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Research paper

Chitosan drug binding by ionic interaction

Yaowalak Boonsongrit [a,b], Ampol Mitrevej [a], Bernd W. Mueller [b,*]

[a] Department of Manufacturing Pharmacy, Faculty of Pharmacy, Mahidol University, Bangkok, Thailand
[b] Department of Pharmaceutics and Biopharmaceutics, Christian-Albrechts-University, Kiel, Germany

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Abstract

Three model drugs (insulin, diclofenac sodium, and salicylic acid) with different pI or pKa were used to prepare drug-chitosan micro/nanoparticles by ionic interaction. Physicochemical properties and entrapment efficiencies were determined. The amount of drug entrapped in the formulation influences zeta potential and surface charge of the micro/nanoparticles. A high entrapment efficiency of the micro/nanoparticles could be obtained by careful control of formulation pH. The maximum entrapment efficiency did not occur in the highest ionization range of the model drugs. The high burst release of drugs from chitosan micro/nanoparticles was observed regardless of the pH of dissolution media. It can be concluded that the ionic interaction between drug and chitosan is low and too weak to control the drug release.

Keywords: Chitosan; Ionic interaction; Microparticles; Nanoparticles; Insulin; Diclofenac sodium; Salicylic acid

1. Introduction

Chitosan, a natural polysaccharide, is an *N*-deacetylated derivative of chitin which can be obtained from crustaceans, insects, fungi etc. Several interesting properties of chitosan such as film forming ability, gelation characteristics, bioadhesion properties and penetration enhancing effects which were explained by opening tight junctions of epithelial cells have been reported [1–3]. Moreover, chitosan is a biodegradable and biocompatible polymer and due to its promising properties it has received great attention in the pharmaceutical field as well as in food science and in cosmetic formulations. Within pharmacy it has been developed to microparticles and nanoparticles for encapsulation of drugs and biological substances [4–6]. Due to its polymeric cationic characteristics, chitosan can interact with negatively charged molecules or polymers. Recently, chitosan micro/nanoparticles were formulated by ionic interaction between the cationic chitosan and anionic counterions as tripolyphosphates leading to interpolymer linkages. With this so-called ionic gelation process nano- and microparticles are produced by flocculation of the chitosan polymer [4,7–13]. In these particulate gel beads the cationic chitosan molecule should still have the capability to bind anionic molecules by an ionic reaction. This method of production of drug loaded chitosan nano- and microparticles has gained much attention because it is simple, non-toxic and involves a mixture of aqueous phases at room temperature without using organic solvents.

Based on several publications, the binding or interaction of oppositely charged molecules of chitosan micro/nanoparticles was demonstrated by IR spectra [9,10], X-ray photoelectron spectroscopy [4], and viscosimetry [11]. The release profiles of chitosan micro/nanoparticles prepared by this method showed a high burst effect within half an hour [10,12,13] and a total release of the drug within a short time. This behaviour already shows that the binding properties of chitosans based on an ionic reaction may be poor and that chitosans are not the optimal excipients in formulation technology as may be seen from the literature. The binding properties by ion exchange is shown by the increase in the solubility of indomethacin released from the kneaded mixture and by the solid complex of low molecular weight chitosan and indomethacin. The interaction between the amino group of chitosan and carboxyl group of indomethacin was mentioned because it was the reason for complexation [14]. Especially, the interaction of chitosans and peptides or proteins is often reported as showing a carrier and stabilizer effect [4,7,12,15]. Alonso and co-workers prepared insulin loaded chitosan nanoparticles base on ionic interaction between both moelcules. The loading capacity was up to

* Corresponding author. Department of Pharmaceutics and Biopharmaceutics, Christian-Albrechts-University Kiel, Gutenbergstr. 76, D-24118 Kiel, Germany. Tel.: +49 431 8801333; fax: +49 431 8801352.
E-mail address: bwmueller@pharmazie.uni-kiel.de (B.W. Mueller).

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55%. These chitosan nanoparticles released insulin in active form and enhanced nasal absorption in rabbits [12]. Nasal vaccine delivery by this kind of nanoparticles was also studied. The anti-tetanus IgG levels elicited by tetanus toxoid loaded nanoparticles and the anti tetanus IgA titers detected at 6 months after administration were higher than that of fluid vaccine [7]. The carrier and stabilizer effect is mostly explained by an electrostatical binding of the loaded drug molecule to the cationic chitosan polymer. Due to the fact that polymers with high molecular weights (70–150 kD) showed an immunogenic side effect after parenteral application the commercially produced chitosans cannot be used for parenteral formulations [16,17]. In order to use the advantageous properties of chitosans as excipients for proteins in the parenteral pathway, short chain chitosans were produced from fungus by a biotechnological method [18,19]. It could be shown that the properties of the short chain chitosans (MW below 5 kD, deacetylation degree above 95%) changed completely e.g. they were soluble even in alkaline solutions. Furthermore, and this was a disappointing result of that reseach, these chitosans showed neither a binding effect to negatively charged molecules nor a stabilizing effect to peptides [20].

Concerning the antimicrobial efficacy of high molecular weight chitosans with a high degree of deacetylation it is always reported in the literature that this is a positive side effect of this group of excipients which may be used in drug formulation technology [21–25]. The reason of the antimicrobial efficiency is explained by the binding of the cationic chitosan to the anionic molecules at the outer surface of the bacterial membrane. Jumaa et al. showed, however, that a chitosan in an aqueous solution (1.5%) has no antimicrobial effect [26]. An emulsification of the same aqueous phase leads surprisingly to formulations which showed antimicrobiological stability. This effect was explained by an increased concentration of the chitosan in the interface and an adsorption and agglomeration of the microorganisms and not by an electrostatical interaction with the excipient.

The aim of this study, is to follow the binding efficiency of high molecular chitosans in oder to show their capacity to act as pharmaceutical carrier and stabilizer in drug formulation technology. Due to the fact that from a chemical point of view chitosan represents a weak base, a peptide (insulin), a weak acid (diclofenac), and salicylic acid as a stronger acid were chosen as model drugs.

2. Materials and methods

2.1. Materials

Chitosan (MW 150 kDa, degree of deacetylation 84.5%) was purchased from Fluka (Buchs, Switzerland). Pentasodium tripolyphosphate (TPP) was provided by Sigma (St Louis, USA). Human insulin was generously supplied by Aventis (Frankfurt, Germany). Diclofenac sodium (USP grade) was purchased from Heumann Pharma (Feucht, Germany). Salicylic acid was bought from Kraemer&Martin (Sank Augustin, Germany).

2.2. Preparation of chitosan microparticles

Drug loaded chitosan microparticles were prepared by ionic interaction. A 0.2% w/v solution of chitosan was prepared in 1% v/v acetic acid solution. Tripolyphosphate (0.1% w/v), diclofenac sodium solution (5 mg/ml) and salicylic acid (2 or 3 mg/ml) were dissolved in purified water.

To prepare an insulin solution (5 mg/ml), insulin powder was dispersed in pH 7.4 phosphate buffer solution. Subsequently, the insulin was dissolved by adding 1 N HCl. An equal amount of 1 N NaOH solution was then added to adjust the pH of the solution to pH 7. Finally, the pH 7.4 phosphate buffer was added to the desired volume [27].

The chitosan solution (25 ml) was stirred at 11,000 rpm with a high speed stirrer (Ultraturrax®T25B, Ika-Werke, Staufen, Germany) at room temperature (25 °C). The insulin solution, diclofenac sodium solution or salicylic acid solution was dropped into the chitosan solution (0.75 ml/min). The tripolyphosphate solution (10 ml) was gently added to the system and micro/nanoparticles were formed. For the release study part of the drug-chitosan micro/nanoparticle suspension was washed three times with purified water by filtration method to remove non-entrapped drug in the suspending medium.

To study the effect of the amount of drug on the physicochemical properties of micro/nano-particles, various amounts (2–28 ml) of drugs were used for preparation of the drug-chitosan micro/nanoparticles.

2.3. Characterization of micro/nanoparticles

Measurements of particle sizes were determined by a laser diffractometer (Helos-Partikelgroessnanalysator Windox4, Sympatec, Clausthal-Zellerfeld, Germany) and photon correlation spectroscopy (Zetaplus®,Brookhaven instruments, Vienna, Austria) was used for detection of submicron particles. The samples were diluted at the ratio of 1:2 to 1:12 with purified water before measurement. With laser diffraction analysis, the suspensions were characterized by X_{50} quantiles of the volumetric distribution.

The charge of micro/nanoparticles was expressed in terms of zeta potential by laser doppler anemometry (Zetaplus®, Brookhaven instruments, Vienna, Austria) and the surface charge per volume of suspension by particle charge detector (PCD 03pH, Muetek analytic, Herrsching, Germany). The samples were diluted with purified water. The dilution factor was 1:30 and 1:12, respectively. The particle charge detector (PCD) provided quantitative determination of the charges of particles by measuring the streaming current. The cylindrical test cell with a fitted displacement piston constituted the centerpiece of the PCD. The oscillating piston forced the sample suspension to flow along the plastic wall of the test cell so that the counter-ions were sheared off and the so-called streaming current were generated. The streaming current was immediately detected and the polyelectrolyte with opposite charge to the sample was titrated to the sample until it reached the point of zero charge. The surface charge was calculated

from the titrant consumption. The titrant used to neutralize positive charge was polyethylensulfonate sodium (PES-Na).

The formula for calculation of the surface charge was:

$$Q = \frac{Vc}{m}F$$

Q = surface charge (C/ml); V = consumption (l)
c = titrant concentration (mol/l); m = active substance of sample (ml)
F = Faraday's constant (9.6485×10^4 C/mol)

2.4. Evaluation of drug entrapment efficiency

The amount of drug entrapped in the micro/nanoparticles was calculated by two methods. The first method was the calculation of the difference between the total amount of drug used to prepare the micro/nanoparticles and the amount of non-entrapped drug remaining dissolved in the aqueous suspending medium. Micro/nanoparticles were separated from the suspending medium by filtration using 0.1 μm membrane filter. The aqueous medium was assayed for drug concentration by HPLC. The drug entrapment efficiency of micro/nanoparticles was calculated as indicated below:

Entrapment efficiency:

$$\frac{(\text{Total amount of drug loading} - \text{free drug in supernatant})}{\text{Total amount of drug loading}} 100$$

The second method for evaluation of drug entrapped in the micro/nanoparticles was an estimation of the drug directly in the micro/nanoparticles. This was used in the release studies to calculate the percentage of the released amount of drug. An accurate volume of micro/nanoparticles suspension was incubated in pH 9.6 alkaline borate buffer at 37 °C and agitated at 100 rpm. At predetermined times, after centrifugation, the supernatant was assayed for drug concentration by HPLC. The total amount of drug was considered as the entrapped amount.

2.5. Analysis of drugs

High performance liquid chromatography was used to assay human insulin, diclofenac and salicylic acid. HPLC analyses were performed using a high precision pump (Model 480, Gynkotek, Munich, Germany), a UV detector (HPLC430, Kontron instruments, Bletchey, UK), and a reverse phase column (LiChroCart 125-4, LiChrospher 100 RP-18 (5 μm), Merck, Darmstadt, Germany) and the column temperature was maintained at 20 °C for analysis of insulin and diclofenac and 40 °C for analysis of salicylic acid.

For human insulin the mobile phase was composed of 27% v/v acetonitrile and 73% v/v of buffer (0.01 M KH_2PO_4 and 0.1 M Na_2SO_4, adjusted to pH 3 with H_3PO_4). The flow rate was 1 ml/min. A 100 μl of sample was injected and the eluent was monitored for the absorbance of insulin by UV detector at 215 nm.

For analysis of diclofenac samples of 100 μl were eluted with a mobile phase comprising methanol (60% v/v) and 40% buffer (0.01 M of KH_2PO_4 and Na_2HPO_4, adjusted to pH 6.5 with H_3PO_4). The flow rate was 1 ml/min and the detection wavelength was set at 282 nm.

For analysis of salicylic acid 30% v/v of acetonitrile and 70% v/v of 50 mM KH_2PO_4 (pH 3.0) were used as a mobile phase. The flow rate was 0.7 ml/min. A 100 μl of sample was injected and the detection wavelength was set at 254 nm.

Microparticle enzyme immunoassay (MEIA) was performed to test the immunological activity of human insulin. An automated analyzer (IMx System, Abbott laboratories, Illinois, USA) and Imx insulin reagents were used. Accurate volumes of both washed and unwashed insulin-chitosan micro/nanoparticles suspension were incubated in pH 9.6 alkaline borate buffer at 37 °C and agitated at 100 rpm. The supernatants were taken at predetermined time intervals after centrifugation. The samples were prepared using a similar method to that described by Mueller and Trenktrog [27]. Samples were diluted with 3.5% w/v bovine serum albumin in isotonic phosphate buffer pH 7.4 before analysis.

2.6. In vitro drug release studies

Both washed and unwashed micro/nanoparticles of the formulations composed of 6 ml of model drugs were subjected to in vitro release studies. Micro/nanoparticle suspensions (0.1 ml) were placed into centrifuge tubes containing 1.4 ml of dissolution media and incubated at 37 °C under agitation (100 rpm) (Julabo SW20C, Julabo Labortechnik GmbH, Seelbach, Germany). The dissolution media were pH 7.4 phosphate buffer and pH 3 HCl solution (2.3×10^{-3} N). After centrifugation of the samples (10,000 rpm, 10 min) at predeterminated time intervals, 1 ml samples were withdrawn and replaced by fresh medium. The amount of drug released from the microparticles was analyzed using HPLC as described above.

3. Results and discussion

In this study chitosan micro/nanoparticles based on ionic interaction between drug and chitosan were prepared and characterized. Three model drugs with different pI or pKa were studied (insulin-pI = 5.3, diclofenac sodium-pKa = 4.0 and salicylic acid-pKa = 2.8) because an increase in binding force to chitosan was expected with decreasing pKa. Tripolyphosphate (pH 9.1) was used as a crosslinking agent in order to form microparticles or to strengthen the microparticle formation. From the literature [4] it can be derived that the opposite charge of chitosan and drug cause a spontaneous forming of particles, which are finally improved by adding tripolyphosphate. Furthermore, from literature [15] it is known that increasing chitosan concentrations as well as increasing tripolyphosphate concentrations will lead to increasing particle diameters and at least to agglomeration of the produced microparticles. Similar results are obtained with the preparations under discussion. An example for the insulin

formulation is shown in Fig. 1. Increasing tripolyphosphate concentrations lead to a decrease in zeta potential and hence to an increase in particle size. Due to the fact that the addition of tripolyphosphate is done only to improve particle formation on a low size level the tripolyphosphate concentration was fixed at 0.1% and the chitosan concentration at 0.2%.

3.1. Effect of drug concentration on the physicochemical properties of the chitosan microparticles

For production of insulin-chitosan microparticles a positive charged chitosan solution (pH 5.0) and a negative charged insulin solution (pH 7.2) were used. During production the pH of the microparticle suspensions gradually increased with increasing insulin concentration or addition of tripolyphosphate. The insulin-chitosan microparticles exhibited a particle size (X_{50}) in the range of 1–7 μm. After addition of tripolyphophate solution the particle size shows values between 2 and 5 μm which means no significant change. Increasing amounts of insulin solution have no influence on the size of the microparticles. Fig. 2(A) and (B) illustrate the change in surface charge of insulin-chitosan microparticles expressed as zeta potential and surface charge per volume of suspension both before and after adding tripolyphosphate, respectively insulin solution into the system. Surface charge and zeta potential were decreased with increasing insulin concentration which led to constant values at insulin concentrations over 12 ml (1.27 mg/ml of suspension) in the formulations. It seems that the binding of insulin even in higher concentrations cannot neutralize the positive charge of the chitosan molecule and even the addition of tripolyphosphate did not result in a zero surface charge.

For the formulation of diclofenac-chitosan microparticles the opposite charged chitosan molecules in solution (pH 5) and diclofenac sodium solution (pH 7) were mixed. Almost the same development of the pH course and of the physicochemical properties as already seen with the insulin-chitosan microparticles was obtained except for the change in surface

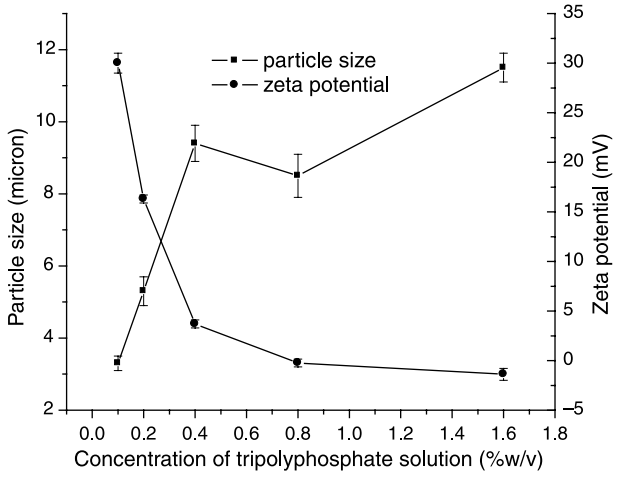

Fig. 1. Influence of tripolyphosphate concentration on particle size and zeta potential of an insulin-chitosan solution (the concentration of insulin and chitosan in the suspension was 0.07 and 0.12% w/v, respectively).

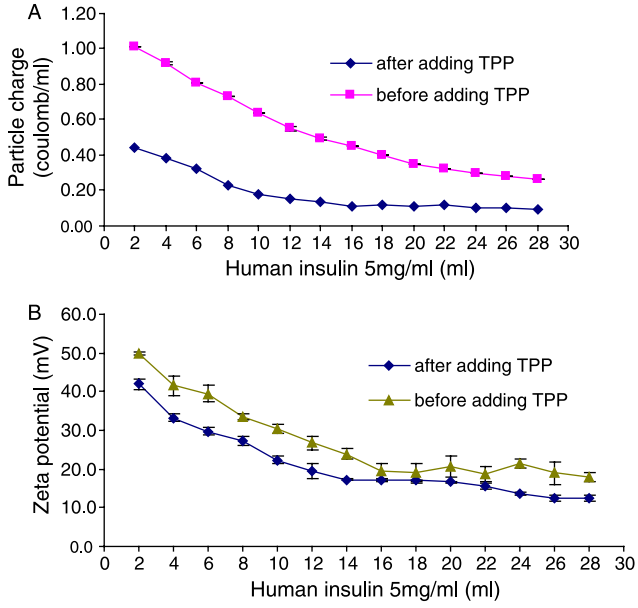

Fig. 2. Particle charge (A) and zeta potential (B) of insulin-chitosan microparticles before and after adding tripolyphosphate with increasing the concentration of insulin ($n=3$).

charge of the microparticles (Table 1 and Fig. 3). The addition of diclofenac solution as well as tripolyphosphate solution did not change the zeta potential of the system significantly. This shows that the colloidal chitosan molecule is not much influenced by the addition of a weak acid. However, the surface charge of the formed microparticles is strongly influenced by the addition of diclofenac sodium or tripolyphosphate.

Mixing chitosan solution (pH 5) with a salicylic acid solution (pH 4) showed no particle formation although a reaction between both components should be expected due to the fact that salicylic acid is a stronger acid. Particles were obtained only after adding tripolyphosphate with a size in the nanometer range. Table 2 shows the pH of the salicylic-chitosan nanoparticles suspensions which were about 5. In fact, at pH 5 most of the salicylic acid is in ionized form and chitosan shows a positive charge. The results in Fig. 4 indicate that there was no marked influence of the salicylic acid concentration on zeta potential and surface charge of

Table 1
pH and particle size (X_{50}) of diclofenac-chitosan microparticles

Volume of diclofenac sodium solution[a] (ml)	PH of suspension		Particle size (μm)[b]	
	Before adding TPP	After adding TPP	Before adding TPP	After adding TPP
2	5.2	5.2	3.6±0.0	4.5±0.1
4	5.2	5.3	4.7±0.0	5.0±0.1
6	5.3	5.3	4.1±0.0	3.5±0.2
8	5.3	5.3	4.2±0.0	3.5±0.1
10	5.3	5.4	3.4±0.1	3.4±0.0
12	5.3	5.4	4.7±0.0	4.1±0.5
14	5.3	5.5	4.3±0.0	4.5±0.2

[a] Diclofenac sodium solution 5 mg/ml.
[b] Mean±SD ($n=3$).

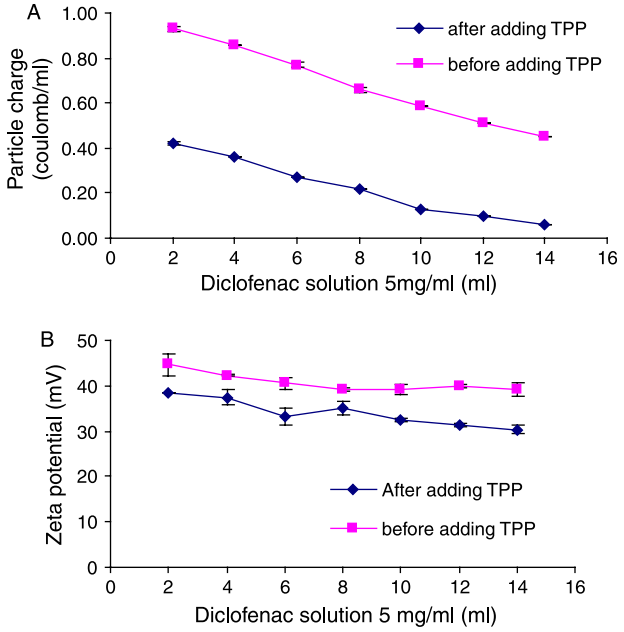

Fig. 3. Particle charge (A) and zeta potential (B) of diclofenac-chitosan microparticles before and after adding tripolyphosphate with increasing the concentration of diclofenac sodium ($n=3$).

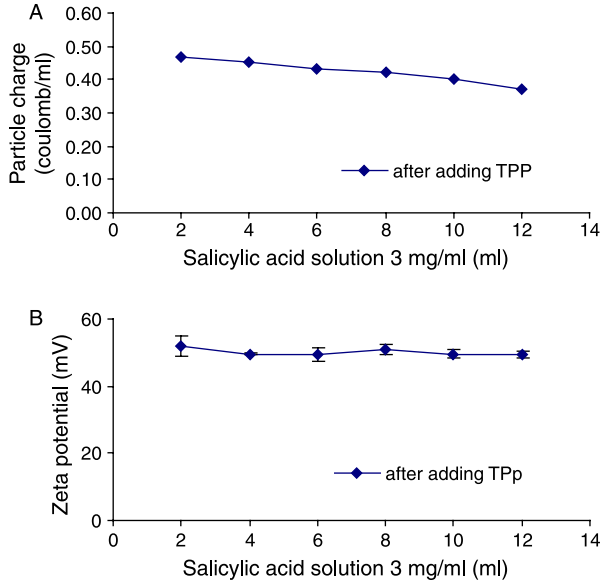

Fig. 4. Particle charge (A) and zeta potential (B) of salicylic acid-chitosan nanoparticles after adding tripolyphosphate with increasing the concentration of salicylic acid ($n=3$).

the chitosan solution after crosslinking with tripolyphosphate and thus particle formation. The zeta potential could not be detected in the formulations without tripolyphosphate solution. The slow decrease of the surface charge itself shows that an unexpectedly low interaction between the moelcules occur and this should lead to low entrapment efficiencies if the entrapment is a function of ionic interaction. Furthermore, the excellent water solubility of salicylic acid in its dissociated form at pH 5 will hinder a higher entrapment because the lack in ionic interaction leads to a decrease in concentration in the polymeric phase.

3.2. The entrapment efficiency of drug-chitosan microparticles

All model drugs in this study were dissolved and adjusted to a pH where most of the molecules were negatively charged before adding to a pH 5 chitosan solution (positive charge). The pH of preparations should allow the model drugs molecule to interact with the chitosan polymer if an ionic interaction exists. By measuring zeta potential and surface charge it could be shown that even at higher counter ion concentration the chitosan polymer has a positive charge. Therefore the negatively charged tripolyphosphate was added in order to complete the microparticle formation.

Table 2
pH and mean particle size of salicylic-chitosan nanoparticles

Volume of salicylic acid solution[a] (ml)	PH of suspension after adding TPP	Particle size (nm)[b] after adding TPP
2	5.2	708.5±35.0
4	5.2	671.4±30.7
6	5.1	699.4±38.1
8	5.1	473.2±20.4
10	5.1	483.7±4.5
12	5.1	476.8±15.1

[a] Salicylic acid solution 3 mg/ml.
[b] Mean±SD ($n=3$).

In the case of insulin microparticles the entrapment efficiency increased with increasing drug concentration, due to incomplete microparticle formation (Table 3). The addition of tripolyphosphate therefore led to a higher entrapment. Equilibrium was reached at 40 mg insulin referring to 50 mg chitosan. The maximum uptake of insulin is reached at 28 ml (=140 mg). Concerning the influence of pH more than 90% of insulin was entrapped in a pH area of 5.8–6.9. This is contrary to the study of Lim and co-workers in which a dominatly high association efficiency was observed only at pH 6.1 [13].

Diclofenac is nearly completely entrapped although the addition of diclofenac did not influence the zeta potential which may indicate that there is no ionic interaction with the chitosan polymer. The particle surface charge was, however, continuously decreased. The addition of tripolyphosphate led to zero charge which is in coincidence with a decrease in entrapment efficiency.

Only a small amount of salicylic acid was encapsulated by the chitosan microspheres which was expected by the charge measurements. Particles in the nano-range are only formed after addition of tripolyphosphate. The small size indicates that only a low amount of drug is integrated in the particle and due to the fact that nearly no change in zeta potential and surface charge is observed no interaction between salicylic acid and chitosan can be assumed at pH 5.

From the Henderson–Hasselbalch equation it can be derived that salicylic acid (pKa=2.8) is in a 99% ionized form if the pH of the system is about two pH units higher than the pKa of the drug. So at pH 5 salicylic acid has a great possibility to bind to chitosan if the ionic interaction really causes entrapment of

Table 3
Entrapment efficiency of drug-chitosan particles made of pH 5 chitosan solution

Volume of drug solutions[a] (ml)	Entrapment efficiency (% ± SD)[b] of				
	Insulin-chitosan microparticles		Diclofenac-chitosan microparticles		Salicylic-chitosan nanoparticles
	Before adding TPP	After adding TPP	Before adding TPP	After adding TPP	After adding TPP
2	0.5 ± 0.4	53.5 ± 2.1	95.0 ± 2.3	98.7 ± 0.1	22.8 ± 1.9
4	10.4 ± 0.9	79.2 ± 1.1	96.9 ± 1.6	98.3 ± 0.3	19.2 ± 1.8
6	55.7 ± 9.8	93.9 ± 1.2	99.5 ± 0.4	96.9 ± 1.0	17.4 ± 1.1
8	91.0 ± 8.4	95.7 ± 1.0	99.0 ± 0.5	97.7 ± 0.3	17.6 ± 0.3
10	97.1 ± 1.0	96.5 ± 0.1	99.5 ± 0.2	95.3 ± 0.4	16.8 ± 1.5
12	95.4 ± 0.8	96.8 ± 0.3	99.2 ± 0.2	90.3 ± 0.9	15.1 ± 0.3
14	98.0 ± 0.3	95.2 ± 0.8	99.3 ± 0.1	84.7 ± 1.1	
28	96.9 ± 1.1	91.1 ± 0.8	87.0 ± 0.4	67.7 ± 1.5	

[a] Insulin solution (5 mg/ml) diclofenac sodium solution (5 mg/ml) salicylic acid solution (3 mg/ml).
[b] $n = 3$.

drug into particles. However, only an entrapment efficiency from 15 to 23% could be reached and this could only be due to physical entrapment. In order to get a more pronounced protonization the pH of the chitosan solution was decreased to pH 3.3 which led indeed to an increased entrapment efficiency of salicylic acid with a maximum of 77% (Table 4). The drug loading of the microparticles decreased, however, with increasing salicylic acid concentration which may be caused by the restricted dissociation of the salicylic acid. Using the pH 3.3 chitosan solution in combination with insulin a limited entrapment efficiency was observed which can be explained by the fact that the solution medium showed a pH below the insulin isoelectric point.

These results indicate that entrapment efficiency could be improved by changing the pH of chitosan solution but the maximum entrapment efficiency did not occur in the highest ionization range of the model drugs. The highest entrapment efficiencies of drugs were achieved when the pH of formulation solution was near the pKa of model drugs. It seems that the ionic interaction between drug and chitosan is not the main mechanism that causes particle formation. The protonated amino group of both pH 3.3 and pH 5 chitosan solution as well as the dissociated functional groups of the model drugs are masked by a water layer. The possibility of an electrostatical interaction is, therefore, low. But because of the preparing conditions (high speed stirring rate and long preparation time) and reducing of electrostatic repulsion by adding counter ions even when the pH of formulation was near pKa of the model drugs a flocculation of the colloidal chitosan in solution occurred. Hence the drug in the microparticle may be partly bound and partly physically entrapped. Due to the fact that at least only some amino groups of the chitosan molecule are involved in an electrostatical binding the addition of tripolyphosphate lead to an increased flocculation and hence to a better microparticle formation.

Table 4
pH and entrapment efficiency (EE) of drug-chitosan particle with tripolyphosphate made of pH 3.3 chitosan solution (mean ± SD, $n = 3$)

Volume of drug solutions[a] (ml)	Insulin-chitosan microparticle suspension		Salicylic acid-chitosan nanoparticle suspension	
	pH	EE (%)	pH	EE (%)
2	3.6	32.1 ± 3.7	3.6	71.6 ± 1.7
4	3.6	23.7 ± 2.2	3.4	77.5 ± 2.8
6	3.5	25.8 ± 0.8	3.4	76.0 ± 4,5
8	3.5	24.6 ± 0.9	3.4	76.0 ± 1,7
10	3.5	18.1 ± 4.3	3.3	67.8 ± 5,3
12	3.5	21.3 ± 0.9	3.3	65.8 ± 0.5

[a] Insulin solution 5 mg/ml salicylic acid solution 2 mg/ml.

3.3. Immunological activity of human insulin-chitosan microparticles

The amounts of human insulin released from both washed and unwashed insulin-chitosan microparticles suspension were assayed for insulin by MEIA test and compared with the amount of insulin obtained by HPLC analysis. The unwashed microparticles contained the adsorbed amount of the non-entrapped insulin fraction. It can be concluded that insulin-chitosan microparticles prepared by high speed stirrer at a speed of 11, 000 rpm, at room temperature (25 °C) still maintained immune activity of human insulin inside the particles (Table 5).

3.4. In vitro drug release studies

The release profiles of insulin from both washed and unwashed particles in a pH 7.4 phosphate buffer and in pH 3 HCl solution were shown in Fig. 5. From the graphs it can be seen that most of the insulin was released within 10 min from the microparticles in pH 7.4 phosphate buffer, in which

Table 5
The amount of insulin released from chitosan microparticles (mean ± SD, $n = 3$)

Samples	Release amount (μg) (by HPLC analysis)	Release amount (μg) (by ELISA test)
Unwashed CH6T[a]	51.4 ± 0.2	42.0 ± 4.3
Washed CH6T[a]	17.4 ± 0.2	18.5 ± 0.9
Human insulin solution (5 ug/ml)	4.8 ± 0.1[b]	6.3 ± 0.4[b]

[a] The formulation was composed of 6 ml of 5 mg/ml of insulin solution.
[b] $n = 2$.

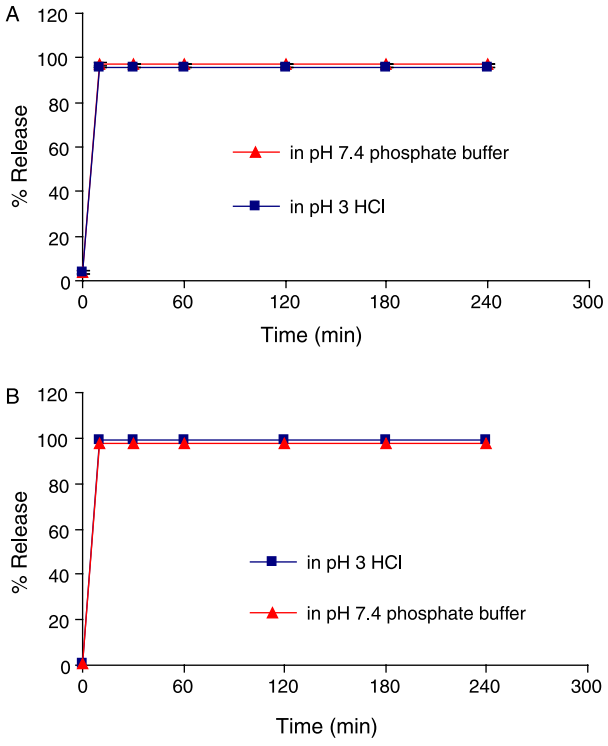

Fig. 5. Release profiles of insulin from unwashed (A) and washed (B) insulin-chitosan microparticles in different media ($n=3$).

the chitosan matrix is not soluble, as well as in the pH 3 HCl solution in which the chitosan should be soluble. Surprisingly, the release profiles are the same and there is no difference between the encapsulated fraction and the adsorbed drug fraction. One should have expected that the entrapped and bound insulin would show a more sustained release. In the case of diclofenac the release in acid medium shows the same profile at a lower concentration level due to its restricted dissociation (Fig. 6(A)). A similar release is observed with salicylic acid from chitosan nanoparticles (Fig. 6(B)).

A drug release with a strong burst effect which is not followed by a further increase in concentration of the drug in the dissolution medium indicates no binding of the drug molecule to the chitosan microparticle matrix. In the case of binding one would have expected that at least in the pH 7.4 medium a release profile could be found which follows the square root time law.

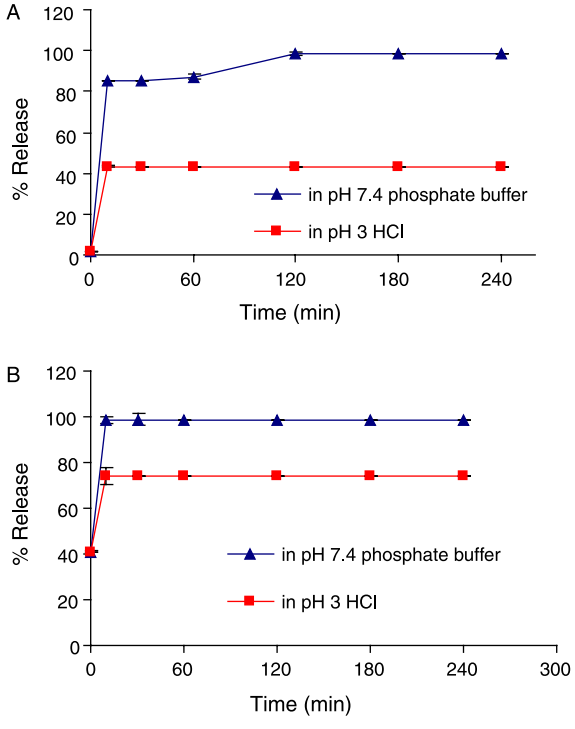

Fig. 6. Release profiles of diclofenac (A) and salicylic acid (B) from washed chitosan micro/nanoparticles in different media ($n=3$).

4. Conclusions

Drug-chitosan micro/nanoparticles with high drug entrapment efficiency could be prepared by careful control of formulation pH. Entrapment efficiency and the amount of drugs inside the particles are affected by zeta potential and surface charge of the produced micro/nanoparticles, respectively. However, no correlation between entrapment efficiency and zeta potential respective surface charge could be found. Furthermore, a drug release in one burst independent of the pH of the dissolution medium indicates that an ionic interaction between a negatively charged drug and the positively charged chitosan polymer is low. The results show that the low chitosan binding capacity to drugs cannot be used in nano-/microparticles for modifying the drug release.

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