Available online at www.sciencedirect.com
SCIENCE DIRECT
International Journal of Pharmaceutics 312 (2006) 113–118
INTERNATIONAL JOURNAL OF PHARMACEUTICS
www.elsevier.com/locate/ijpharm

Preparation and in vitro evaluation of mucoadhesive properties of alginate/chitosan microparticles containing prednisolone

Sakchai Wittaya-areekul [a,*], Jittiporn Kruenate [b], Chureerat Prahsarn [b]

[a] *Department of Pharmaceutical Technology, Faculty of Pharmaceutical Sciences, Naresuan University, Phitsanulok 65000, Thailand*
[b] *National Metal and Materials Technology Center, National Science and Technology Development Agency, Prathumthani 12120, Thailand*

Received 6 January 2006; accepted 6 January 2006
Available online 21 February 2006

Abstract

This study describes the preparation of mucoadhesive alginate/chitosan microparticles containing prednisolone intended for colon-specific delivery. Two methods have been used for the preparation of the particles: the one-step method is the method in which prednisolone was dispersed within sodium alginate solution and this dispersion was then dropped in a solution containing both calcium chloride and chitosan. The two-step method consisted also of the dispersion of prednisolone in alginate solution and then dropping this dispersion into a solution containing calcium chloride, the particles were then transferred to a chitosan solution. The concentration of sodium alginate solution at 2% (w/v), various concentrations of calcium chloride solution (0.5–1.0%, w/v), chitosan solutions (0.5, 1.0 and 1.5%, w/v) and prednisolone drug load (2, 5, 10 and 15%, w/v) have been used. The results for both preparation methods show that the particle size and drug content were mainly depending on the amount of the drug concentration and not the amount of chitosan and calcium chloride. The in vitro mucoadhesive tests for particles prepared from both methods were carried out using the freshly excised gut of pigs. The particles prepared by the one-step method exhibited excellent mucoadhesive properties after 1 h test. Increased chitosan concentrations from 0, 0.5, 1.0 to 1.5% (w/v) resulted in 43, 55, 82 and 88% of the particle remaining attached on the gut surface after 1 h, respectively. However, the particles prepared by the two-step method showed significant less mucoadhesion under the same experimental conditions. At chitosan concentrations of 0, 0.5, 1.0 and 1.5% (w/v) the amount of particles remaining attached to the mucosal surface of the pig gut after 1 h was 43, 3, 11 and 11%, respectively. The prednisolone release at a pH of 6.8 after 4 h was between 63 and 79% for the particles prepared by the one-step method and between 57 and 88% for the particles prepared by the two-step method with a prednisolone drug load of 5 and 10% (w/v), respectively. The results show that depending on the preparation method these chitosan coated alginate particles show different mucoadhesiveness whereas their other properties are not statistically significant different.

Keywords: Alginate/chitosan; Prednisolone; Microparticles; Mucoadhesive

1. Introduction

Colonic drug delivery systems have been widely studied for the last decade due to the recently recognized importance of this region of the gastrointestinal tract for drug absorption. The benefit of those delivery systems are that they can be applied both for local and systemic drug delivery (Kinget et al., 1998; Calabresi and Chanber, 1992). Among the different approaches to achieve colon-specific drug delivery, the use of chitosan provides great promise due to its non-solubilty at pH values higher than 6.5 which prevail in the jejunum and the ileum of the gut whereas the colonic pH value is in the range of 5.5–6.0 and chitosan then gets soluble again and will release the incorporated drug substances. A suitable dosage form then could be an enteric coated gelatin capsule containing chitosan or chitosan coated microparticles which are released in the colon. Because it is well known that such enteric coated delivery systems for colon targeting release their drug content often before reaching the colon, chitosan due to its insolubility in the ileum will prevent undesired drug release in this part of the gut. In its hydrated form, chitosan also shows good mucoadhesive properties (Fiebrig et al., 1994, 1995; Takeuchi et al., 1996). There has been increasing interest in the study on alginate/chitosan microparticles as carriers for controlled release of proteins and drugs for its biocompatible, biodegradable and

* Corresponding author. Tel.: + 66 5526 1000x3619; fax: +66 5526 1057.
E-mail addresses: sakchaiw@nu.ac.th, sakchai99@yahoo.com (S. Wittaya-areekul).

0378-5173/$ – see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.ijpharm.2006.01.003

mucoadhesive properties (Vandenberg and De La Noue, 2001; Mi et al., 2002).

Chitosan has been shown to interact with mucin (Fiebrig et al., 1994, 1995), and liposomes coated with chitosan have also been shown in vivo to have a prolonged residence time in the GI tract of rats in comparison to uncoated liposomes (Takeuchi et al., 1996). Microparticles with a chitosan gel core and a polycation–polyanion membrane have been widely investigated in connection with various applications. Alginate, a negatively charged polysaccharide with high charge density is the most commonly used polyanion for microparticles production and has been reported to be mucoadhesive as well (Craig and Tamburic, 1997; Gomez and Wee, 1998). The alginate/chitosan microparticles can be produced by different methods (Huguet and Dellacherie, 1996; Daly and Knorr, 1988). However, most of the reports focused on preparation methods of microparticles and characteristics of drug release. The mucoadhesive properties, one of the important feature of alginate/chitosan microparticles used in the drug delivery system, were rarely included in these studies.

This study focuses on the development of microparticles with an alginate core and chitosan coatings containing prednisolone as a model drug. The developed microparticles consist of prednisolone microcrystals entrapped within sodium alginate and coated with chitosan as an outer layer. Alginate/chitosan microspheres were prepared by complex coacervation using sodium alginate as a gel core. Two principally different procedures were used: a one-step method where a complex coacervate membrane is formed at the interface between the alginate and chitosan solutions when the alginate solution is dropped directly into a solution of calcium chloride mixed with chitosan. The other method is a two-step method which comprises the formation of alginate beads stabilized with calcium alginate, followed by a membrane forming second step where the beads are suspended in a solution of chitosan. This work also focuses on the in vitro evaluation of the mucoadhesive properties of alginate/chitosan microparticles containing prednisolone using a pig intestine device attached to the USP disintegration apparatus.

2. Materials and methods

2.1. Materials

High molecular weight chitosan (MW 474 kDa) with a deacetylation degree of 96% was purchased from Aqua Premier Co., Ltd. (Chonburi, Thailand). Low viscosity sodium alginate (viscosity 250 cps, 2%, w/v) was purchased from Sigma–Aldrich Inc. (St. Louis, MO, USA). Micronized prednisolone, Cibacron brilliant red 3B-A, and calcium chloride were purchased from Fluka AG (Switzerland).

2.2. Preparation of microparticles

Alginate/chitosan microparticles were prepared by complex coacervation using sodium alginate as a gel core. All alginate solutions (2%, w/v) were prepared by dissolving sodium alginate in de-ionized water. Micronized prednisolone powder (2–15%, w/v) was then suspended thoroughly into the alginate solutions by a homogenizer. The alginate/prednisolone mixture was dropped through a 0.45 mm syringe needle at a dropping rate of 1.0 ml/min. Two different procedures were used: the one-step method was by dropping the alginate/prednisolone mixture directly into a solution of calcium chloride (0.5–1.0%, w/v) mixed with chitosan (0.5–1.5%, w/v). The two-step method was by dropping the alginate/prednisolone mixture into calcium chloride solution (0.5–1.0%, w/v), followed by a membrane forming step where the particles were suspended in a solution of chitosan (0.5–1.5%, w/v). The microparticles were allowed to harden for at least 2 h before washing them twice with distilled water, and were then dried at room temperature.

2.3. Characterization of the prednisolone microparticles

2.3.1. Determination of prednisolone content in microparticles

The prednisolone content was determined by dissolving 100 mg of microparticles in 0.2 M phosphate buffer pH 6.8 under sonication for at least 1 h until the microparticles were completely dissolved. After dissolution, the samples were filtered and analyzed spectrophotometrically at 242 nm (Model Gray 1E, Australia). The results are expressed as the amount of prednisolone in 100 mg of microparticles.

2.3.2. Scanning electron microscopy

The surface morphology of the microparticles was examined using scanning electron microscopy (SEM LEO 1455VP, Cambridge, UK). The samples were mounted directly onto the SEM sample holder using double-sided sticking tape and were gold spray-coated.

2.3.3. Particle size determination

Measurements of the particles size distributions and mean diameters of the microparticles were carried out with an optical microscope (Olympus, Germany). Fifty randomly chosen microparticles were taken to measure their individual shape and size. Microparticles were visualized under 4× magnification and the diameter noted using a microscope equipped with a calibrated slide-mounted tracing device permitting accuracy of ±50 μm. The Feret's diameters were registered for irregular sized microparticles.

2.3.4. Colorimetric determination of chitosan in aqueous solution

A colorimetric method for the determination of chitosan in an aqueous solution was described by Muzzarelli (1998). A solution of Cibacron brilliant red 3B-A dye was prepared by dissolving 150 mg of the powder in 100 ml deionized water. Five milliliter of the stock solution were diluted to 100 ml with 0.1 M glycine hydrochloride buffer. The final concentration of the dye solution was 75 μg/ml. To prepare the standard curve, a stock solution of 0.5% (w/v) chitosan (15, 30, 45, 60, 80, 100, 150, 200 and 250 μl) was filled into test tubes, followed by addition of different volumes of buffer to reach 300 μl. Then 3 ml aliquots of dye solution were added to each tube. The absorbance values

were measured spectrophotometrically at 575 nm. The aqueous chitosan solutions were tested before and after the preparation of microparticles by both preparation methods. The difference amount of chitosan before and after preparation of the microparticles was calculated as the amount of chitosan bound to the alginate cores of the microparticles.

2.4. In vitro release studies

Drug release profiles from alginate/chitosan microparticles containing prednisolone with an accurately weighed amount of microparticles were obtained using USP dissolution apparatus II (Erweka, Germany). The study conditions were set at a stirring speed of 75 rpm, dissolution medium volume of 500 ml at 37 °C. The dissolution medium was 0.2 M phosphate buffer pH 6.8 as a representative of the intestinal pH. At fixed intervals, an aliquot of 5 ml was withdrawn for analysis of prednisolone. The medium was replaced with fresh dissolution medium at each interval. The samples were filtered and analyzed spectrophotometrically at 242 nm. The results measured in triplicate are expressed as a percentage of the drug released.

2.5. Measurement of in vitro mucoadhesive properties

The in vitro evaluation of the mucoadhesive properties of the microcapsules was carried out using the proximal portion of pig's large intestine. The freshly slaughtered large intestine was washed with physiological saline and attached on a microscopic slide. Thirty pre-swollen microcapsules were brought in contact to the intestine using the pressure of 25 g on the microscopic slide for 2 min. The mucoadhesiveness of the microcapsules was measured by connecting the prepared slide with the gut to the USP disintegration apparatus (Van Kel Inc., NC, USA). The particles were forced to wash off under the reciprocating motion of disintegration apparatus in 900 ml phosphate buffer pH 6.8. The number of particle remaining attached was counted after 15, 30, 45 and 60 min.

2.6. Statistical analyses

All of the experiments were done in triplicate. One-way analysis of variance (ANOVA) was performed to determine the significant difference in each property among the formulations. The differences were considered to be significant at a level of $P < 0.05$.

3. Results and discussion

3.1. Particle size and prednisolone content

Figs. 1 and 2 show that increasing concentration of prednisolone from 2, 5, 10 to 15% (w/v) in sodium alginate 2% (w/v) without chitosan coating resulted in increasing particle sizes These results were expected since at the constant concentration of sodium alginate of 2% (w/v), an increasing concentration of prednisolone resulted in increased overall viscosity of the dispersed system. The increased concentration of calcium chloride

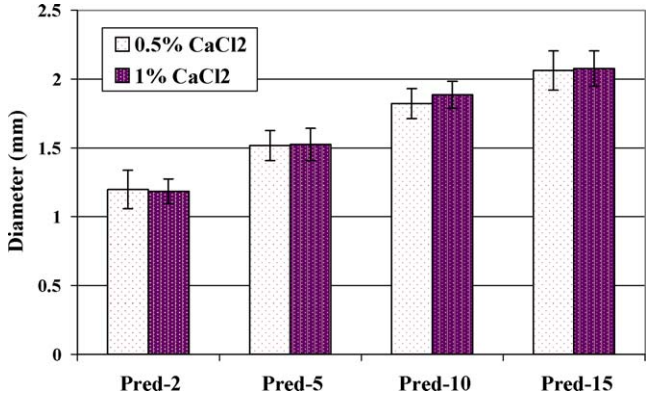


Fig. 1. The average Feret's diameter of alginate microparticles prepared with 2% (w/v) sodium alginate, 0.5 and 1.0% (w/v) $CaCl_2$, and 2, 5, 10, and 15% (w/v) prednisolone.

from 0.5 to 1% (w/v) resulted in comparable particle size and drug content. These results revealed that the calcium chloride concentration of 0.5% (w/v) was sufficient to form the gel network.

The particles prepared by the one-step method gave approximately the same particle size and drug content as those prepared by the two-step method ($P < 0.05$). In the one-step method, the alginate network is formed by the reaction with both chitosan and calcium ions, allowing also the diffusion of chitosan molecules into the alginate gel core. The concentration of calcium ions in the chitosan solution during the particle preparation had a large effect on the ability of the microparticles to bind chitosan (Gaserod et al., 1998). Their results suggested a higher diffusion rate of chitosan molecules into the alginate core in the presence of calcium chloride concentrations and resulting in a higher porosity of alginate core. In the two-step method, the alginate network is formed during the reaction with calcium ions only followed by the wall formation with chitosan molecules.

The quantitative determination of unbound chitosan in the supernatant was done with Cibacron brilliant red 3B-A in diluted aqueous solutions after removal of the microparticle residues. This method proved to be suitable for the determination of

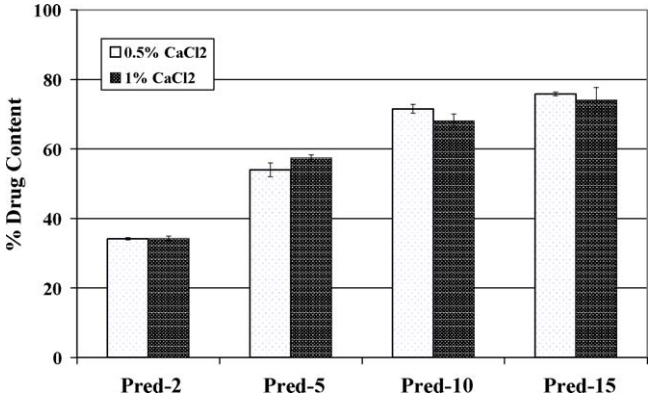


Fig. 2. The amount of incorporated prednisolone in alginate microparticles prepared with 2% (w/v) sodium alginate, 0.5 and 1.0% (w/v) $CaCl_2$, and 2, 5, 10 and 15% (w/v) prednisolone, respectively.

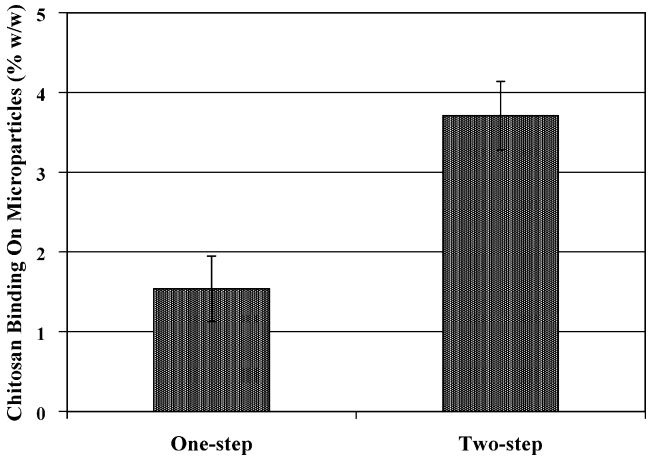
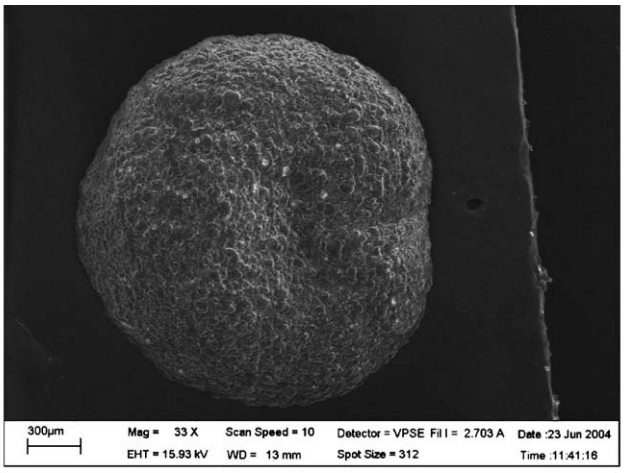


Fig. 3. The amount of chitosan bound to microparticles prepared by the one-step and two-step method using 2% (w/v) sodium alginate, 0.5% (w/v) $CaCl_2$, 0.5% (w/v) chitosan, and 5% (w/v) prednisolone, respectively.

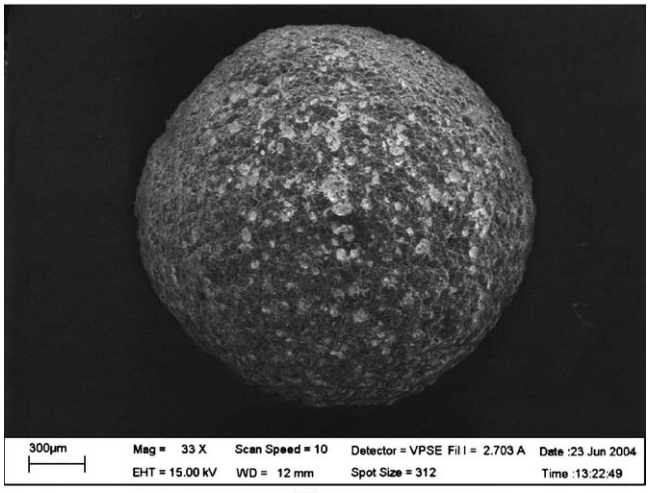
unknown concentrations of chitosan in the course of technical operations where chitosan is consumed, for instance during the preparation of polyelectrolyte complexes (van der Merwe et al., 2004). A formulation of 5% (w/v) sodium alginate, 5% (w/v) prednisolone, 0.5% (w/v) calcium chloride and 0.5% (w/v) chitosan was used to study the effect of the amount of chitosan able to be attached around the alginate/predinosolone core depending on the preparation method one or two. Fig. 3 shows the amount of chitosan bound to the microparticles. These results reveal that the particle prepared by the one-step method has significantly less chitosan binding (1.54%, w/w) to its alginate core compared to those prepared by the two-step method (3.71%, w/w). These results can be explained by the fact that when the one-step method is used, both the positively charged chitosan molecules and the positively charged calcium ions are competing with the negative charges on the surface of the alginate core. Using the two-step method firstly the calcium ions can crosslink the alginate core surface. In the second step, the chitosan molecules can attach themselves to the left positive charge of the alginate core and the adhering chloride ions at the particle surface. By means of this second step process a thicker and well packed chitosan layer around the alginate particle is formed in comparison to the one-step method where the chitosan molecules retain their flexibility.

3.2. Surface morphology of the microparticles

The SEM analysis revealed that the microparticles prepared in this study were mostly spheres with rough surfaces. Fig. 4 shows the appearance of the white spots on the surface of alginate/chitosan microparticles when increasing the concentration of prednisolone from 5 to 10% (w/v). Presumably, those spots represent predinisolone crystal precipitation at the particle surface. However, this drug precipitation could only be seen at the sodium alginate particle's surface, whereas the particles coated with chitosan either with the one-step method or with the two-step method did not show prednisolone precipitation.



(a)



(b)

Fig. 4. SEM micrograph of a microparticle prepared with 2% (w/v) sodium alginate, 0.5% (w/v) $CaCl_2$, and 0.5% (w/v) chitosan; (a) 5% (w/v) prednisolone and (b) 10% (w/v) prednisolone.

3.3. In vitro release behavior of the microparticles

The in vitro release profiles of prednisolone from microparticles containing 2% (w/v) sodium alginate, 5 and 10% (w/v) prednisolone and 1% (w/v) of chitosan prepared by the one-step and the two-step method were investigated in phosphate buffer pH 6.8. Fig. 5 showed that after 4 h, the release of prednisolone from the microparticles with the prednisolone loading of 5 and 10% (w/v) were 79 and 63% for the one-step method, respectively, and 88 and 57% for the two-step method, respectively. At pH 6.8, the alginate/chitosan microparticles containing prednisolone were swollen but did not dissolve. The results suggested that the microparticles containing 5% (w/v) prednisolone exhibited faster water uptake and swelling of the microcores than those containing 10% (w/v) prednisolone in the formulations. Independent of the preparation method the microparticles showed controlled release of prednisolone. At a loading of 5% (w/v) prednisolone 75–80% was released after 4 h. With a loading of 10% (w/v) about 60% of the drug was released at the same time.

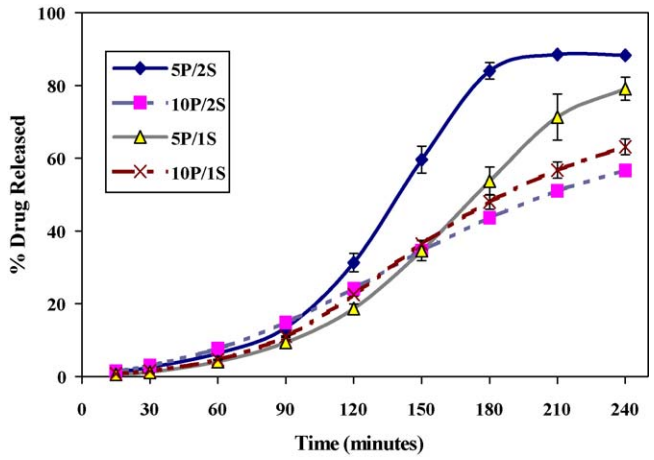


Fig. 5. The release profiles of prednisolone from microspheres prepared from 2% (w/v) sodium alginate and 1% (w/v) chitosan; (5P/2S = 5% (w/v) prednisolone with two-step; 10P/2S = 10% (w/v) prednisolone with two-step; 5P/1S = 5% (w/v) prednisolone with one-step; 10P/1S = 10% (w/v) prednisolone with one-step).

3.4. In vitro mucoadhesive properties of the microparticles

The in vitro mucoadhesive test was carried out using the formulation of 5% (w/v) sodium alginate, 5% (w/v) prednisolone, 0.5% (w/v) calcium chloride, and various concentrations of chitosan. The results of in vitro mucoadhesive tests are shown in Fig. 6 where the particles prepared by the one-step method exhibited excellent mucoadhesive properties after 1 h. It should be mentioned that the pressure on the particles to adhere at the gut wall is not realistic for the in vivo situation and the results are only for the purpose of comparison of different formulation to reveal some basic knowledge for the mucoadhesion behavior of the particles. This study revealed that preparing alginate/chitosan microparticles containing prednisolne by the one-step method produced particles with sufficient amount of

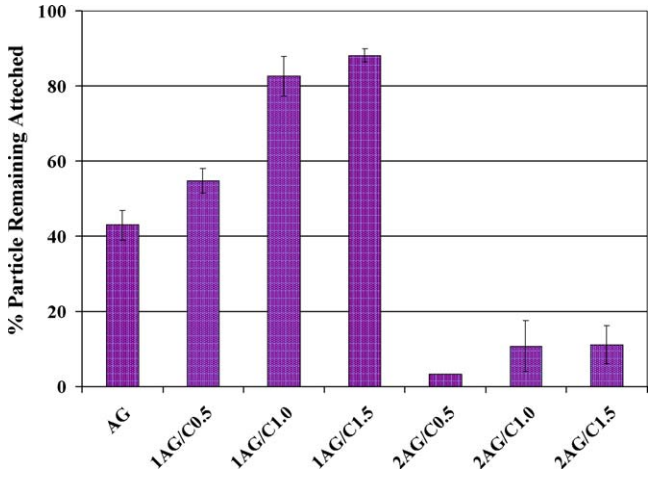


Fig. 6. The in vitro mucoadhesive tests of microparticles (AG = alginate, 1AG/C0.5 = alginate with 0.5% chitosan, 1AG/C1.0 = alginate with 1.0% chitosan, 1AG/C1.5 = alginate with 1.5% chitosan prepared by the one-step method; 2AG/C0.5 = alginate with 0.5% chitosan, 2AG/C1.0 = alginate with 1.0% chitosan, 2AG/C1.5 = alginate with 1.5% chitosan prepared by the two-step method).

chitosan on the surface of the alginate particle cores. Blank calcium alginate microparticles without a chitosan coating exhibited by themselves some mucoadhesive properties as 43% of the particles remained attached to the mucosal surface after 1 h. The one-step coating of the particles with 0.5, 1.0 and 1.5% (w/v) of chitosan enhanced the mucoadhesive properties to 52, 82 and 88% of the particles remaining attached after 1 h, respectively. Increasing the chitosan concentration to more than 1.5% (w/v) resulted in a too highly viscous solution not suitable for a good coating of the alginate core of the particles.

On the other hand, the microparticles containing prednisolone prepared by the two-step method exhibited poor mucoadhesive properties with chitosan concentration from 0.5, 1.0 to 1.5% (w/v) resulting in 3, 11 and 11% of the particles remaining attached in the isolated gut after 1 h, respectively.

There are several theories described in the literature that might explain the mechanism of mucoadhesion between adhesive materials and mucin. They included the theory of electrostatic adsorption (van der Waals, hydrogen bonds), wetting, diffusion and fracture theories (Mortazavi and Smart, 1995; Burjak et al., 2001; Dobrozsi et al., 1999). Several in vitro and in vivo tests were designed to evaluate the mucoadhesiveness, such as the rinsing method developed by Rangna Rao and Buri (1989), the measurement of detachment forces in vitro and measurements of the GI transit using radio-opaque microparticles, gamma scintigraphic technique, and the measurements of the residence time of particles in the isolated intestinal loop in rats. Most of the studies showed that the prerequisite for a good mucoadhesiveness of a polymer is its high flexibility of its polymer backbone structure and of its polar functional groups. Such a flexibility of the polymer chains, however, is reduced if the polymer molecules are crosslinked either with each other or with coagulation agents like calcium ions. Those microparticles may show poor mucoadesiveness. This situation may be the case with the two-step preparation methods where the chitosan molecules forming a wall around the alginate core are tightly bound with each other and the calcium ions. Another situation prevails at the one-step method: here both chitosan molecules and calcium ions are competing which each other at the same time with the negatively charged groups of the alginate molecules and this competition may result in that chitosan molecules are only slightly bound and hence keep their flexibility when the particles are suspended in aqueous milieu. As a result of this, they are able to interact with the mucus chains and show good mucoadhesiveness. This is supported by a study of Huang et al. (2000) demonstrating that the adhesive capabilities of hydrogels can be improved by tethering of long flexible chains to a particle surface. The resulting hydrogels exhibit increased mucoadhesive properties due to enhanced anchoring of the flexible chains with the mucosa.

4. Conclusion

This work demonstrates the effects of formulation and process variables on particle size, drug content and drug release and especially on the mucoadhesiveness of microparticles made from alginate as a core and chitosan as the outer coating. The

in vitro mucoadhesive study revealed that the one-step method is advantageous to fabricate alginate/chitosan microcapsules with excellent mucoadhesive properties for colonic drug delivery whereas the two-step method is not. More research will be necessary to elucidate the mechanisms of chitosan particle coating, however the important message of this investigation is that changes in the manufacturing process of such chitosan coated alginate particles can result in strong and even unexpected changes in the particle's surface properties. In order to obtain an effective delivery system for colon delivery, these microparticles have to be incorporated in a suitable drug carrier which releases the microparticles in the colon with intact mucoadhesive properties.

Acknowledgements

This work was supported by a grant from the National Metal and Materials Technology Center (MTEC), National Science and Technology Development Agency of Thailand. Special thanks to Professor Hans E. Junginger from Faculty of Pharmaceutical Sciences, Naresuan University for his valuable advice in preparing this manuscript.

References

Burjak, M., Bogataj, M., Velnar, M., Grabnar, I., Mrhar, A., 2001. The study of drug release from microspheres adhered on pig vesical mucosa. Int. J. Pharm. 224, 123–130.

Calabresi, P., Chanber, B.A., 1992. Chemotherapy of neoplastic disease. In: Hardman, J.G., Limbird, L.E., Perry, B.M., Ray-mond, W.R. (Eds.), Goodman and Gilman's The Pharmacological Basis of Therapeutics, ninth ed. McGraw-Hill, pp. 1225–1232.

Craig, D.Q.M., Tamburic, E., 1997. Dielectric analysis of bioadhesive gel systems. Eur. J. Pharm. Biopharm. 44, 61–70.

Daly, M.M., Knorr, D., 1988. Chitosan-alginate complex coacervate capsules: Effect of calcium chloride, plasticizers, and polyelectrolytes on mechanical stability. Biotechnol. Prog. 4, 76–81.

Dobrozsi, D.J., Smith, R.L., Sakr, A.A., 1999. Comparative mucoretention of sucralfate suspensions in an everted rat esophagus model. Int. J. Pharm. 189, 81–89.

Fiebrig, I., Harding, S.E., Davis, S.S., 1994. Sedimentation analysis of potential interactions between mucins and a putative bioadhesive polymer. Prog. Colloid Polym. Sci. 94, 66–73.

Fiebrig, I., Harding, S.E., Rowe, A.J., Hyman, S.C., Davis, S.S., 1995. Transmission electron microscopy on pig gastric mucin and its interactions with chitosan. Carbohydr. Polym. 28, 239–244.

Gaserod, O., Smidsrod, O., Skjak-Braek, G., 1998. Microcapsules of alginate-chitosan—I: A quantitative study of the interaction between alginate and chitosan. Biomaterials 19, 1815–1825.

Gomez, W.R., Wee, S.F., 1998. Protein release from alginate matrices. Adv. Drug Deliv. Rev. 31, 267–285.

Huang, Y., Leobandung, W., Foss, A., Peppas, N.A., 2000. Molecular aspects of muco- and bioadhesion: tethered structures and site-specific surfaces. J. Control. Release 65, 63–71.

Huguet, M.L., Dellacherie, E., 1996. Calcium-alginate beads coated with chitosan: Effect of the structure of encapsulated materials on their release. Process Biochem. 31, 745–751.

Kinget, R., Kalala, W., Vervoort, L., Mooter, G.V., 1998. Colonic drug targeting. J. Drug Target. 6, 129–149.

Mi, F.L., Sung, H.W., Shyu, S.S., 2002. Drug release from chitosan-alginate complex beads reinforced by a naturally occuring crosslinking agent. Carbohydr. Polym. 48, 61–72.

Mortazavi, S.A., Smart, J.D., 1995. An investigation of some factors influencing the in vitro assessment of mucoadhesion. Int. J. Pharm. 116, 223–230.

Muzzarelli, R.A.A., 1998. Colorimetric determination of chitosan. Anal. Biochem. 260, 255–257.

Rangna Rao, K.V., Buri, B.A., 1989. Novel in situ method to test polymers and coated microparticles for bioadhesion. Int. J. Pharm. 52, 265–270.

Takeuchi, H., Yamamoto, H., Nuwa, T., Hino, T., Kawashima, Y., 1996. Enteral absorption of insulin in rats from mucoadhesive chitosan-coated liposomes. Pharm. Res. 13, 896–900.

van der Merwe, S.M., Verhoef, J.C., Kotze, A.F., Junginger, H.E., 2004. N-trimethyl chitosan chloride as absorption enhancer in oral peptide drug delivery. Development and characterization of minitablet and granule formulations. Eur. J. Pharm. Biopharm. 57, 85–91.

Vandenberg, G.W., De La Noue, J., 2001. Evaluation of protein release from chitosan-alginate microcapsules produced using external or internal gelation. J. Microencap. 18, 433–441.