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Research Article

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Chitosan–Sodium Lauryl Sulfate Nanoparticles as a Carrier System for the *In Vivo* Delivery of Oral Insulin

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Abstract. The present work explores the possibility of formulating an oral insulin delivery system using nanoparticulate complexes made from the interaction between biodegradable, natural polymer called chitosan and anionic surfactant called sodium lauryl sulfate (SLS). The interaction between chitosan and SLS was confirmed by Fourier transform infrared spectroscopy. The nanoparticles were prepared by simple gelation method under aqueous-based conditions. The nanoparticles were stable in simulated gastric fluids and could protect the encapsulated insulin from the GIT enzymes. Additionally, the *in vivo* results clearly indicated that the insulin-loaded nanoparticles could effectively reduce the blood glucose level in a diabetic rat model. However, additional formulation modifications are required to improve insulin oral bioavailability.

KEY WORDS: chitosan; insulin; nanoparticles; oral delivery system; sodium lauryl sulfate.

INTRODUCTION

Insulin is administered by subcutaneous injection to treat diabetic type I patients. In addition to the psychological barriers for the use of insulin in injectable form, its use is accompanied by different complications such as hypoglycemia, lipoatrophy at the injection site and all other risks associated with injections. These complications make the search into alternative routes for insulin delivery is a necessity (1–4). The most studied routes are oral, nasal, buccal, and pulmonary. Buccal route is based on micellar solubilization commercialized in spray form. Nasal route was fully investigated but no commercial exploitation is taking place (5,6). Pulmonary route was commercially exploited but unfortunately was withdrawn from the market (7). Nevertheless, oral route is the most desired and has been investigated thoroughly (8,9).

In order to make oral route delivery possible, two main obstacles must be overcome namely, lack of insulin stability in gastrointestinal tract (GIT) and its absorption hindrance (8). Consequently, many formulation strategies have been attempted. Among those formulation strategies is the use of ionic interaction between positively and negatively charged polymeric materials. Due to favorable nature of chitosan polymer as a positively charged biocompatible, nontoxic, and mucoadhesive polymer, many researchers selected chitosan as an oral drug carrier. Unfortunately, in many cases, characterization of chitosan used was not elaborated. In these studies, chitosan was treated as single component while in reality it is a mixture of different molecular weights (10–12). However, low molecular weight chitosans (LMWC) can be more beneficial than high molecular weight chitosans due to their higher water solubility, and their ability to form nanoparticles (13). Thus, LMWC would be able to entrap insulin and could stabilize insulin within their nanostructure. The interaction between chitosan and poly-γ-glutamic acid was used and proved to be a suitable delivery system (14). Other systems consisting of insulin with chitosan and alginate showed reasonable reduction in glucose level following oral delivery (15). These studies suffered from their inability to deliver enough insulin concentration into the blood stream. However, it was observed that the addition of chitosan to different preparations intended for insulin oral delivery could lead to the reduction in particle size down to nano-level range (16–18). In these studies, it was evident that insulin absorption

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was dependent on particle size. Thus, the priority in this research was shifted towards production of nanosized particulate system.

Indeed, different reports explored this matter using self-emulsifying systems. For example, it was reported by Elsayed *et al.* that LMWC and oleic acid can be used as a nanovesicle-forming material and may be included within a micelle formed from glycerol-6-dioleate and PEG8 caprilic/capric glyceride mixed in 1:1 ratio and dispensed in oleic acid (19). This system proved its suitability as an oral route for insulin delivery in rats (20,21). Further, this oral insulin delivery system was also used in "proof of concept" studies conducted on healthy human volunteers, where it seems to offer a base for further clinical studies (22). Although this system was practically successful, still its encapsulation efficiency needs improvement. This system could open the door for the use of other aggregate forming materials, which are able to protect the interior of the solubilized system in cooperation with chitosan. Consequently, this may suggest the suitability of these systems to deliver other protein drugs orally.

Other systems based on building up of micelles in aqueous medium through the use of an ionic surfactant, where oligo-chitosan can be included with insulin and solubilized in the interior of the micelle, is worth the investigation. In this study, sodium lauryl sulfate is selected as a micelle former concurrent with a polyelectrolyte complex (PEC) formed between chitosan and insulin. The integrity, stability, and bioactivity of the system are going to be evaluated.

MATERIALS AND METHODS

Materials

Recombinant human (rh) insulin powder was purchased from Biocon, India. Low-molecular-weight chitosan (LMWC) of an average molecular weight about 13 kDa and degree of deacetylation of 81% was prepared in-house and analyzed within the Jordanian Pharmaceutical Manufacturing Company facilities, as described in previous work by Qandil *et al.* (19). Sodium lauryl sulfate (SLS) was purchased from Cognis, GmbH, Germany. Strepotozotocin and pepsin were obtained from Sigma-Aldrich, USA.

Methods

Preparation of Nanoparticles Containing Insulin

Preparation of insulin–chitosan complex was carried out as has been previously described in details earlier (20,21). Briefly, 0.5 g of chitosan (13 kDa) was placed in a glass vial, dissolved in 10 ml deionized water and its pH was adjusted to 5.5 using 0.2 M NaOH, the final volume was completed to 20 ml using deionized water. In another vial, 100 mg of rh-insulin powder was dissolved in 1 ml of 0.1 M HCl, followed by the addition of 3 ml of 1 M Tris (hydroxymethyl)-aminomethane buffer pH 7. Chitosan–insulin complexes were prepared by adding 1 ml of chitosan solution to an equal volume of insulin solution in a glass vial under gentle magnetic stirring, and incubating for a further 15 min at room temperature. All experiments were carried out in triplicate at room temperature. Freshly prepared solutions were used in each experiment.

Three milliliters of the PEC was added in drop-wise manner into 20 ml of SLS aqueous solution (1% *w*/*v*) with pH adjustment up to 5 using sodium hydrogen phosphate. Following its gentle mixing, the solution was homogenized at 500 bars (5 cycles) using high-pressure homogenizer (Emulsiflex-C5 Avestin Inc Canada). Five milliliters of this homogenate was mixed with equal volume of aqueous chitosan solution (6% *w*/*v*) having pH 5.5.

The mixture was stirred at 300 rpm for 15 min.

Characterization of the nanoparticles

Hydrodynamic Diameter of the Produced Nanoparticles. The particle size distribution of the resulted particles was determined with Zetasizer Nano ZS (Malvern Instruments, UK) at 25°C. The angle of the scattering light used for particle size determination was 173°. Sample analysis was based on water viscosity (0.88 mPa s) and refractive index (1.33) at 25°C. Solution containing particles were diluted 1:10 *v*/*v* with pure deionized water, simulated gastric fluid (SGF), or simulated intestinal fluid (SIF) and samples were measured for three times and 11 reading per run. The average hydrodynamic diameter was determined automatically.

Zeta Potential Measurement. The zeta potential was measured with Zetasizer Nano ZS (Malvern Instrument, UK) at 25°C. The preparation was diluted 1:10 *v*/*v* with pure deionized water, SGF, or SIF. The viscosity and dielectric constant of pure water were used for Zeta potential calculation.

Nanoparticle Morphology. The particle morphology was examined by transmission electron microscope (TEM; Zeiss EM 10 CR, Germany). Different drops of the solution were applied to Formvar-coated grid and left to dry at room temperature to be studied under the TEM. Different particle size was observed and the photograph was taken for a representative sample.

Encapsulation Efficiency

The method of determination of the amount of insulin entrapped within nanoparticles has been described in a previous work (23). Briefly, the nanoparticles were centrifuged at 15,000 rpm for 30 min at 15°C and the insulin content in the supernatant was assayed by reversed-phase high-pressure liquid chromatography (RP-HPLC). The encapsulation efficiency equation has been applied as described elsewhere (24)

FTIR

Appropriate amount of chitosan, SLS, and dried nanoparticles were used. Fourier transform infrared spectroscopy (FTIR) was conducted on 360 FTIR Avatar spectrometer (Nicolet, USA) in the range between 4,000 and 400 cm^{-1} using eight scans with a resolution of 2 cm^{-1}. The results were compared with reference to a physical mixture.

Modeling and Calculation of Binding Energy

Computations in water were performed with Hyperchem® (release 8.0.6). Force field used in these computations was MM+ (atomic charges) method implemented in Hyperchem. Atomic charges were obtained by performing AM1 semi-empirical calculations (0.30 gradient). Energy minimizations were obtained using the conjugate gradient algorithm (0.01 kcal/mol Å) gradient. All molecules were built up from natural bond angles, as defined in the accompanied software. The structures were then minimized using different force fields as mentioned above.

Aqueous solvation effects were achieved by using the Polak-Rebiere algorithm (PR), which rapidly packs solvents molecules around the system. The implementation of the PR into Hyperchem builds a periodic box by adding solvent molecules such that the solvent van der Waals surfaces do not overlap with the van der Waals surface of the solute. Periodic boundary conditions were employed using a cubic box. The dimension of the box was, in each case, limited to the minimum dimension where the potentials fall to zero.

These calculations were done in order to explore the nature and strength of the interactions between chitosan and insulin, chitosan and SLS, and insulin and SLS. For each case, the minimum interaction between the two species mentioned was found and their binding was calculated accordingly.

Assessment of Insulin Stability using RP-HPLC

Nanoparticles were dissolved completely in order to release insulin into the solution using 0.01 M HCl containing 1% Polysorbate 80 (Tween 80) and vortexed for 1 min prior to testing. However, insulin content was analyzed by high-pressure liquid chromatography (HPLC, Thermospectra HPLC using TSP 1000 pump system with TSP 1000 UV-VIS detector and a TSP AS 3000 autosampler, Spectra System, USA) as described previously (23).

Protection Against SGF

To assess the protective effect against gastric degradation, 2 g of the nanoparticles were incubated (37°C) and shaken with 5 ml of simulated gastric fluid pH 1.2 for 1 h in a water bath shaker (100 strokes per minute). Insulin samples were centrifuged at 15,000 rpm for 30 min at 15°C and insulin content in the samples and supernatant were assayed by RP-HPLC as mentioned before. Preliminary studies showed that there is no significant difference in the assay ($p<0.5$) between fresh insulin standard and insulin incubated in SGF for 1 h.

In vivo Studies on Diabetic Rats

All experiments were carried out on adult male Wister rats in compliance with the European Community council directives of November 24, 1986 (86/609/EEC).

Diabetic rats have been prepared via conducting two intra-peritoneal injections of streptozocin each containing 80 mg/kg. Diabetes was confirmed in rats when glucose in their blood was higher than 200 mg/dl and measured by glucose meter (One Touch Sure Step-life Science Inc., USA). Diabetic rats were divided into three groups. The first group was injected with rh-insulin (1 IU/kg) subcutaneously. The second group was given oral insulin while the third group was give rh-insulin loaded in nanoparticles. The second and third group received rh-insulin (50 IU/kg) dose. Blood glucose was monitored at different time intervals using glucose meter. The relative pharmacological availability (PA%) was calculated through comparing the areas above the curve for oral and sc preparations taking into consideration the difference in the dose (21).

Results and Discussion

Formulation and Characterization of the Nanoparticles

In the present work, the concentration of SLS was much higher than its critical micelle concentration. Such high concentration facilitates the process of gelation. Consequently, the solution of chitosan was dropped into negatively charged counterion of SLS solution and gelled particles were produced instantaneously due to the formation of water insoluble sulfonate salt of chitosan (25).

The resultant gelled particles were homogenized and characterized in terms of size. Dynamic light scattering data indicated the formation of nanoparticles with an average particle size of 253 nm, as shown in Table I. In previous studies, it was illustrated that chitosan can form microspheres with sodium sulfate (26,27). However, these were not utilized according to literature screening in the delivery of peptides or proteins. In this study, high-pressure homogenizer was used to reduce the particle size in order to produce nanoparticles, since the size of the drug carrier is considered to be the major parameter in determining its efficiency as drug delivery system.

Compared to micron-sized particles, nanoparticles have higher surface area that can lead to higher drug loading. They can maintain a more intimate contact with the biological tissues (28).

The nanoparticles of chitosan–SLS were evaluated following their dispersion in three different media *i.e.* water, SGF, or SIF. The data are summarized in Table I. Nanoparticles dispersed in water or SGF presented a mean particle size of 253 and 297 nm respectively, while nanoparticles

Table I. Characterization of Chitosan–SLS Nanoparticles

Dispersion medium	Z average (nm)	Polydispersity index	Zeta potential (mV)
Water	253±2.5	0.408	38.9±1.2
SGF	291±7.2	0.575	29.06±1.32
SIF	625±5.7	0.418	13.93±0.31

dispersed in SIF presented a mean diameter of 625 nm. The significant increase in particle size in SIF may be due to forced aggregation of particles, which was also confirmed by zeta potential measurements. This is important in terms of physical stability of the nanoparticles in the SIF environment. This indicates the suitability of such preparation to withstand the acidic environment while it aggregate in SIF medium may hinder its liberation and delivery.

The measurement of zeta potential allows predictions of the colloidal stability in aqueous dispersions. Usually, particle aggregation is less likely to occur for charged particles with optimum zeta potential (ζ>30 mV) due to electrostatic repulsions. The zeta potential and standard deviation of the nanoparticles diluted with water, SGF, or SIF are shown in Table I. The high zeta potential values revealed that the nanoparticles formed are stable in water and SGF. A significant decrease in zeta potential (p<0.05) was observed in samples diluted with SIF, possibly due to interactions of phosphate ions with chitosan or might be due to the change in pH that could affect charge distribution on the nanoparticles since the pKa of chitosan is around 6.5, which could lead to the nanoparticle aggregation (29).

An important character of the nanoparticles is the presence of a positive charge, which could be attributed to the excessive chitosan molecules entangled onto the surface of the obtained nanoparticles. Thus, the resulting nanoparticles displayed a positive surface charge as shown in (Fig. 1). The presence of positive charges on the nanoparticle surface is important for their interaction with the cellular membrane components and the tight junctions, which could ultimately trigger their paracellular permeation (30,31).

In order to illustrate the morphology of the nanoparticles under the microscope, a 2-dimensional image of the nanoparticles was obtained. TEM micrographs (Fig. 2) indicated clearly that the nanoparticles were almost spherical in shape.

To confirm the formation of a complex between SLS and chitosan, the precipitates obtained upon addition of SLS to chitosan solution were analyzed using FTIR, peaks of SO_2 stretch (asymmetric 1,222 cm^{-1} and symmetric 1,085 cm^{-1}) in the complex were shifted to the lower field relative to unbound SLS (1,215 and 1,067 cm^{-1}), which is attributed to the salt formation of sulfate groups as shown in Fig. 3. Dai and Dong (32) found similar shifts with SLS salts. The FTIR result suggests that the sulfate group in SLS binds to chitosan forming the complex.

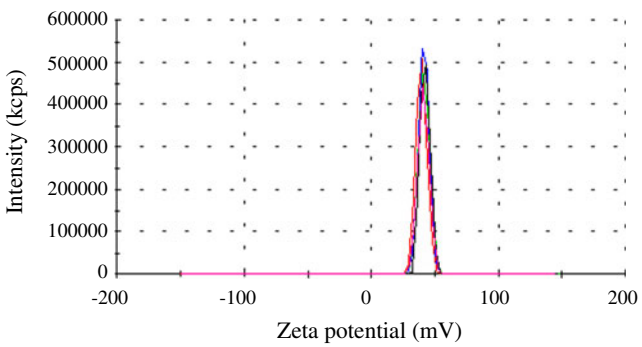

Fig. 1. Zeta potential of chitosan–SLS nanoparticles dispersed in water

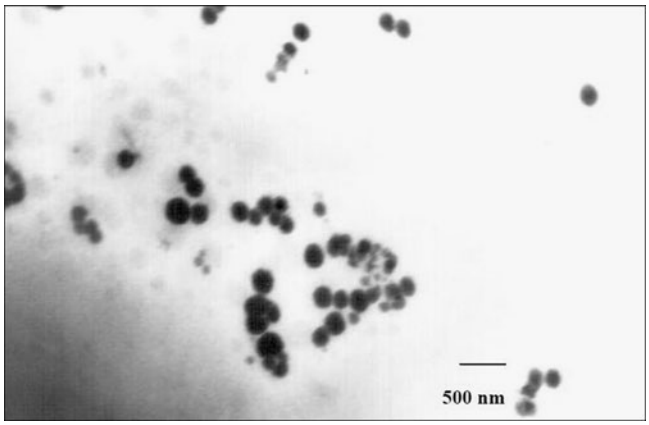

Fig. 2. TEM images of insulin-loaded nanoparticles

It is worth mentioning that due to the presence of chitosan in molar excess, it is suggested that chitosan will have a dual function in terms of complexation, *i.e.*, chitosan will complex with both insulin and SLS at the same time. The initial stage of complexation between insulin and chitosan was reported previously (21). However, the resultant complexation process is weak and easily dissociated in the acidic environment. In addition, the resultant chitosan–insulin complex did not result in any protection against gastric enzymes (21). Thus, the addition of SLS as an anionic surfactant could be beneficial, where it may result in stronger association with chitosan and so providing a more protection of insulin against the harsh environment of the stomach.

Due to the possibility of many types of interactions with the nanoparticles, it would be beneficial to get more insight about the most influencing interaction in the nanoparticle formation of the delivery system based on the binding energy. The simulated interactions (models) are summarized in Fig. 4. In Fig. 4a, chitosan/insulin complex has a binding energy of −32.5 kCal/mol, while in case of chitosan/SLS, the model was constructed using 1:1 molecule ratio (Fig. 4c); the complex the binding energy is very large (−1,276 kcal/mol), the reason may be due to the formation of intensive hydrogen bonds between glucosamine units of chitosan with SLS. The insulin and SLS interaction was calculated the model was built using 1:1 molecule ratio, where SLS was introduced to the insulin in different orientation to get the minimum energy (highest binding energy). The binding energy was found to be minimal (−6.44 kCal/mol), indicating a weak interaction between insulin and SLS. This highest binding energy was attributed to the interaction between SLS and chitosan. This could suggest that the larger contribution in formation of strongly bound nanoparticles of such delivery system is due to chitosan–SLS interaction while keeping insulin ready to be delivered.

Stability and Encapsulation Efficiency of Insulin in Nanoparticles

Reversed-phase HPLC chromatograms of insulin-loaded particles dissolved in 1% Tween 80 manifest the same retention time and peak shape as those of standard insulin.

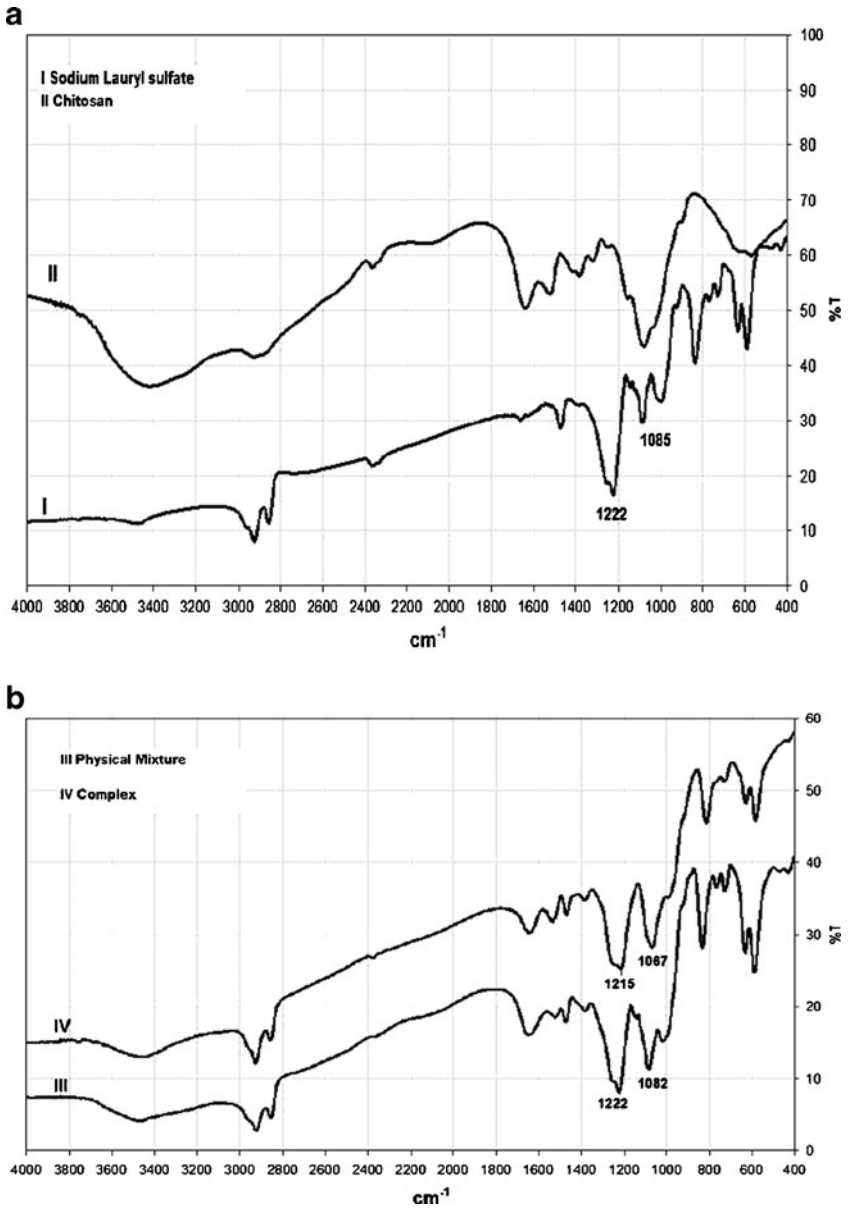

Fig. 3. FTIR spectra of: **a** *I* sodium lauryl sulfate (SLS), *II* chitosan; **b** *III* chitosan SLS physical mixture, *IV* chitosan SLS complex

Furthermore, no additional peaks were observed (Fig. 5). These results may suggest that there was no significant insulin degradation upon incorporating into chitosan–SLS nanoparticles. Once more, the interaction between chitosan–SLS forms a suitable barrier for insulin protection *in vitro*.

Nanoparticles displayed high encapsulation efficiency as 82.04±1.95% of insulin was encapsulated. The content of insulin in nanoparticles was 1.28 IU/ml. This assay followed the measurement of insulin content in standard insulin powder where HPLC method yielded 1.39 IU/ml.

Release in SGF Medium

Around 10% of total loaded insulin was released from nanoparticles following their incubation at pH 1.2 after 1 h. This indicates that chitosan–SLS could protect insulin from the aggressive gastric environment compared to nanoparticles prepared by ionic gelation of cationic chitosan and a polyionic substance. For example nanoparticles prepared from chitosan and poly(γ-glutamic acid) became unstable at pH 1.2 and broke apart (17) and nanoparticles composed of chitosan and tripolyphosphate rendered the protein more susceptible to acid and enzymatic hydrolysis (33). This may be attributed to the fact that most of the polyionic hydrogels lack stability especially at extreme pHs. Chitosan nanoparticles may take a randomly coiled conformation at alkaline/neutral pH, because of their unionized amino groups (34). At lower pH region, chitosan acquires a net positive charge due to protonation of amino groups and swelling can occur as a result of electrostatic repulsion of charged ionic group. Such hydrogels display extremely poor retention at gastric pH. Intermixing of chitosan and SLS leads to strong complex formation and this may restrict the mobility of chitosan chains. Sajeesh and Sharma (28) observed the same stabiliz-

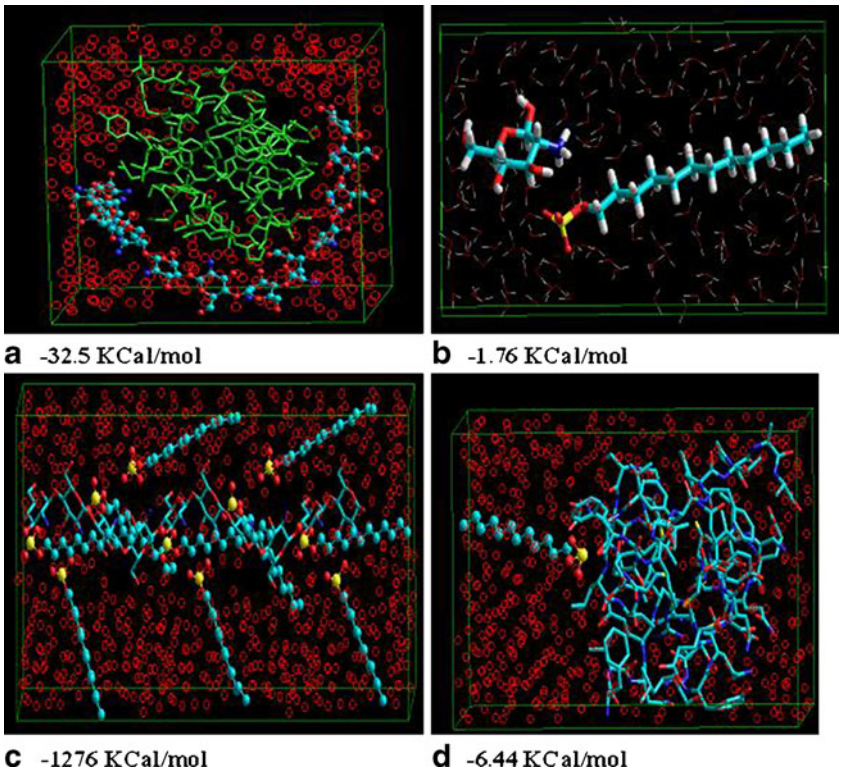

Fig. 4. Modeling of chemical interactions between **a** insulin and chitosan, **b** glucosamine (monomer of chitosan) and SLS, **c** chitosan and SLS, and **d** insulin and SLS

ing effect with polymethacrylic acid–chitosan-polyether nanoparticles. It can be concluded that chitosan–SLS complex is able to protect insulin from aggressive stomach environment.

Pharmacological Activity of Insulin-loaded Chitosan–SLS Nanoparticles

In order to confirm the potential use of nanoparticles loaded with insulin for oral delivery, the pharmacological effects were evaluated in diabetic rats. Figure 6 illustrates changes in blood glucose after oral administration of insulin-loaded nanoparticles. A significant difference in plasma glucose reduction (percentage relative to the initial value) between control and nanoparticle group was observed especially 2 h after administration ($p<0.05$). As anticipated, non-protected insulin did not show any activity, this allows the conclusion that this system may be useful in insulin oral

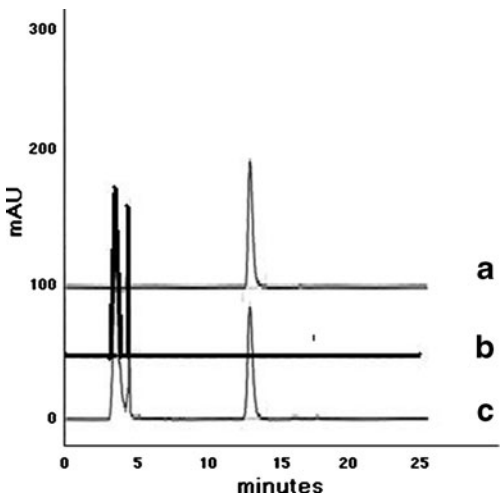

Fig. 5. RP-HPLC chromatograms of insulin fresh standard (*A*), blank (*B*) and insulin extracted from chitosan–SLS nanoparticles using 1% Polysorbate 80 (*C*)

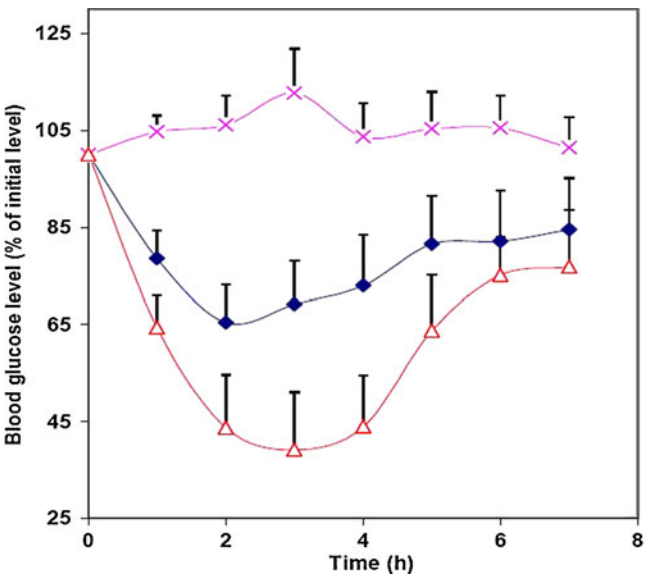

Fig. 6. Changes in blood glucose level *versus* time profiles after a single oral administration of chitosan–SLS nanoparticles (*diamond*) to STZ-diabetic rats compared to free insulin solution given orally as a control (*multiplication sign*) and s.c injection (*triangle*). Results are expressed as mean±S.E.M ($n=7$ per group). The oral nanoparticles were statistically different when compared with the control at time intervals up to 5 h ($p<0.05$)

delivery due to its ability to protect insulin in GIT tract and facilitated its absorption resulting in a pharmacological activity.

CONCLUSION

A successful attempt to deliver insulin orally is carried out. The system used applied solubilization of polyelectrolyte complex made from insulin-chitosan in SLS micelles. Excess of SLS interacted with excess chitosan-forming gelled particles having sizes in the vicinity of 250 nm. These particles can protect insulin in acidic medium but tend to aggregate in SIF. Although their delivery system was to protect insulin, the activity of the absorbed insulin is modest when compared to SC. This system can be developed to use less insulin and a reduction in the particle size may improve insulin availability.

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