The Influence of Chitosan on *in Vitro* Properties of Eudragit RS Microspheres

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Eudragit RS microspheres containing chitosan hydrochloride were prepared by the solvent evaporation method using acetone/liquid paraffin solvent system and their properties were compared with Eudragit RS microspheres without chitosan, prepared in our previous study. Different stirring rates were applied (400—1200 rpm) and drug content, Higuchi dissolution rate constant, surface and structure characteristics of the microspheres were determined for each size fraction. An increase in average particle size with a reduction of stirring rate appeared in limited interval in both series. The average particle size of microspheres without chitosan, prepared at the same stirring rate, was smaller. Pipemidic acid content increased with increasing fraction particle size, but not with increasing stirring rate as it was observed for microspheres without chitosan. We presume that high pipemidic acid content in larger microspheres is a consequence of cumulation of undissolved pipemidic acid particles in larger droplets during microspheres preparation procedure. Pipemidic acid release was faster from microspheres with chitosan and no correlation between Higuchi dissolution rate constant and stirring rate or fraction particle size was found, though it existed in the system without chitosan. Structure and surface characteristics of microspheres observed by scanning electron microscope (SEM) were not changed significantly by incorporation of chitosan. But in contrast with microspheres without chitosan, the surface of chitosan microspheres was more porous after three hours of dissolution. It is supposed that the influence of particle size fraction and stirring rate on release characteristics is expressed to a great extent through porosity and indirectly through total effective surface area, but the incorporation of highly soluble component *i.e.* chitosan salt hides these effects on drug release. In conclusion, changes in biopharmaceutical properties due to varying stirring rate and fraction particle size exhibited the same direction as those reported for the microspheres without chitosan, although they are less expressed because of increased experimental variability, likely caused by chitosan.

Key words microsphere; chitosan; solvent-evaporation method; stirring rate; pharmaceutical property

Numerous microspheres preparation methods and materials, which can be incorporated in microspheres, enable precise optimisation of controlled release in different physiological conditions. Due to microscopic size, microspheres can be used alone or incorporated in other drug delivery systems and are suitable for various routes of application.

Chitosan is a linear polysaccharide consisting of β-1,4 linked monomers of D-glucosamine and *N*-acetyl-D-glucosamine. It dissolves in aqueous media at acidic pH, as polyelectrolyte with high positive charge density, but is insoluble in most organic solvents.[1,2] There exist several techniques which enable incorporation of chitosan in microspheres. In a review of Kaş[2] the following procedures are presented: ionotropic gelation, extrusion/spheronization, spray drying method, multiple emulsion method, solvent evaporation technique and precipitation/coacervation method. Chitosan microspheres are frequently prepared by the solvent evaporation[3,4] and other methods[5] using crosslinking reaction with glutharaldehyde. In these microspheres chitosan is a matrix polymer, where the degree of crosslinking may affect its bioadhesive properties and release of the drug. A modified solvent evaporation method for chitosan microspheres preparation avoiding crosslinking reaction was also developed.[6] Furthermore, in our previous studies, uncrosslinked chitosan hydrochloride was incorporated in microspheres as an additional mucoadhesive component. Microspheres were prepared by the solvent evaporation method and matrix polymer was Eudragit RS.[7,8]

The stirring rate of emulsion system is one of the frequently studied process parameters in solvent evaporation procedure. The effect of this parameter on biopharmaceutical properties of microspheres containing drug and matrix polymer was often observed.[9—11] However, the presence of other components, which are incorporated to attain site-specific delivery and controlled release, could also influence microsphere properties and could affect process parameters.

In the scope of our work we are preparing mucoadhesive microspheres for local intravesical administration. Chitosan is one of the components which we use to achieve mucoadhesivity of microspheres. After the instillation into urinary bladder microspheres are expected to adhere on the surface of vesical mucosa and release the drug for a prolonged time interval independently of micturition. This drug delivery system could be used in treatment of severe infections in urinary bladder. Pipemidic acid was chosen as a model drug because it has suitable physico-chemical properties and is also frequently used in systemic treatment of urinary tract infections. To prepare microspheres with controlled properties, all parameters during preparation procedure have to be well defined and the present article represents a contribution to better knowledge of the system we use.

The purpose of this work was to determine how the stirring rate of emulsion system during solvent evaporation procedure affects the physico-chemical and biopharmaceutical properties of microspheres: particle size, drug content, release rate and kinetic, morphology and structure of the microspheres. It was also examined how these properties depend upon particle size. For this purpose, two series of microspheres were prepared by the solvent evaporation method using system of liquid paraffin/acetone solvents. Micro-

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spheres from the first series consisted of following components: matrix polymer (Eudragit RS), model drug (pipemidic acid) and droplets stabilizers (magnesium stearate and Span 80). The results were presented in article, published recently.[12] In the present work we prepared a series of microspheres, which included a bioadhesive component chitosan; the other substances and their amounts were the same as in the first series. The microspheres were prepared by the same method and under the same conditions as microspheres without chitosan. This article presents the results obtained from a series of bioadhesive microspheres and a comparative analysis of *in vitro* properties of both types of microspheres.

Experimental

Materials The following materials were used. Pipemidic acid trihydrate and magnesium stearate were a gift from Lek, d.d. Slovenia. Chitosan hydrochloride (Protasan Cl 212, viscosity 82mPas, deacetylation 73%) was produced by Pronova, Norway and Eudragit RS by Röhm GmbH, Germany. Span 80 was purchased from Aldrich, Germany. Other solvents and substances used for preparation and evaluation of microspheres were of analytical grade. Pipemidic acid was used in anhydrous form obtained by drying.

Preparation of Microspheres Microspheres were prepared by w/o emulsification and solvent evaporation technique. Eudragit RS 100 (1.5 g) was dissolved in acetone (5 ml). Chitosan hydrochloride (0.5 g) was dispersed in Eudragit RS solution and the mixture was stirred in a water bath on magnetic stirrer at 5 °C for 10 min. Then a dispersion of pipemidic acid (0.5 g) and magnesium stearate (0.2 g) in acetone (3 ml), which had been stirred on magnetic stirrer at room temperature for 5 min, was added to the polymer dispersion. A resulting mixture—the inner phase—was stirred under unchanged conditions for 5 min. Then it was rapidly poured into the continuous phase consisting of Span 80 (0.4 g) and liquid paraffin (80 ml), previously cooled to 5 °C, during stirring at the selected rate (400, 600, 800, 1000 or 1200 rpm). The temperature of the emulsion system was gradually increased to 40 °C and maintained at 40 °C for 40 min, so that the solvent could evaporate. The solidified microspheres were filtered, washed with *n*-hexane and dried under vacuum at room temperature overnight. All experiments for microspheres evaluation were performed next day.

Sieve Analysis The obtained microspheres were sieved through screens with the following mesh sizes: 500, 400, 315, 250, 200, 160, 125, 100 and 80 μm. Sifting time was 15 min. Average particle diameters and size distributions were determined by sieve analysis. The selected fractions: 80—100, 100—125, 125—160, 160—200, 200—250 and 250—315 μm were used for further evaluations.

Drug Content Determination Pipemidic acid content was determined for each size fraction separately. Approximately 10 mg of accurately weighed microspheres were dispersed in 25 ml of methanol and stirred for 40 min. The methanol dispersion was filtered and 200 μl of filtrate dried in a vacuum drier. The residue after drying was dissolved in PBS (phosphate buffer saline, pH 7.4, Ph.Eur.3rd). Drug concentration was determined spectrophotometrically at 330 nm.

Dissolution Studies Drug release tests were performed according to USP XXIV paddle method for each size fraction separately. Accurately weighed amounts (45—65 mg) of microspheres were introduced into 1 l of PBS (phosphate buffer saline, pH 7.4, Ph.Eur.3rd) and stirred with 100 rpm at 37 °C. Six milliliters samples were withdrawn and filtered at selected time intervals. The concentrations of pipemidic acid were determined spectrophotometrically at 330 nm.

Electron Microscopy Surface and shape characteristics were evaluated by scanning electron microscope. The microspheres were carbon-coated and additionally sputtered with gold. Samples were examined by scanning electron microscope (JEOL JSM 5800) at accelerating voltage 14 kV using secondary electron imaging.

The following microsphere size fractions were examined: 80—100 μm (stirring rate: 800 rpm), 125—160 μm (stirring rate: 400, 800, 1200 rpm) and 200—250 μm (stirring rate: 800 rpm). Microsphere size fractions of 125—160 μm (stirring rate: 400 and 1200 rpm) were evaluated by scanning electron microscope also after 3 h of dissolution test.

Results and Discussion

Microspheres containing matrix polymer Eudragit RS, bioadhesive component chitosan hydrochloride, droplet stabilizer magnesium stearate and pipemidic acid as model drug were prepared by the solvent evaporation technique using acetone/liquid paraffin solvent system. Microspheres were prepared at 400, 600, 800, 1000 and 1200 rpm stirring rate. All batches of microspheres were spherical, free-flowing particles and no agglomeration was observed. The recoveries of microencapsulation were 73 to 88%; comparing to microspheres without chitosan the loss of product was slightly higher. The same *in vitro* propeties of all microspheres were determined, except of the batch prepared at 1200 rpm which was analyzed only by a scanning electron microscope.

Effect of Different Stirring Rates on Particle Size Average particle sizes of microspheres prepared at different stirring rates are shown in Fig. 1. As can be seen from results obtained at stirring rates 600, 800 and 1000 rpm, the average particle size reduces with increasing stirring rate. To explain this phenomenon, the following mechanism was suggested[9—12]: by increasing the shear force, which disperses the inner phase into a finer initial emulsion and at the same time prevents agglomeration of "immature" microspheres more successfully, smaller microspheres are formed. On the other hand, the average particle size of both chitosan microspheres and microspheres without chitosan[12] prepared at 400 rpm was smaller than expected according to the trend obtained from the values at higher stirring rates. This indicates that the increase of average particle size with reduction of stirring rate is limited in investigated systems.

Chitosan microspheres had a bigger average diameter than microspheres without bioadhesive component prepared at the same stirring rates.[12] Chitosan, which is dispersed in acetone in the form of particles, increases the total amount of solid substances dispersed in the same volume of the inner phase. Therefore, chitosan microspheres which are formed from droplets of the same size in acetone/liquid paraffin emulsion have a greater average diameter than microspheres without chitosan. Similar results were obtained when the total solid content of the inner phase has been increased by a dissolved polymer,[9] although in the latter case the increase of particle size was probably a consequence of a greater viscosity of the inner phase.

The statistical analysis was performed by one-way ANOVA. The influence of the stirring rate on chitosan microspheres' diameter was found to be non-significant ($p=0.31$), while for microspheres without chitosan this effect was significant. The values for relative standard deviations (average RSD: for microspheres with chitosan 12.7% and without chitosan 6.3%) indicate that the incorporation of chitosan increased experimental variability and for this reason

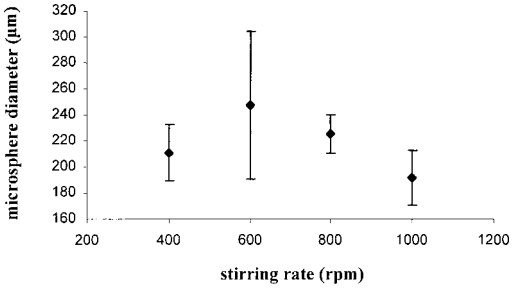

Fig. 1. Effect of Stirring Rate on Average Particle Size and Standard Deviation of Chitosan Microspheres

Means of at least three experiments are superimposed.

the influence of stirring rate on particle size of chitosan microspheres could not be confirmed statistically.

Effect of Different Stirring Rates on Drug Content The drug content was determined for all selected size fractions of microspheres prepared at different stirring rates. Results are shown in Fig. 2. The drug content (9—21%) increases with an increasing particle size for each sample of microspheres prepared at different stirring rates; such trend was also seen at microspheres without chitosan.[12] Furthermore, drug content determined for the biggest size fractions was higher than theoretical drug content in both series, although this effect was more expressed in the system without chitosan. Significance of the influence of particle size on drug content was also statistically confirmed (analysis of variance (ANOVA), $p<0.001$)). We propose a similar explanation for the observed phenomenon as described for the microspheres without chitosan[12]: a diffusion of pipemidic acid and a loss of magnesium stearate[13] from the inner phase of initial emulsion and a cumulation of undissolved particles of pipemidic acid and chitosan in larger microspheres.

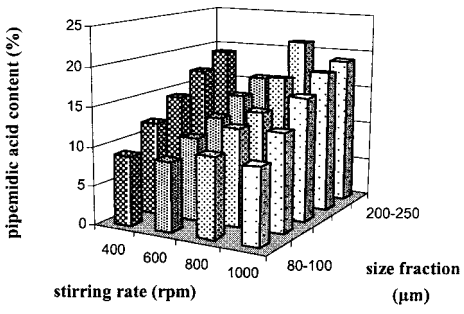

Fig. 2. The Dependence of Pipemidic Acid Content in Chitosan Microspheres on Their Fraction Particle Size and on the Stirring Rate Used for Preparation of Microspheres

Size fractions 80—100, 100—125, 125—160, 160—200 and 200—250 μm were tested. Means of six to eight determinations are superimposed.

On the other hand, the correlation between stirring rate and drug content, which exists at microspheres without chitosan,[12] can not be observed in this series (Fig. 2), although ANOVA shows significant influence of stirring rate on pipemidic acid content ($p<0.001$).

The drug content for microspheres with and without chitosan[12] was also compared (Fig. 3). The highest values of drug content for microspheres with chitosan were significantly smaller than the values for microspheres without chitosan at the same size fraction and stirring speed, the difference was increasing with an increasing size fraction and stirring rate. According to proposed hypothesis,[12] microspheres with the highest values of drug content contain crystals of pipemidic acid. In inner phase of system with chitosan not only crystals of pipemidic acid but also insoluble chitosan particles were dispersed. We presume that greater droplets are formed by more intensive incorporation of both, chitosan and pipemidic acid particles. That is why greater chitosan microspheres, which are formed from droplets with higher amount of particles, contain less pipemidic acid than microspheres of the same size without chitosan. As seen on Fig. 3, the significant difference in drug content appears in fraction sizes 200—250 and 160—200 μm which confirms the upper hypotheses.

Effect of Different Stirring Rates on Drug Release During *in vitro* dissolution test 81—100% of pipemidic acid

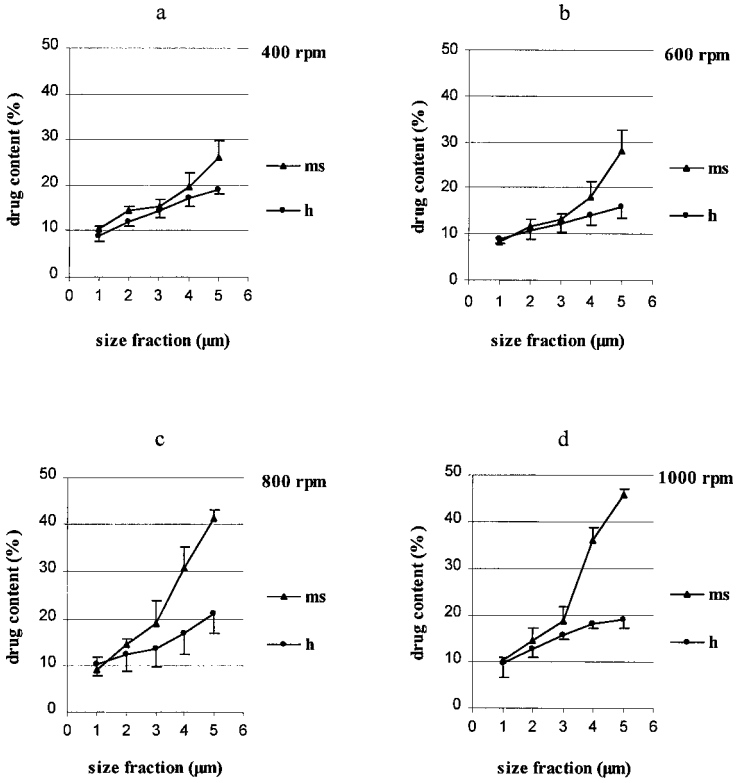

Fig. 3. The Dependence of Pipemidic Acid Content in Microspheres without Chitosan (ms) and in Chitosan Microspheres (h) on Particle Size

Size fraction: (1) 80—100 μm, (2) 100—125 μm, (3) 125—160 μm, (4) 160—200 μm, (5) 200—250 μm; for microspheres prepared at stirring rates: (a) 400 rpm, (b) 600 rpm, (c) 800 rpm and (d) 1000 rpm. Means and standard deviations of six to eight determinations are shown.

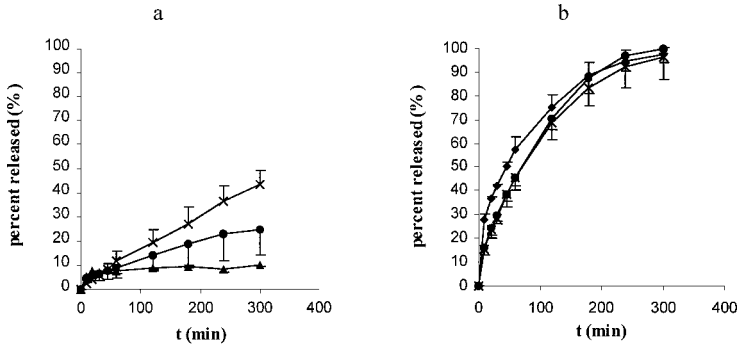

Fig. 4. Drug Release Profiles from Microspheres (a) without Chitosan and from Microspheres (b) with Chitosan

Typical shapes of dissolution curves are presented. Means and standard deviations of six to eight determinations are shown. ×: 1200 rpm (a), or 1000 rpm (b) and 200—250 μm; ●: 800 rpm, 125—160 μm; ▲: 400 rpm, 80—100 μm.

content was released in 300 min from all selected size fractions of chitosan microspheres prepared at different stirring rates. The shape of all dissolution curves was parabolic (Fig. 4b).

Since matrix structure of microspheres was supposed and no significant swelling was observed, profiles were fitted to Higuchi model in the interval from 20 to 90% of released pipemidic acid. Values of the correlation coefficients, which evaluate the fitting of chosen model with experimental data, were in the range of 0.968 to 0.997. Higuchi constants were in the range of 5 to 7 $min^{-1/2}$ and no significant differences due to varying stirring rate or particle size were observed.

As it is evident from Fig. 4, the drug release profiles from microspheres with and without chitosan differ considerably. For the microspheres without chitosan the amount of released pipemidic acid in 300 min ranged from 5 to 52%. Dissolution curves exhibited linear (greater size fractions and higher stirring rates) or parabolic shape (smaller size fractions and lower stirring rates) and burst effect was observed. Higuchi constants (0.1—3 $min^{-1/2}$) increased with increasing stirring rate and particle size, which was explained with larger total effective surface area seen on scanning electron micrographs where bigger microspheres prepared at higher stirring rates had greater porosity.[12] Considering the composition of microspheres with and without chitosan, which is very similar, we assume that evident changes in release (shape of dissolution curve and rate of release) appeared due to chitosan. Chitosan was incorporated as a hydrochloride salt, which dissolves in phosphate buffer saline. No significant swelling of microspheres during dissolution procedure was observed. Swelling of microspheres is almost completely prevented by Eudragit RS matrix, because this polymer is insoluble in aqueous media and exhibits very low degree of swelling. However, chitosan hydrochloride might swell on the surface of microspheres, but we suppose that it dissolves relatively fast and does not contribute to the swelling of microspheres significantly. Due to the dissolution of chitosan on the surface and inside the particles, matrix structure of chitosan microspheres became more porous, diffusion from microspheres was facilitated and release of pipemidic acid was accelerated. Additionally, the wettability of microspheres is probably increased by chitosan which might increase the dissolution rate, especially in early phase of dissolution process. On the contrary, matrix of microspheres without chitosan represented only polymer Eudragit RS, which exhibits low swelling and permeability. As expected, scanning electron microscope (SEM) showed hardly any changes in porosity of microspheres without chitosan after 3 h of dissolution test,[12] whereas the surface of microspheres with chitosan seems to be changed. We suppose that differences in the surface of chitosan microspheres, which are well seen on Fig. 5, are due to very small pores found after dissolution of chitosan.

Therefore, it is presumed that in the tested systems the influence of particle size fraction and stirring rate on release characteristics is expressing mostly through porosity and thus total effective surface area. Incorporation of another soluble component (chitosan) could change structure of microspheres and increase the porosity during dissolution and thus covers the described influences on drug release.

Effect of Different Stirring Rates on the Surface and Structure of Microspheres The influence of stirring rate on the surface and structure of microspheres was studied on scanning electron micrographs of microspheres prepared at stirring rates 400 and 1200 rpm, fraction size 125—160 μm (Figs. 5a, b). The differences were obvious. Microspheres prepared at stirring rate 1200 rpm were mainly hollow (on Fig. 5b only one hollow microsphere is seen, but many of them can be observed at lower magnifications) with relatively smooth surface, while microspheres prepared at stirring rate 400 rpm were seldom hollow and had rough surface thickly covered with small flat particles.

Dependence of surface and structure of microspheres prepared at 800 rpm on their size was also seen on SEM (Fig. 6). In contrast to small microspheres (80—100 μm), the microspheres of the size fraction 200—250 μm were mainly hollow.

No significant differences in surface and structure between microspheres prepared with and without chitosan at the same preparation conditions and of the same size fractions were observed. We assume that this is a consequence of the same mechanism, involved in their formation. Hypothesis which explains hollow microspheres in greater size fractions was proposed in our previous article[12]; solidification of bigger emulsion droplets is non-uniform and starts by forming the wall, thus for further evaporation from the inside, wall must be broken.

This process could also explain hollow structure of microspheres produced at higher stirring rates. Faster agitation causes faster evaporation of acetone from liquid paraffin and diffusion of acetone from the emulsion droplets into liquid paraffin. Thus, at higher stirring rates there is a greater possi-

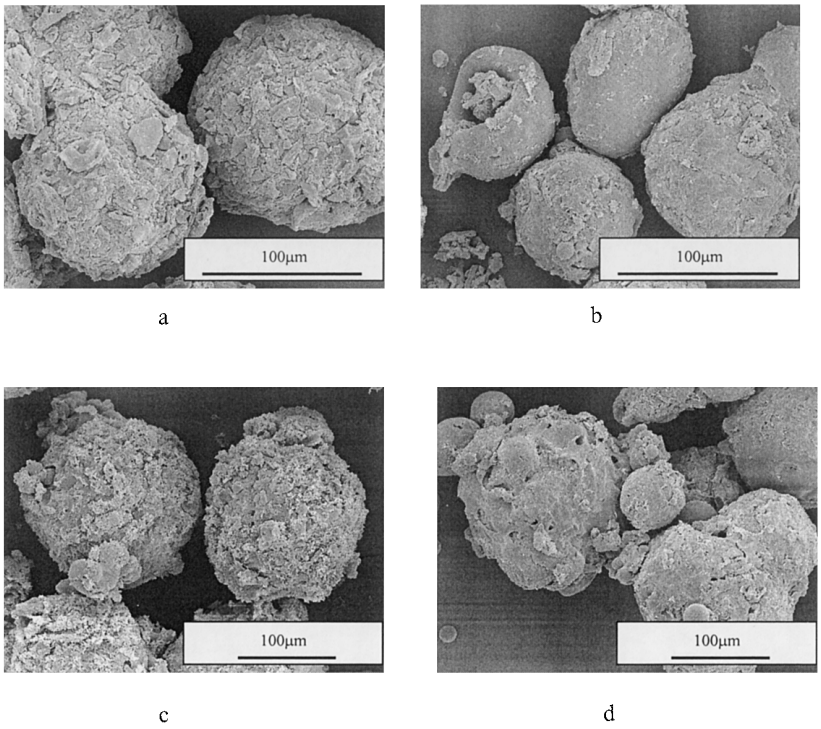

Fig. 5. Scanning Electron Micrographs of Chitosan Microspheres, Size Fraction 125—160 μm, Prepared at Stirring Rates: (a) 400 rpm, (b) 1200 rpm, (c) 400 rpm—after 3 h of Dissolution Test, (d) 1200 rpm—after 3 h of Dissolution Test

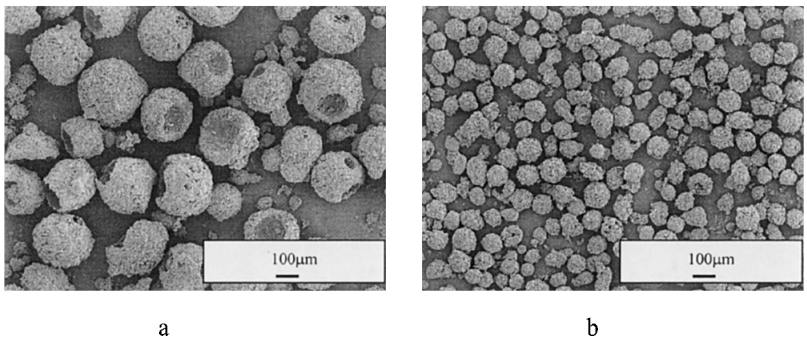

Fig. 6. Scanning Electron Micrographs of Chitosan Microspheres Prepared at Stirring Rate 800 rpm, Size Fraction: (a) 200—250 μm and (b) 80—100 μm

bility that the solidification is not homogeneous and that wall is formed before acetone from the inside of "immature" microspheres evaporates. This could be the reason that hollow microspheres appear more frequently at stirring rate 1200 rpm than 400 rpm.

Particles, which were observed on the surface of both series of microspheres could be chitosan (in the case of chitosan microspheres), magnesium stearate or pipemidic acid; the latter would explain burst effect, which was seen in release profiles of microspheres without chitosan.

Conclusion

Eudragit RS microspheres containing chitosan were prepared by the solvent evaporation method and their properties were compared with Eudragit RS microspheres without chitosan.[12] Evident effect of manufacturing parameter, *i.e.* stirring rate on particle size and surface characteristics of microspheres was found. The dependence of pipemidic acid content and surface characteristics on fraction particle size was also observed. Changes in pharmaceutical properties due to varying stirring rate and fraction size were exhibited in the same direction as those reported for the microspheres without chitosan,[12] although they are less expressed because of increased experimental variability likely caused by chitosan.

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