solution into the receiving beaker. In a typical run, the applied electrostatic voltage and the pump flow rate were kept at constant at 7 kV and 0.2 mL/h, respectively, while the gap distances between the two electrodes varied from 0.5 to 1.8 cm. As shown in Figure 5, the resulting microsphere sizes varied irregularly with the electrode gap distance. Interestingly, when the distance was increased to 1.4 cm, minimum microspheres (185.8 ± 13.8 μm) were obtained in all experiments tested. Incidentally, arc discharges were observed at a gap distance of 0.5 cm or smaller. In a situation of great electrode distance, the microsphere size had a wider size deviation. In relationship to electrostatic field strength, the microspheres sizes varied quite irregularly with a change of gap distance. This problem could remain unsolved until we acquire better knowledge of the interaction between charged droplets and locally fluctuated electrostatic fields. However, we realized the potential to optimize the conditions by manipulating the parameters in producing varieties of microspheres and some preliminary data are being presented elsewhere.[24]

Effect of crosslinking/gelating agent on microsphere morphology and properties

The morphology of chitosan microspheres prepared by this electrostatic system and the effect of crosslinking/gelating agent to the microspheres were examined by light microscopy as well as SEM. The sphericity of the microspheres was generally good and each exhibited a membrane structure of distinct boundary, by treatment with $Na_5P_3O_{10}$/NaOH mixed in different ratios (Fig. 6). We found a complementary functional relationship of $Na_5P_3O_{10}$ and NaOH solutions. Each reagent alone will not act properly in the crosslinking process. Microspheres treated with just an aqueous NaOH solution became flattened and lost the spherical shape over time. On the other hand, microspheres treated with only an aqueous $Na_5P_3O_{10}$ solution exhibited a thinner and more fragile membrane structure. However, with a volume ratio of $Na_5P_3O_{10}$/NaOH at 19, the thickness of the outer boundary membrane of microspheres increased and exhibited distinct bilayer structure. These microspheres could be shrunk at room temperature, after immersion in an acetic acid (0.1*N*) solution for 8 h (Fig. 7), while retaining a bilayer structure. This result indicates that the outer layer became crosslinked with $Na_5P_3O_{10}$ in a modified basic medium and is therefore insoluble in an acetic acid solution. Furthermore, in this case, it is believed that chitosan droplets could be first precipitated by gelation in NaOH (being a nonsolvent solution) to form a gelating microsphere. Subsequently, the ionic crosslinking reaction with $Na_5P_3O_{10}$ occurs on the outer part upon treatment with $Na_5P_3O_{10}$/NaOH mixture to form a skin structure. Afterwards, the masses of chitosan gelating droplets were gradually exposed to further infiltration of $Na_5P_3O_{10}$/NaOH solution. Finally, the chitosan solution was totally neutralized and crosslinked and this resulted in the precipitation of chitosan polymer to form a core structure. Thus, we witnessed a one-step crosslinked chitosan microsphere fabricating process. Furthermore, the mechanical strength of the microspheres was also improved at higher ratios of $Na_5P_3O_{10}$/NaOH reagent (Fig. 8). This result could be due to a more efficient crosslinking reaction with higher concentration of $Na_5P_3O_{10}$ to chitosan, which strengthened the polymer network of the outer part of microsphere to yield better mechanical strength. From SEM observation, the chitosan microsphere showed good sphericity and smooth surface morphology (Fig. 9).

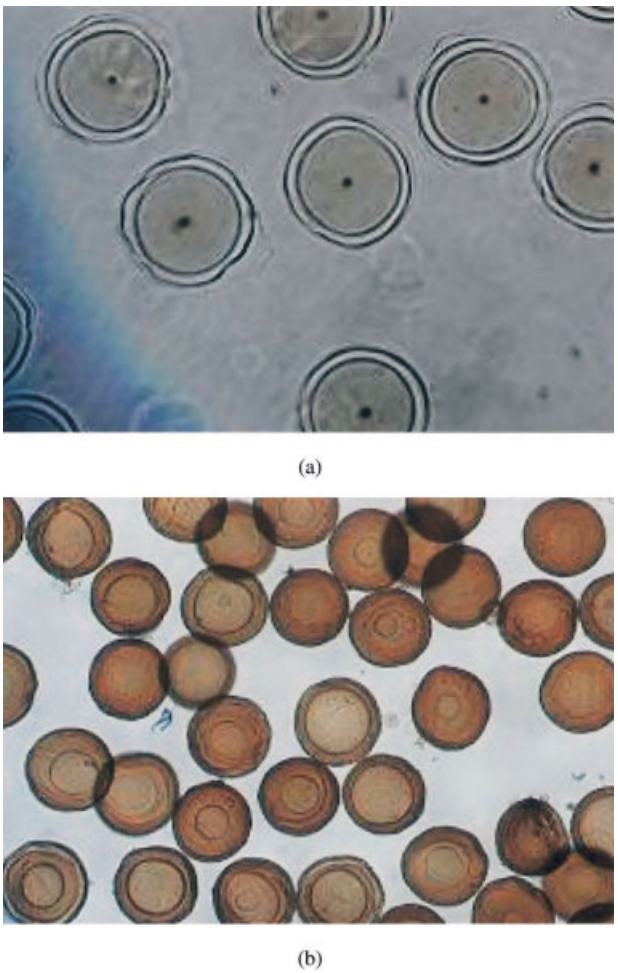


Figure 7 Chitosan microsphere (crosslinked by $Na_5P_3O_{10}$/NaOH: 19/1). (a) Bilayer structure; (b) after immersion in acetic acid (0.1*N*) for 8 h.

In summary, we found that the combination of pump extrusion rate, applied electrostatic field, surface chemistry of chitosan solutions, and needle gauge all influence the size and characteristics of chitosan

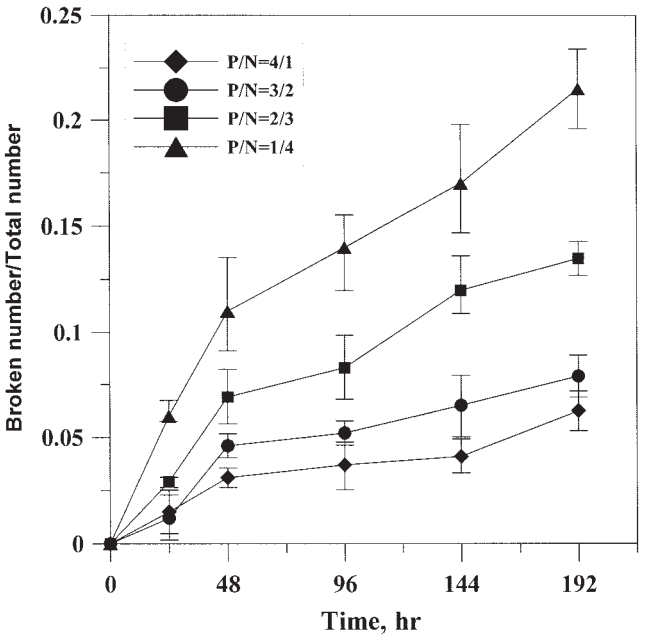


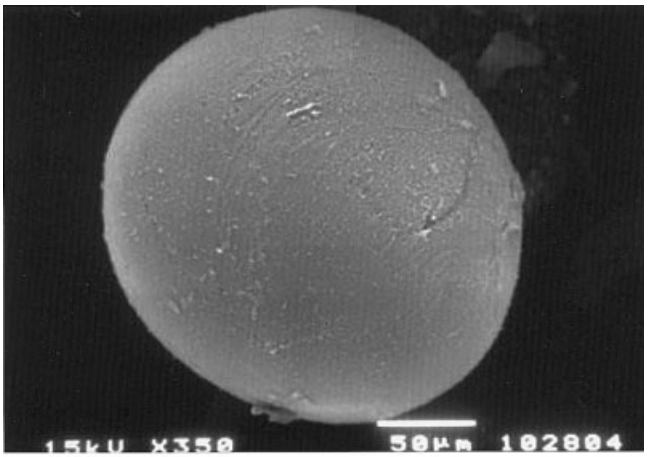
Figure 8 Mechanical strength of crosslinked chitosan microsphere. (Corresponding to Fig. 6.)

microspheres. Systematic and expanded experiments to find out the interrelationship of all the parameters discussed here and beyond will lead us to in-depth understanding of the role that chitosan may play in the encapsulation of synthetic and natural biomaterials.

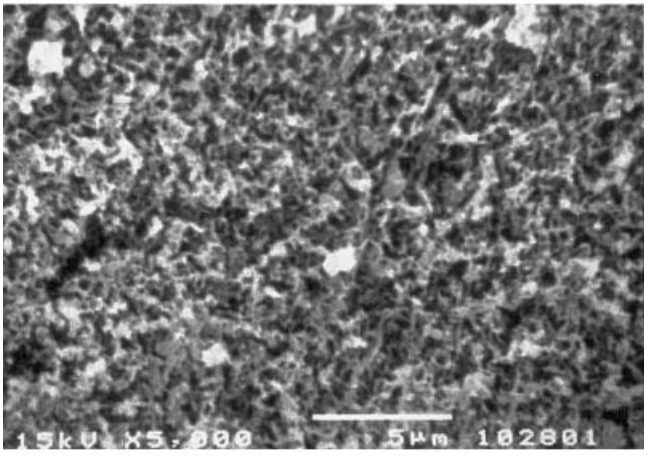
CONCLUSION

In the present study, we developed a process of continuously injecting chitosan microdroplets through a high-voltage electrostatic system and into a $Na_5P_3O_{10}$/NaOH solution to produce chitosan microspheres. The results show that there are three parameters determining the microsphere size, namely, electrostatic field strength, pump flow rate, and electrodes gap distance. The microsphere size can be predicted within a certain range by a linear regression equation: Microsphere diameter, $D = -(75.48) + 45.67 \times$ (Electrostatic Field Strength, E) [$7 \leq$ (Electrostatic Field Strength) ≤ 10] ($R^2 = 0.956$, $P < 0.001$), with the pump flow rate and the electrode gap distance set at a constant 0.2 mL/h and 1 cm, respectively. From this equation, we can produce chitosan microspheres at a specific diameter by simply adjusting the voltage applied. By modulating these parameters, it was possible to produce chitosan microspheres as small as 185.8 $\pm$ 13.8 μm and as large as 380.9 $\pm$ 11.5 μm. The chitosan microspheres obtained by this method are very homogeneous in size with small deviation in most conditions. Moreover, these sizes were very reproducible between experiments. It is therefore possible to predetermine a set of conditions to produce microspheres of determinate size.

An important improvement of this study was that we could prepare different membrane structures and with wider range of properties of chitosan microspheres simply by using different ratios of crosslinking/gelating agent ($Na_5P_3O_{10}$/NaOH). Consequently, the selection of crosslinking/gelating ratio could provide an additional degree of freedom by permitting simultaneous variations in mechanical properties and membrane permeability without the need for extra manipulation. Furthermore, this nontoxic reagent meets the biocompatibility requirement of crosslinked chitosan microspheres. The study on applicability of this high-voltage electrostatic field system to encapsulate various materials such as β-TCP (with preliminary results shown in Fig. 10) and live cells is currently underway in our laboratories.



(a)



(b)

Figure 9 SEM micrographs of crosslinked chitosan microsphere. (a) Single sphere; (b) detail surface of a sphere.

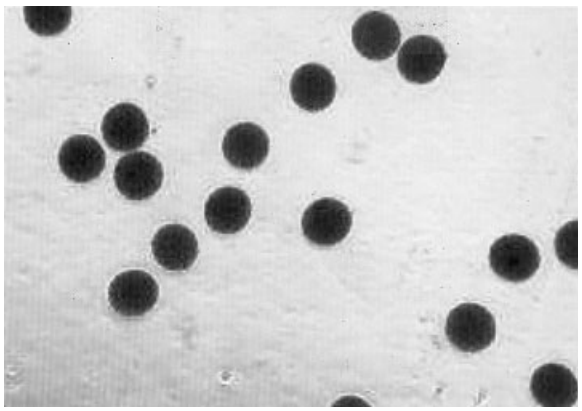


Figure 10 Optical micrographs of encapsulation of β-TCP by microsphere of chitosan.

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References

1. Genta, I.; Costantini, M.; Asti, A.; Conti, B.; Montanari, L. Carbohydr Polym 1998, 36, 81.
2. Kuo, S. M.; Chang, S. J.; Lin, L.-C.; Chen, C. J. J Appl Polym Sci 2003, 89, 3897.
3. He, P.; Davis, S. S.; Illum, L. Int J Pharm 1999, 187, 53.
4. Mi, F.-L.; Shyu, S.-S.; Chen, C.-T.; Schoung, J.-Y. Biomaterials 1999, 20, 1603.
5. Mi, F.-L.; Tan, Y.-C.; Liang, H.-F.; Sung, H.-W. Biomaterials 2002, 23, 181.
6. Fan, M. Y.; Lum, Z. P.; Fu, X. W.; Lévesque, L.; Tai, L. T.; Sun, A. M. Diabetes 1990, 39, 519.
7. Hallé, J. P.; Chevalier, S.; Beaudry, M.; Chapdelaine, A.; Cousineau, S.; Saintonge, J.; Yale, J. F. Transplantation 1993, 55, 350.
8. Lum, Z. P.; Krestow, M.; Tai, I. T.; Vacek, I.; Sun, A. M. Transplantation 1992, 53, 1180.
9. Genta, I.; Perugini, P.; Conti, B.; Pavanetto, F. Int J Pharm 1997, 152, 237.
10. Suzuki, T.; Mizushima, Y.; Umeda, T.; Ohashi, R. J Biosci Bioeng 1999, 88, 194.
11. Amiji, M. M. J Biomater Sci Polym Ed 1996, 8, 281.
12. Gutowska, A.; Jeong, B.; Jasionowski, M. Anatom Rec 2001, 263, 342.
13. Mi, F.-L.; Shyu, S.-S.; Wu, Y.-B.; Lee, S.-T.; Shyong, J.-Y.; Huang, R.-N. Biomaterials 2001, 22, 165.
14. Wang, Y.-C.; Lin, M.-C.; Wang, D.-M.; Hsieh, H.-J. Biomaterials 2003, 24, 1047.
15. Acikgoz, M.; Kas, H. S.; Orman, M.; Hincal, A. A. J Microencapsul 1996, 13, 141.
16. Thanoo, B. C.; Sunny, C. M.; Jayakrishnan, A. J Pharm Pharmacol 1992, 44, 283.
17. Mi, F.-L.; Kuan, C.-Y.; Shyu, S.-S.; Lee, S.-T.; Chang, S.-F. Carbohydr Polym 2000, 41, 389.
18. Ohya, Y.; Takei, T.; Kobayashi, H.; Ouchi, T. J Microencapsul 1993, 10, 1.
19. Illum, L.; Farraj, N. F.; Davis, S. S. Pharm Res 1994, 11, 1186.
20. Lorenzo-Lamosa, M. L.; Remuñán-López, C.; Vila-Jato, J. L.; Alinso, M. J. J Controlled Release 1998, 52, 109.
21. Hsu, B. R.-S.; Chen, H.-C.; Fu, S.-.H.; Huang, Y.-Y.; Huang, H.-S. J Formos Med Assoc 1994, 93, 240.
22. Etuk, B. R.; Weatherley, L. R.; Murray, K. R. J Microencapsul 1995, 12, 173.
23. Hallé, J.-P.; Leblond, F. A.; Pariseau, J.-F.; Jutras, P.; Brabant, M.-J.; Lepage, Y. Cell Transplant 1994, 3 (5), 365.
24. Su, C. H.; Kuo, C. H.; Chang, S. J.; Kuo, S. M. 8th Biochemical Engineering Conference; Taiwan, 2003.