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Research paper

Characterization of drug–chitosan interaction by ^{1}H NMR, FTIR and isothermal titration calorimetry

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Abstract

Electrostatic interaction between opposite charge of drugs (insulin and benzoic acid) and chitosan was studied by ^{1}H NMR, FTIR and isothermal titration calorimetry (ITC). No ionic interaction between the carboxyl group of benzoic acid and the amine group of chitosan could be detected. There was a minor change in the FTIR spectra of insulin–chitosan microparticles made of different concentrations of insulin. Exothermic heat of reaction between insulin and chitosan was obtained by ITC. However, the measured interaction enthalpy change (ΔH) was possibly due to the conformational changes and the adsorption phenomena of insulin onto the surfaces of the particles but not to a binding interaction. The binding of tripolyphosphate, a widely used cross-linking agent, to pH 3.3 and pH 5 chitosan was also studied by ITC. The interaction enthalpy change of the binding between tripolyphosphate and chitosan indicated that tripolyphosphate provided a stronger interaction to pH 5 chitosan than to pH 3.3 chitosan. However, it can be stated that the electrostatical interaction forces between the tested molecules insulin, benzoic acid, and tripolyphosphate and chitosan are found to be very weak.

Keywords: Chitosan; Ionic interaction; Insulin; Benzoic acid; ^{1}H NMR; FTIR; Isothermal titration calorimetry

1. Introduction

Chitosan is one of the most abundant natural polymers consisting of (1 → 4)-2-acetamido-2-deoxy-β-D-glucopyranosyl and (1 → 4)-2-amino-2-deoxy-D-glucopyranosyl units. Chitosan exhibits a large number of amine groups which are believed to be involved in the interaction between chitosan and many other substances [1–3]. There is a remarkable interest in food science and cosmetics concerning the application of chitosan due its property to act as natural preservative. Chitosans showed antimicrobial activity on the hypothesis that protonated amine groups of chitosan at C-2 interact with anionic constituents on the surface of microorganisms causing the damage to the cells [4]. Unexpectedly, chitosan in lipid emulsions showed better antimicrobial activity than chitosan solution [5]. Surfactant, stearic acid and zinc ions promoted the antimicrobial ability of chitosan [6–8]. In technical chemistry the interaction of chitosan films with lithium triflate was studied for the application in solid state batteries [9]. In the pharmaceutical field chitosan gel bead micro/nanoparticles prepared by the ionotropic gelation method were developed as a drug carrier for controlled release formulations. The ionic interaction of chitosan and drug via the amine group of chitosan and the carboxyl group of a drug has been reported to be a key factor of particle formation [10,11]. Several ways have been used to demonstrate the binding between both functional groups such as viscosimetry [12], turbidity measurement [13], X-ray photoelectron spectroscopy [10] and FTIR spectroscopy [8,14,15].

From our previous study [16] drug–chitosan micro/nanoparticles produced by ionic gelation method showed high entrapment efficiency. But the model drugs were

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released from the particles with a strong burst effect within a short time regardless of the pH of the dissolution medium. Similar results were shown in the literature [2,11], however, the high drug burst did not lead to a discussion about the drug binding. It is doubtful if positively charged chitosan can interact with opposite charged molecules. The purpose of this work is to investigate the possible interaction between model drugs (human insulin and benzoic acid) and chitosan by means of ^{1}H NMR, FTIR spectroscopy and isothermal titration calorimetry (ITC).

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) and 18-crown-6-ether were purchased from Sigma (St. Louis, USA). Human insulin was generously supplied by Aventis (Frankfurt, Germany). Benzoic acid, sodium benzoate, barium chloride dehydrate and potassium bromide were obtained from Merck KGaA (Darmstadt, Germany) and were of analytical grade.

2.2. Preparation of drug–chitosan micro/nanoparticles

A 0.2% w/v solution of chitosan was prepared in a 1% v/v acetic acid solution. Tripolyphosphate (0.1% w/v) and benzoic acid (2 mg/ml) were dissolved in purified water. In order to prepare an insulin solution (5 mg/ml), insulin powder was dispersed in pH 7.4 phosphate buffer solution and 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 7.4. Finally, the pH 7.4 phosphate buffer was added up to the desired volume [17].

For preparation of drug–chitosan micro/nanoparticles, 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). Insulin solution or benzoic acid solution (6 ml of various concentrations) was dropped into the chitosan solution. Then 0.1% triphosphate solution (10 ml) was added to the system while stirring was continued to complete micro/nanoparticles formation. The rate of adding drugs and tripolyphosphate was 0.75 ml/min. Stirring was continued for 1 min after all compounds were added.

In order to determine FTIR spectra various concentrations of insulin (1–6 mg/ml) and benzoic acid (0.5–2.5 mg/ml) were used to prepare micro/nanoparticles. The micro/nanoparticle suspensions were washed 3 times with purified water by centrifugation at 10,000 rpm to remove the non-entrapped drug and the non-precipitated chitosan which remained in the suspending medium. Then the micro/nanoparticles were precipitated by centrifugation (10,000 rpm) and dried by vacuum dryer at 20 °C over 12 h.

2.3. ^{1}H NMR spectroscopy

To prove the existence of ionic interaction between benzoic acid and chitosan, the benzoic acid solution (0.3–0.03% w/v) was mixed with 0.2% w/v chitosan solution, purified water or 0.2% w/v ammonium acetate solution at a ratio of 1:2. Then the chemical shift was followed by ^{1}H NMR spectroscopy. A 0.3% sodium benzoate in the above-mentioned solutions was also analyzed to show the chemical shift of 100% ionized drug. The sample measurements were carried out in a Bruker ARX 300 NMR spectrometer (Bruker, Rheinstetten, Germany) at temperature of 300 K and a frequency of 300.13 MHz with water suppression technique. Dimethyl sulfoxide-d_6 with 0.1% tetramethylsilane (TMS) was used as the external standard.

2.4. FTIR spectroscopy

An accurate amount of drug–chitosan micro/nanoparticles was dried and the peak shifts were studied by FTIR spectroscopy (Perkin-Elmer 16 PC spectrometer, PerkinElmer, Boston, USA). The infrared spectra were detected in KBr disks over a range of 4000–600 cm^{-1} using eight scans with a resolution equal to 4 cm^{-1}.

2.5. Isothermal Titration Calorimetry (ITC)

The heats of drug or tripolyphosphate binding to chitosan or chitosan micro/nanoparticles were measured by calorimetric titration using an LKB microcalorimeter, connected to a Thermal Activity Monitor (TAM 2277). Data evaluation was carried out with the computer program Digitam 4.1 for Windows (Thermometric AB, Sweden). The electrical calibration was carried out by the standard reaction of an 18-crown-6-ether with $BaCl_2$ in order to check the accuracy of the microcalorimeter. During the calorimetric titration a solution of a drug or of tripolyphosphate was added to the chitosan solution or chitosan micro/nanoparticles suspension in the titration cell. In Table 1 the experiments which were carried out by ITC are summarized. In a single experiment 15 injections of 30 μl of tripolyphosphate solution were titrated into 2.5 ml of chitosan solution/suspension by a motor-driven 1000 μl syringe. The solution or suspension in the titration cell was stirred at 80 rpm throughout the experiments and its temperature was maintained at 25 °C. The reference cell of the microcalorimeter was filled with 3 ml of purified water. The area under the peak of each injection is proportional to the resulting heat of interaction. The blank experiments were performed to obtain the heat of dilution which was subtracted from the heat of interaction to obtain the actual heat of binding. All measurements were carried out in duplicate.

3. Results and discussion

Based on ionic interaction the positively charged chitosan solution (pH 3.3 or pH 5.0) was mixed with the nega-

Table 1
The experiments by isothermal titration calorimetry

Experiment no.	Titrant	Titrand
I	Insulin pH 7.2	Chitosan pH 5.0
Blank exp.	Phosphate buffer pH 7.2	Chitosan pH 5.0
II	Benzoic acid pH 3	Chitosan pH 3.3
Blank exp.	Benzoic acid pH 3	Dilute acetic acid pH 3.3
III	Tripolyphosphate	Chitosan pH 5.0
Blank exp.	Tripolyphosphate	Dilute acetic acid pH 5.0
IV	Tripolyphosphate	Chitosan pH 3..3
Blank exp.	Tripolyphosphate	Dilute acetic acid pH 3.3
V[a]	Tripolyphosphate	Insulin–chitosan microparticles
VI[b]	Tripolyphosphate	Benzoic-chitosan solution

[a] Titration of tripolyphosphate to dilute acetic acid pH 5.0 was a blank experiment.
[b] Titration of tripolyphosphate to dilute acetic acid pH 3.3 was a blank experiment.

tively charged drug molecule in solution. As a cross-linking agent, tripolyphosphate was added to form micro/nanoparticles or to strengthen the particle formation. Because of the high burst release of the drug entrapped in the micro/nanoparticles a strong ionic binding between drug and carrier seems to be doubtful [16].

3.1. ^1H NMR spectroscopy

Due to the low entrapment efficiency of benzoic acid in chitosan nanoparticles made of pH 5 chitosan solution (24–32%) as compared with benzoic acid–chitosan nanoparticles made of pH 3.3 chitosan solution (68–80%) only the chitosan pH 3.3 was analyzed. A clear solution was obtained when chitosan solution pH 3.3 was mixed with benzoic acid solution (pH 3.0). The particle size of benzoic acid–chitosan particles after adding the cross-linking agent was in the nanometer range. The ^1H NMR analysis of the clear solutions of different concentrated benzoic acid–chitosan mixtures was performed in order to prove the ionic interaction between chitosan and benzoic acid. Fig. 1 shows the ^1H NMR spectra of the aromatic region (7.6–6.8 ppm) of benzoic acid and sodium benzoate in chitosan solution. The peaks at about 7.5, 7.1 and 7.0 ppm represent the ortho-, para-, and meta-protons, respectively. A very small shift of signal of benzoic acid to the lower direction was observed with decreasing the benzoic acid concentration in the mixtures. The same direction of the chemical shift was observed by proton NMR analysis of different concentrations of benzoic acid and its sodium salt in 0.2% ammonium acetate solution and in purified water. Assuming that the ortho-proton signal was an average signal for benzoic acid and benzoate, Fig. 2 shows a plot of the chemical shift for this ortho-proton signal in the three media mentioned above. It can be

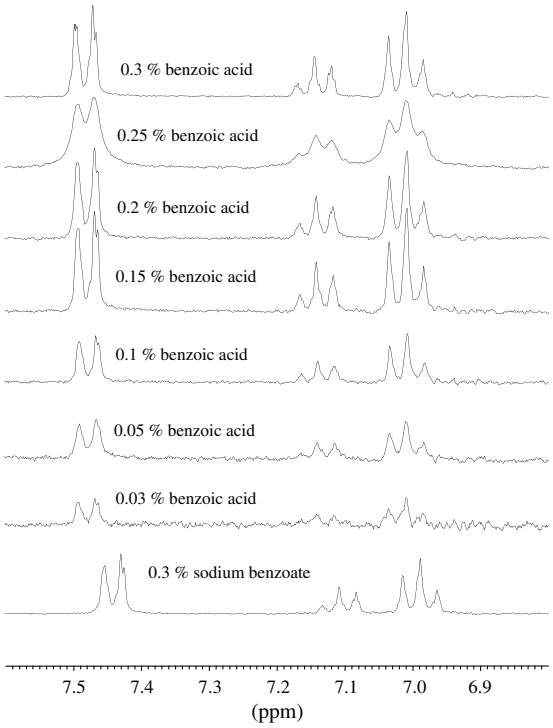

Fig. 1. ^1H NMR spectra of benzoic acid and sodium benzoate in 0.2% chitosan solution (pH 3.3).

stated that there is no binding or interaction between benzoic acid and chitosan because the small shift of the signal in the same direction was detected in all the mixtures with benzoic acid and even in purified water. The dissociation of benzoic acid increased when the total concentration of benzoic acid in the mixtures was decreased and this effect leads to the shift of the proton signal.

3.2. FTIR spectroscopy

FTIR analysis is proposed in many references as the possible way to investigate the interaction between substances [18–20]. In this study dried drug–chitosan micro/nanoparticles were analyzed by FTIR to observe the possible interaction of the functional groups of both the molecules. Chitosan exhibits main characteristic bands of carbonyl (C=O-NHR) and amine group($-NH_2$) at 1654 cm^{-1} and 1540 cm^{-1}, respectively [9,14]. The broad band due to the stretching vibration of $-NH_2$ and –OH group can be observed at 3400–3500 cm^{-1} [7,21]. The bands at 1000–1200 cm^{-1} are attributed to the saccharide structure of chitosan [22]. In the FTIR spectra of insulin–chitosan microparticles (Fig. 3) no band shifts could be detected. But it seems that some bands of chitosan overlapped with those of insulin which resulted in a widening of the carbonyl (1654 cm^{-1}) and amine bands (1540 cm^{-1}). This band widening is more distinct with increasing concentrations of insulin (1 mg/ml to 6 mg/ml solution). In addition, the change of the three small bands at wavenumbers about 1400–1500 cm^{-1} to two bands of

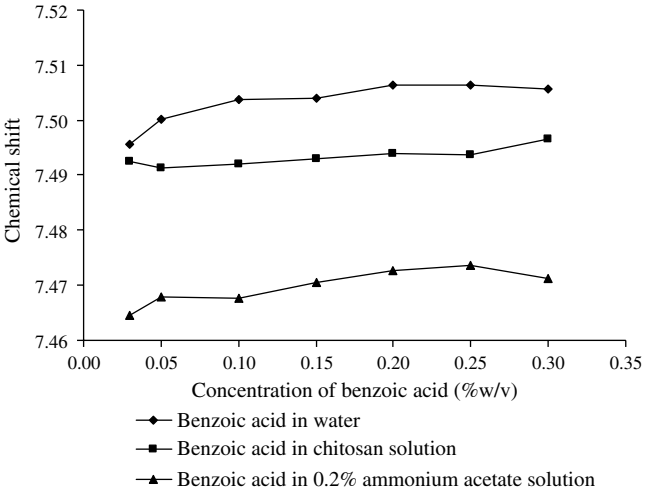

Fig. 2. Chemical shift of ortho-proton signal of various concentrations of benzoic acid in different media.

insulin was observed. These observations show the possibility of a weak interaction between insulin and chitosan [9,14].

Fig. 4 represents the spectra of benzoic acid and benzoic acid–chitosan nanoparticles with increasing benzoic acid concentration from 0.5 mg/ml to 2.5 mg/ml. There was no noticeable shift of the major bands of chitosan. This indicates once more that there was no interaction between benzoic acid and chitosan [9].

3.3. Isothermal Titration Calorimetry (ITC)

The interaction between opposite charged molecules resulted in the change of enthalpy which could be detected by microcalorimetry. The measured enthalpy change (ΔH) for the titration of $BaCl_2$ to the 18-crown-6 ether (standard experiment) was −31.1 kJ/mol with a $\log K$ of 3.8 (K is the stability constant or equilibrium constant of the reaction) and was in accordance with the literature [23,24]. Table 1 shows the different experiments done by ITC. The interaction of pH 7.2 human insulin (5 mg/ml, 860 μM) and pH 5 chitosan (0.2%w/v, 13.30 μM) was performed. The insulin solution in phosphate buffer (0.05 M phosphate buffer with 1 N HCl and 1 N NaOH) was titrated into a titration cell containing 2.5 ml chitosan solution. The raw data for the titration of insulin to chitosan are shown in Fig. 5A. A gradual increase of exothermic heat was observed with the increasing number of injections. The heat of dilution was measured by titrating phosphate buffer to the chitosan solution. The raw calorimetric data in Fig. 5B show exothermic peaks in the beginning of the injection and endothermic peaks in the following course. This heat of dilution was then subtracted from the heat interaction of the titration between insulin and chitosan (Fig. 5A) to obtain the enthalpy of binding between them. In Fig. 6A the integrated data of the enthalpy change (ΔH) per mole of insulin are plotted versus the concentration of insulin in titration cell after subtracting the heat of dilution. The interaction enthalpy change was not very high (<2000 kJ/mol of insulin) when compared with binding studies of other macromolecules or polymers. For example an enthalpy change of about 6,300 kJ/mol was observed for the reaction between the sugar units of chitosan and β-lactoglobulin [13]. The enthalpy change of the titration between insulin and chitosan seems to be only partly due to the ionic interaction between both molecules. Part of the enthalpy change may be due to conformational changes, ionization of polar groups, and especially due to adsorption phenomena of insulin at the surface of the polymer carrier [13,25]. The conformational changes can be

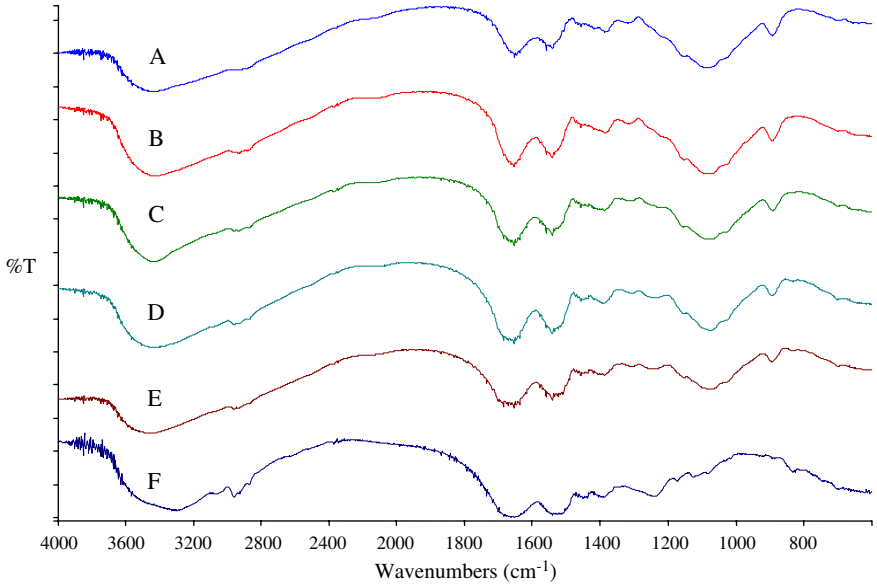

Fig. 3. IR spectra of insulin (F) and insulin–chitosan nanoparticles with different concentrations of insulin solution in the formulations. (A) 1 mg/ml, (B) 2 mg/ml, (C) 3 mg/ml, (D) 5 mg/ml, (E) 6 mg/ml.

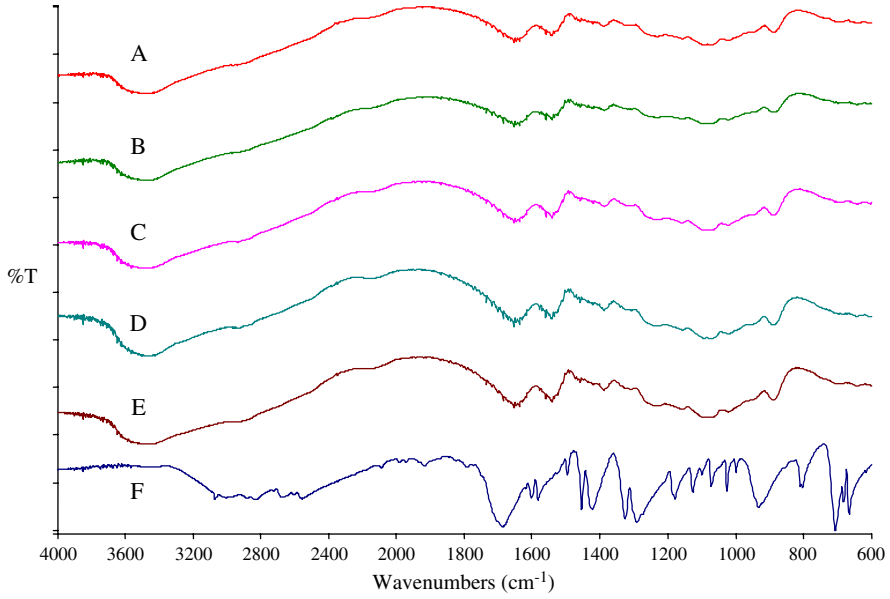

Fig. 4. IR spectra of benzoic acid (F) and benzoic-chitosan nanoparticle with different concentrations of benzoic acid solution in the formulations. (A) 0.5 mg/ml, (B) 1 mg/ml, (C) 1.5 mg/ml, (D) 2.0 mg/ml, (E) 2.5 mg/ml.

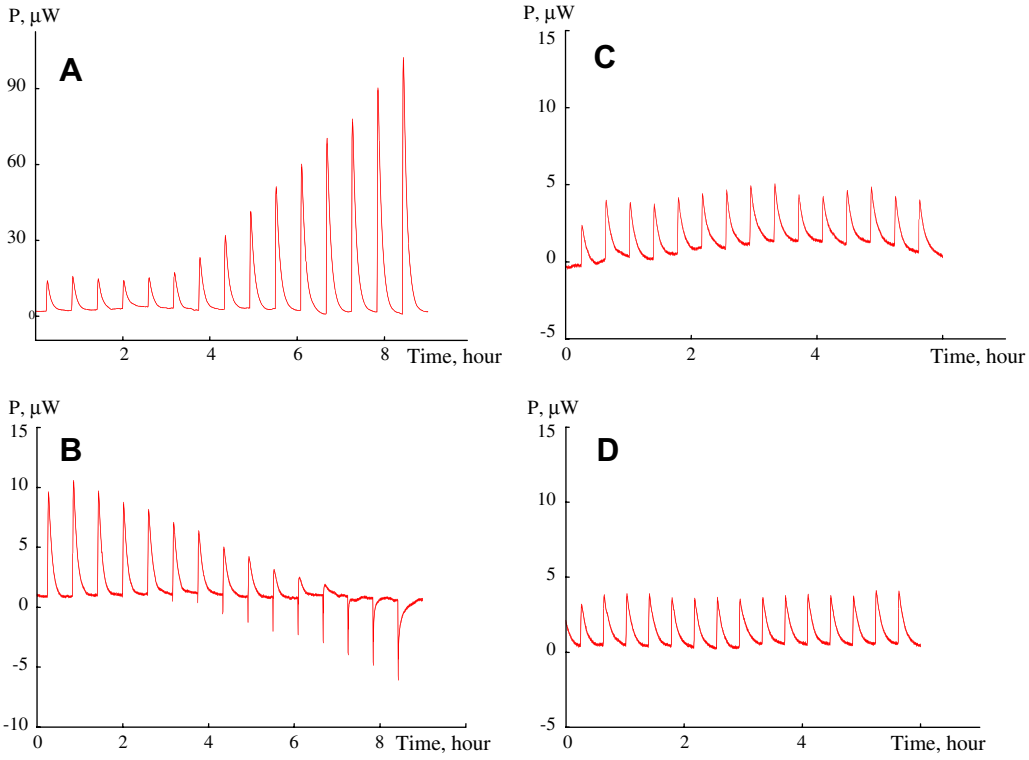

Fig. 5. Heat flow versus time profiles for the sequential titrations of insulin solution into chitosan solution (A), phosphate buffer solution into chitosan solution as a blank experiment (B), benzoic acid solution into chitosan solution (C) and benzoic acid into dilute acetic acid solution as a blank experiment (D).

caused by electrostatic interaction and hydrogen bonds between amino acid groups with charged side chains or hydrophobic interaction of amino acid groups with nonpolar side chains. In our previous study [16] great attention was paid to the adsorption of insulin on the surface which can be detected by surface charge measurement of the drug microparticles. The surface charge of insulin–chitosan microparticles decreased with increasing insulin in formulation and accompanied by the increase of entrapment efficiency. However, the surface charge could not be reduced to the zero level. So the addition of tripolyphosphate as crosslinker led to further precipitation of the chitosan

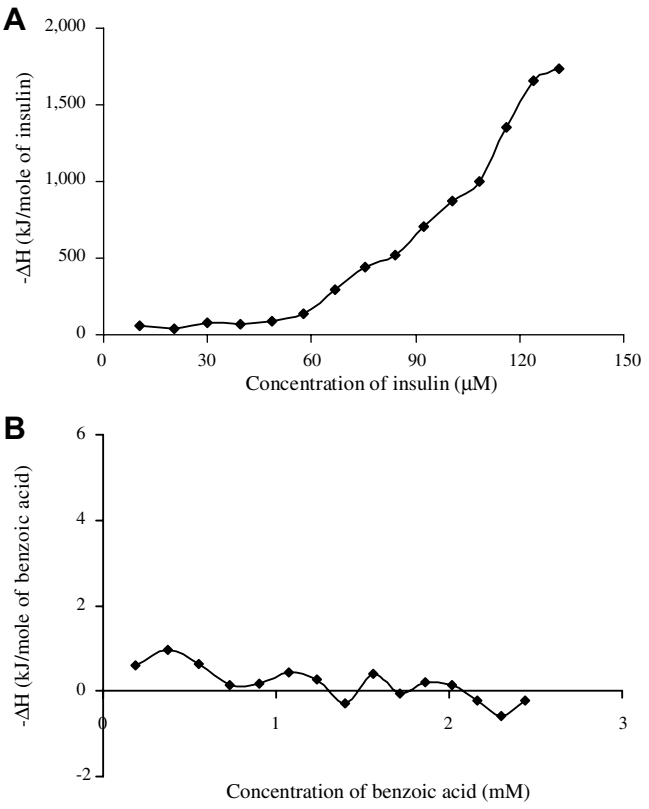

Fig. 6. Integrated data obtained from the previous raw data for the titration of insulin solution into pH 5 chitosan solution (A) and benzoic acid solution into pH 3.3 chitosan solution (B) in the reaction cell after subtracting the blank experiments.

and to an entrapment of the drug without any binding to the polymer. This can be shown by the high burst release of insulin in the in vitro dissolution test. The results reveal that only a small amount of insulin interacts with the positively charged chitosan. Adsorption of most insulin on the surface of the particles or the polymer and physical entrapment during particle formation seems to be the main reason for the high entrapment efficiency and the high burst release.

Benzoic acid was expected to bind tightly to chitosan which represents a weak base. Benzoic acid–chitosan nanoparticles with an entrapment efficiency of about 70% were formed at pH conditions of about 3.5 (data not shown) after addition of tripolyphosphate. Rapid release of benzoic acid from chitosan nanoparticles in pH 7.4 phosphate buffer and purified water was observed. The expected ionic interaction between benzoic acid and chitosan was followed by ITC. Fig. 5C and D show the calorimetric titration curves of benzoic acid solution into pH 3.3 chitosan solution and into pH 3.3 dilute acetic acid solution (blank experiment), respectively. In both experiments the same exothermic heat change was obtained. The interaction enthalpy change (ΔH) per mole of benzoic acid was less than 1.3 kJ/mol (Fig. 6B). So it can be concluded that there was no interaction between benzoic acid and pH 3.3 chitosan, although a high entrapment efficiency was determined. This deduction agrees with the analysis by ^{1}H NMR and FTIR. The result with benzoic acid is the same as with insulin: No binding and a fast drug release from the particles.

In addition the interactions between tripolyphosphate (pH 9.1) and pH 3.3 or pH 5 chitosan were investigated. From the integrated data (Fig. 7) the interaction enthalpy change was calculated as fairly small (<33 kJ/mol tripolyphosphate). This low value is generally due to the low molecular weight of tripolyphosphate [26,27]. The interaction enthalpy change for the interaction between tripolyphosphate and chitosan pH 3.3 was less than 23 kJ/mol and the interaction enthalpy change for the interaction between tripolyphosphate and chitosan pH 5 was less than 33 kJ/mol. In conclusion, the interaction enthalpy change measured by isothermal titration calorimetry of tripolyphosphate and chitosan (pH 3.3 and pH 5) was below 33 kJ/mol.

Tripolyphosphate was used to complete or strengthen the particle formation. From the former study the zeta potential of insulin–chitosan microparticles was decreased only about 5–7 mV after adding tripolyphosphate [16]. The interaction of tripolyphosphate to the surface of insulin–chitosan microparticles was studied by ITC. The interaction enthalpy change was not very different from that between tripolyphosphate and chitosan pH 5 without insulin (<44 kJ/mol; Fig. 8). This indicates that the negatively charged functional groups of tripolyphosphate show only a weak binding to the positively charged amino groups of chitosan during microparticle formation. Tripolyphosphate seems to have only an influence on the hydration of the chitosan polymer although the zeta potential undergoes a minor change only. This zeta potential is in the case of insulin microparticles influenced by adsorption of the insulin molecules to the surface. To summarize one can say that the insulin molecule shows only a weak binding to the amino groups of the chitosan molecule and that the entrapment in chitosan microparticles is more a question of adsorption.

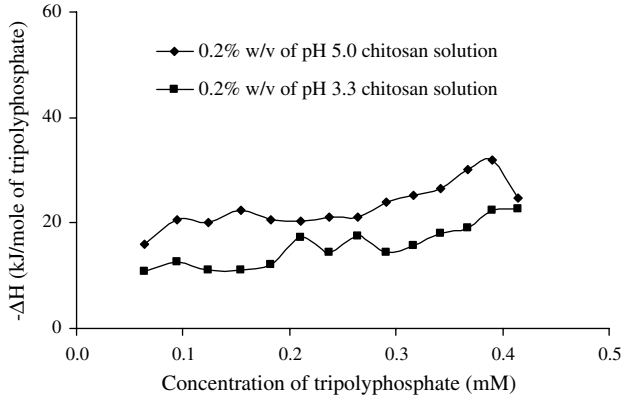

Fig. 7. Integrated data obtained from the titration of tripolyphosphate solution into pH 5 chitosan solution and pH 3.3 chitosan solution after subtracting the blank experiments.

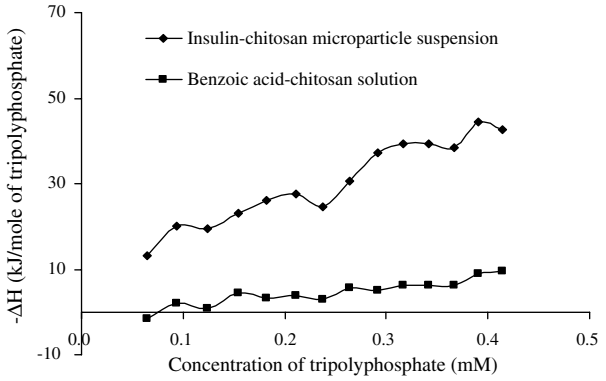

Fig. 8. Integrated data obtained from the titration of tripolyphosphate solution into insulin–chitosan microparticles and into benzoic acid–chitosan solution after subtracting the blank experiments.

The interaction of tripolyphosphate with the mixture of benzoic acid–chitosan solution was also performed. The interaction enthalpy change (ΔH) was quite small (<10 kJ/mol) demonstrating that there is no strong interaction between tripolyphosphate and chitosan in presence of benzoic acid (Fig. 8).

4. Conclusions

The interaction between insulin and chitosan was investigated by FTIR and isothermal titration calorimetry. A weak interaction between insulin and chitosan was revealed by ITC. The low interaction enthalpy change (ΔH) observed can not only be explained by an ionic binding but also by adsorption or conformational change of the insulin molecule. Most of the insulin was adsorbed at the chitosan polymer or physically entrapped during microparticle formation. This result is confirmed by the high burst release of the drug from chitosan microparticles.

Benzoic acid–chitosan nanoparticles made of pH 3.3 chitosan showed similar properties as insulin–chitosan microparticles: High entrapment efficiency and a high burst release profile of the drug. Therefore the ionic interaction between benzoic acid and chitosan (pH 3.3) was analyzed by ^{1}H NMR, FTIR and ITC. With none of these methods an electrostatic interaction between benzoic acid and chitosan could be detected. Benzoic acid was physically entrapped in chitosan nanoparticles which caused the rapid release of the drug. Furthermore the ionic interaction of tripolyphosphate with chitosan was analyzed with the same methods because tripolyphosphate was used in all experiments to strengthen particle formation. The results reveal that tripolyphosphate showed a difference in enthalpy change for the binding to chitosan at pH 5 and pH 3.3, respectively, but the binding is still poor. Tripolyphosphate influences the status of hydration of the chitosan polymer. In general these results led to the conclusion that large molecules as insulin as well as small ones as benzoic acid are not bound by ionic interaction with chitosan. The ionic binding capacity for these molecules is poor. Their entrapment in chitosan nano/microparticles is mainly due to physical entrapment.

Acknowledgements

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