**INTRODUCTION:** Since the parenteral administration is the only route of insulin delivery, alternative routes of administration (oral, nasal, rectal, pulmonary and ocular) have been extensively investigated. Among them, the oral route seems to be the most convenient and physiological because insulin undergoes a first hepatic bypass, thus warranting a primary effect by inhibiting hepatic glucose output. However, insulin is strongly degraded by proteolytic enzymes in the gastrointestinal tract.

In addition, this peptide is very poorly absorbed after oral administration. In order to protect it from biodegradation and to improve its intestinal absorption, insulin has been associated to antiproteases, hydrogels, or combined with absorption enhancers such as cyclodextrins, bile salts and surfactants. Therefore, a carrier system is needed to protect protein drugs from the harsh environment in the stomach and small intestine, if given orally. In recent years, many strategies have been developed to enhance oral protein delivery. Among these approaches, nanoparticulate systems have attracted interest in drug delivery due to the following reasons. Firstly, nanoparticles are able to protect active agents from degradation, secondly they can improve the drug mucosal transport and thirdly particulate systems provide controlled release.

**ABSTRACT:** The overall objective of this research is to improve the oral bioavailability of insulin through encapsulation in nanoparticles formulated by "ionotropic pre-gelation followed by polyelectrolyte complexation technique". The preparation variables such as initial drug concentration, polymer: polymer ratios, crosslinker concentration, stirring speed, stirring time, pH of drug / polymer mixture were investigated to study the effect of variables on nanoparticles size and drug entrapment efficiency. The optimum formula of insulin-loaded nanoparticles was tested for insulin release in three different pH media. The pharmacological activity of insulin-loaded nanoparticles was evaluated following oral dosage in diabetic rats and then study whether insulin-loaded nanoparticles would induce hypoglycemic effect after oral administration to diabetic rats. The optimum formula of nanoparticles improved insulin release characteristics. Thus, the polymer matrix provided protection for insulin in acidic gastric medium and allowed prolonged insulin release in alkaline intestinal medium. *In-vivo* results indicated that nanoparticles kept insulin bioactivity and its hypoglycemic effect after oral administration of insulin-loaded nanoparticles to diabetic rat model. It was found that natural biodegradable nanoparticles are a promising device for oral insulin delivery.

**Keywords:** Ionotropic pre-gelation, Nanoparticles, Insulin, Alginate, Chitosan

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properties for encapsulated drugs. In recent years, ion gelation or polyelectrolyte complex formation (PEC) has drawn increasing attention for producing nanoparticles containing peptides. The nanoparticles prepared by this method have several characteristics favorable for cellular uptake and colloidal stability, including suitable diameter and surface charge, spherical morphology, and a low polydispersity index indicative of a relatively homogeneous size distribution.

In addition, this method has the advantage of not necessitating aggressive conditions such as the presence of organic solvents and/or sonication during preparation; therefore, minimizing possible damage to proteins and peptides during ion gelation formation. Insulin was also encapsulated in polymeric biodegradable nanocapsules, nanospheres or microparticles associated or not to surfactants or antiproteases. Among controlled release formulations, polymeric nano and microparticles have shown interesting promise for protein delivery. Nanoparticulate hydrogels consisting of alginate, agar, agarose, chitosan, or synthetic polymers have been developed and tested over the past two decades. Nanoparticulate delivery systems have the potential to improve protein stability, increase the duration of the therapeutic effect and permit administration through non-parenteral routes. Special attention has been given to mucoadhesive particles which maintain contact with intestinal epithelium for extended periods, promoting penetration of active drug through and between cells due to the concentration gradient between nanoparticles and intestinal membrane.

In fact, insulin was observed to be directly internalized by enterocytes in contact with intestine, and retention of drugs at their absorptive sites by mucoadhesive carriers is a synergic factor. Furthermore, uptake of nanoparticles by the M cells of the Peyer’s patches was demonstrated, being absorbed transcellularly, serving as a major gateway for nanoparticle absorption as well as absorption through the much more numerous gut enterocytes. Polymers such as alginate and chitosan have been described as biocompatible, biodegradable and mucoadhesive, enabling numerous pharmaceutical and biomedical applications including the design of controlled release devices.

The purpose of this study was to evaluate the influence of calcium chloride and the addition of dextran sulfate on physicochemical and biological parameters involved in optimization of alginate–chitosan nanoparticles for oral delivery of insulin.

Physicochemical factors were analyzed in terms of reducing particle size to increase nanoparticle uptake, obtaining stable nanoparticles in suspension, increasing insulin entrapment efficiency to increase process efficiency and controlling insulin release in simulated digestive fluids. Differential scanning calorimetry (DSC), Fourier transform infrared spectroscopy (FTIR) and x-ray diffraction were used to confirm the presence of various formulation components in the nanoparticle structure, and to determine interactions between alginate and chitosan, and between chitosan and dextran sulfate. After physico-chemical characterization of formulated nanoparticles, the biological efficacy of insulin-loaded nanoparticles was determined after oral administration in diabetic rats.

**MATERIALS AND METHODS:**

**Materials:** Recombinant human insulin dry powder (Sigma Aldrich Chemie, France) was used as a model drug, sodium alginate (Al-Shark Al-Awsat Co. Cairo, Egypt) and chitosan (50 KDa) (Gama group Co. Cairo, Egypt) were used as dispersion base and polymer matrix for the drug, dextran sulphate (Al-Shark Al-Awsat Co. Cairo, Egypt) was used as drug entrapment enhancer, calcium chloride dehydrate (Gama group CO. Cairo, Egypt) was used as cross-linking agent, streptozotocin (NCER center, Mansoura, Egypt) was used to make rats diabetic. Lyophilized insulin powder (Sigma Aldrich Chemie, France) was used as reference formulation for comparison in in-vitro insulin release.

Actrapid® INN- insulin human (rDNA) (Novo Nordisk145 A/S, Denmark) was used as reference formulation for the relative bioavailability test. All solvents used were of analytical grade.

**Methods:**

**Drug-Polymer Compatibility Study by DSC**

**Thermal Analysis:** The powder samples (4 mg) (drug alone, 1:1 drug - polymer ratio in physical mixture) or 4 mg of blend film (1:1 drug-polymer...
ratio in blend film) were hermetically sealed in perforated aluminum pans and heated at constant rate of 10 °C/min over a temperature range of 30°C to 350 °C. The system was purged with nitrogen gas at the rate of 100 ml/min to maintain inert atmosphere. All samples were run in triplicate 4.

Fourier Transform Infrared (FTIR) Spectroscopy: The powder samples (physical state or film sample) were gently mixed with 300 mg of potassium bromide (KBr) powder compressed into disks at a force using a manual tablet presser. For each spectrum a 16-scan interferogram was collected with 4 cm⁻¹ resolution in the mid-IR region at room temperature. All samples were run in triplicate and the data presented are the average of the three measurements 5.

X-Ray Diffraction (XRD): The powder samples (4 mg) (drug alone, 1:1 drug – polymer ratio in physical mixture) or 4 mg of blend film (1:1 drug-polymer ratio in blend film) were exposed to a monochromatic nickel-filtered copper radiation (45 kV, 40 mA) in a wide-angle X-ray diffractometer (advanced diffraction system, Sci. 008/ntag Inc., USA) with 2θ angle 6.

Characterization of Optimum Conditions Affecting Nanoparticles Size: Nanoparticles were prepared based on ionotropic pre-gelation followed by complexation of biomaterials carrying opposite charges under controlled pH conditions. The formulation was prepared at room temperature under magnetic stirring at (600, 800 and 1000 rpm) for (30, 60 and 90 min). Ionotropic pre-gelation involves dropwise extrusion of 7.5 ml of (0.18, 0.2, and 0.22% w/v) calcium chloride solution into 117.5 ml of a solution containing alginic sodium salt. Complexation then involves dropwise addition of 25 ml of a solution of chitosan for stabilization of pre-gel nuclei into nanoparticles. The final ratio between two polymers (alginate / chitosan) was kept at (4.3:1, 3:1, 2.5:1 and 2:1) and the final pH of the medium was kept at (4.2, 4.7 and 5.2). The nanoparticle suspension was then centrifuged at 4 °C in the Amicon_ Ultra-15 (Ultracel-100K) centrifuge tube with 100 kDa cut off at 15,000 rpm for 20 min to separate free polymers from nanoparticles. Nanoparticles in the dialysis tube were evaluated for their size and zeta potential. The solution collected in the outer tube was analyzed for loading efficacy 8.

In-vitro Release of Insulin from Insulin–Loaded Nanoparticles: Quantity of dried nanoparticles equivalent to about 2 mg of insulin was then re-dispersed in 5 mL of ultrapure water and placed in a dialysis membrane bag Fig. 1 with molecular cut-off of 12 kDa, tied and placed into 50 mL of dissolution media. The entire system was kept at 37 ± 0.5 °C with continuous magnetic stirring (100 rpm) and the study was carried out in three dissolution media: acidic medium, pH 1.2 (simulated gastric fluid), mild acidic medium pH 4.5 and phosphate-buffered solution (PBS), pH 7.4 (simulated intestinal fluid). At appropriate time intervals, 3 mL of the release medium was removed and 3 mL fresh medium was added into the system to maintain sink conditions. As the control, 6 mg insulin powder was finely dispersed in 300 μl distilled water to form a suspension, 100 μl aliquots (i.e. an equivalent of 2 mg insulin) of which were mixed with 5 ml of ultrapure water and placed in the dialysis bag under the same conditions of insulin-loaded nanoparticles. The withdrawn samples were centrifuged at 15000 rpm for 15 min to remove the precipitate before insulin determination by ELISA 9.
Analysis of Kinetic Model of Drug Release from the Prepared Nanoparticles: Different mathematical functions have been used to model the observed data. Both the linear and non-linear models are being used in practice for dissolution modeling. Linear models include Zero order, Higuchi; whereas the nonlinear models include First order, Korsmeyer-Peppas. Data obtained from in vitro release studies are fitted to various kinetic equations.

Evaluation of Nanoparticles Stability after Electrolyte and Non-Electrolyte Addition: The stability of the nanocomplexes was evaluated in the presence of NaCl (0.9%) and mannitol (2%), by adding both additives to the dispersion immediately after their preparation and on the next day after stirring for over 24 h. The samples were characterized by dynamic light scattering (DLS).

In-vivo Pharmacological Activity of Insulin-Loaded Nanoparticles: The research adhered to the “Principles of laboratory Animal Care” (NIH publication no. 8023, revised 1978). Methodology was approved by the faculty of pharmaceutical sciences and pharmaceutical industries ethics Committee, Future University (REC-FPSPI-7/45).

Rats were rendered diabetic by a single intraperitoneal injection of streptozotocin (65 mg/mL in pH 4.5 citrate buffer). After 2 weeks, rats with fasted blood glucose levels above 300 mg/dl were randomly grouped (n = 6) and used for experiments. These rats were fasted 12 h before and 24 h during the experiment, but were allowed water ad libitum. Test samples (1.0 mL) were administered intragastrically by gavage and blood samples (0.2 mL) collected from the tail vein. Sample was separated in two volumes, one to determine plasma glucose level and the other for insulin determination. For insulin determination, serum was separated from the blood samples by centrifugation (5000 rpm for 10 min) and stored at -80 °C until analysis.

Rats (42 rats after being diabetic and still alive) were divided into seven groups (n = 6 for each group) as follow:

- Nanoparticles were administered at insulin doses of 25, 50 and 100 IU/kg.

- Control rats were similarly administered with equivalent volume of an insulin solution (50 IU/kg), a solution of insulin (50 IU/kg) and empty nanoparticles and a dispersion of blank nanoparticles (empty nanoparticles) and then a control using subcutaneously injected insulin (2.5 IU/kg) was used.

The volume of dispersion and controls administered was 1.0 ml. Also, Aliquots were collected before and during experiment period following administration.

Plasma glucose levels were plotted against time to evaluate the cumulative hypoglycemic effect over time after insulin administration. Pharmacological availability (PA) of peroral insulin-loaded into nanoparticles and in solution was determined as the relative measure of the cumulative reduction in glucose blood levels compared to a 100% availability of the control insulin administered subcutaneously at a dose of 2.5 IU/kg.

Plasma glucose level was determined using the glucometer and expressed as a percent of the baseline plasma glucose level. Serum insulin concentration was measured by a solid two-side enzyme immunoassay (ELISA test kit, Mercodia, Uppsala, Sweden) based on the direct sandwich technique in which two monoclonal antibodies are directed against separate antigenic determinants on the insulin molecule. Serum was separated from blood by centrifugation at 5,000 rpm for 10 min and then added to each well of a 96 well microplate and reading spectroscopically at 450 nm.

Statistical Analysis: The cumulative hypoglycemic effect and the cumulative amount of insulin delivered to plasma were calculated for
each rat, and a one-way analysis of variance (ANOVA) used to evaluate treatment differences. All statistical analyses were performed with the SPSS software package (SPSS for Windows 14.0, SPSS, USA)\textsuperscript{14}.

RESULTS AND DISCUSSION:
Drug-Polymer Compatibility Study by DSC Thermal Analysis: Form Fig. 2: DSC thermogram of insulin showed one broad endothermic peak at 71.3 °C and one exothermic peak at 264.7 °C. Endothermic peak represents denaturation process and water loss temperature. The exothermic peak represents decomposition or degradation process\textsuperscript{4}.

FIG. 2: DSC THERMOGRAMS OF ALGINATE, CHITOSAN, DEXTRAN SULFATE, INSULIN, PHYSICAL MIXTURE AND BLEND FILM

DSC thermogram of insulin / polymers (physical mixture) showed broad endothermic peak at 80.9 °C and two exothermic peaks at 245 °C and 305.6 °C. Endothermic broad peak represents the coalescence of both isolated endothermic polymers peaks and denaturation of insulin while exothermic peaks represent decomposition temperature of alginate and chitosan, respectively\textsuperscript{15}.

DSC thermogram of insulin / polymers (blend film) showed one broad endothermic peak at 77.3 °C. This endothermic peak represents the coalescence of both isolated endothermic polymers peaks and denaturation of insulin\textsuperscript{15}.

From above results, Peaks of physical mixture appeared to be combination of each material but they were different from those of molecular state (blend film) probably because of complexation of polyelectrolytes resulted in new chemical bonds. Endothermic peak of blend film was recorded at 77.3 °C, an intermediate and broader peak value compared with individual polyelectrolytes, which was interpreted as an interaction between both components\textsuperscript{16}. Absence of characteristic peaks of the drug in molecular state (blend film) could indicate that drug was not in crystalline state, but is in amorphous state after entrapment with the polymer because drug crystals completely dissolve inside the polymer matrix during the scanning of temperatures or because the drug remained dispersed at molecular level inside the solid dispersion after the formation of drug/polymers blend film\textsuperscript{16}.

Drug-Polymer Compatibility Study by FTIR: From Fig. 3: FTIR spectrum of insulin showed amino acids characterized by asymmetrical \( N–H \) bending band near 1655 cm\(^{-1}\), a symmetrical bending band near 1548 cm\(^{-1}\).\textsuperscript{17}

FTIR spectrum of insulin/polymers (physical mixture) revealed simple shifts of characteristic band of insulin from 1655 to 1654 cm\(^{-1}\), for alginate from 1415 to 1437 cm\(^{-1}\) and from 1324 to 1322 cm\(^{-1}\), for chitosan from 1554 to 1548 cm\(^{-1}\) and from 1655 to 1654 cm\(^{-1}\), for dextran sulphate from 1235 to 1251 cm\(^{-1}\) and from 1020 to 1027 cm\(^{-1}\).\textsuperscript{4,17}

FTIR spectrum of insulin/polymers (blend film) revealed shifts of characteristic bands as follow: (I) insulin characteristic bands from 1655 to 1654 cm\(^{-1}\) and from 1548 to 1553 cm\(^{-1}\) (II) alginate bands shifted from 1607 to 1619 cm\(^{-1}\), from 1415 to 1436 cm\(^{-1}\), 1324 to 1322 cm\(^{-1}\) (III) chitosan characteristic bands from 1554 to 1553 cm\(^{-1}\), from 1061 to 1060 cm\(^{-1}\), from 1029 to 1027 cm\(^{-1}\) (IV) dextran sulphate characteristic bands from 1235 to 1254 cm\(^{-1}\), from 1020 to 1027 cm\(^{-1}\). From above results, it is well established that the carbonyl and sulphate groups of the anionic polymers (alginate & dextran sulphate)
may interact with the amino group of chitosan and form an ionic complex. Observed changes in the absorption bands of the amino groups, carboxyl groups, amide bonds and sulphate groups could be attributed to an ionic interaction (intramolecular and intermolecular hydrogen bonds) between the carbonyl group of alginate and the amide group of chitosan. The characteristic absorption bands of insulin appeared in drug/polymer blend film with significant changes in bands, which probably indicate that insulin molecule was filled in the polymeric network. These results indicate that the carboxylic groups of alginate associate with amino groups of chitosan through electrostatic interactions to form the polyelectrolyte complex 17.

Drug-Polymer Compatibility Study by XRD: From Fig. 4: X-ray diffraction pattern of alginate showed two peaks at 13° and 21°, pattern of chitosan Fig. 4 showed two strong peaks at 10.3° and 21.8°, pattern of dextran sulphate showed one broad peak at 18.3° and pattern of insulin showed partial sharp crystalline peaks which are characteristics of a macromolecule with some crystallinity 18.

FIG. 4: X-RAY DIFFRACTION PATTERN OF ALGINATE, CHITOSAN, DEXTRAN SULFATE, INSULIN, PHYSICAL MIXTURE AND BLEND FILM

X-ray diffraction pattern of physical mixture showed no changes in the crystalline nature of the individual components 19. X-ray diffraction pattern of blend film showed sharp reduction in crystalline peaks, this observation indicated that the crystalline structure of the polymers and insulin was disrupted after being combined in molecular state because of electrostatic interaction between polymers and between polymers with insulin 19. These results indicated that insulin was molecularly dispersed in the polymeric matrix though drug / polymers electrostatic interaction 20.

Characterization of Optimum Conditions Affecting Nanoparticles Size: From Table 1: The effect of alginate: chitosan mass ratio on particle size showed an increase of size from (348 ± 12) to (1329 ± 31) nm with decreasing alginate: chitosan mass ratio from 4.3:1 to 2.0:1. These relationships provide a processing window for manipulating particles in both nano and microsize range and optimizing the nanosize for intended applications 21. The viscosity of polymer solution was found to affect the nanoparticle size prepared by ionic pre-gelation method. Decreasing the viscosity of polymer solution caused the mean particle size to shift towards a lower particle size. Increasing the viscosity of polymer solution, larger droplets would be formed and consequently, microparticles with large particle size would be formed 22.

The zeta potential value of chitosan / alginate complex at weight ratio 2:1 was further increased to (+ 10.2 ± 1.1) mV because of the accumulation of excessive positive charge from chitosan 23. Polydispersity index increased with increasing chitosan concentration may be due increased aggregation of prepared particles 23.

Calcium chloride used to form the pre-gel with alginate had an important effect on particle size, with diameters increasing from (324 ± 19) to (518 ± 23) nm with increasing calcium chloride concentration. During formation of the pre-gel state, alginate polyanions nucleate around calcium ions, forming distributed nuclei, which increase in size with further addition of the cation due to lateral association of a number of alginate chains 22, 23. Zeta potential was lower than -30 mV for all conditions which shows higher electrostatic stabilization of nanoparticles in suspension, suggesting low aggregation tendency. Also, it was found that higher concentration of calcium chloride caused higher neutralization of negative charge of alginate anions and then increasing of zeta potential occurred 24.

A mean particle diameter of (634 ± 31) nm was obtained at 600 rpm. At 800 rpm this value remained statistically similar at (448 ± 17) nm but increased to (1045 ± 30) nm at 1000 rpm. Mixing intensity seems to influence and promote interaction between Ca²⁺ and alginate G-residues at higher speed intensity, increasing alginate inter-
molecular contact. The mean particle size was increased from \((518 \pm 20)\) nm to \((879 \pm 27)\) nm with stirring time from 30 to 90 min, it was concluded that increasing mixing time would increase the time of exposure of G-residues of alginate to \(\text{Ca}^{+2}\) of the crosslinker which induce the diameter of the "egg box" structure of the pre-gel and when the mixing time induced to 90 min would allow the formation of microparticulate structures instead of nanosize range.

Zeta potential and surface charge of nanoparticles was negative at all batches but at high stirring time (90 min) and stirring speed (1000 rpm), positive charges of both chitosan and calcium moieties would be permitted to complex with anionic moieties of alginate which reduced negative surface charge of alginate core and then increase zeta potential.

<table>
<thead>
<tr>
<th>Parameter / Variation</th>
<th>Effect of parameter variations on nanoparticle size</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alginate: chitosan Ratio</td>
<td>Microsize 875 ± 16 nm Medium 412 ± 10 nm High 518 ± 23 nm Very high 348 ± 12 nm</td>
</tr>
<tr>
<td>Crosslinker conc.</td>
<td>(2:1, 2.5:1, 3:1, 4:3:1)</td>
</tr>
<tr>
<td>(0.18, 0.20, 0.22% w/v)</td>
<td>324 ± 19 nm</td>
</tr>
<tr>
<td>Stirring speed</td>
<td>634 ± 31 nm 448 ± 17 nm (1045 ± 30) nm</td>
</tr>
<tr>
<td>(600, 800, 1000 rpm)</td>
<td>518 ± 20 nm 467 ± 13 nm 897 ± 27 nm</td>
</tr>
<tr>
<td>Stirring time</td>
<td>518 ± 20 nm 467 ± 13 nm 897 ± 27 nm</td>
</tr>
<tr>
<td>(30, 60, 90 min.)</td>
<td>Microsize 542 ± 12 nm 961 ± 19 nm</td>
</tr>
<tr>
<td>pH of the medium</td>
<td>518 ± 20 nm 467 ± 13 nm 897 ± 27 nm</td>
</tr>
<tr>
<td>(4.2, 4.7, 5.2)</td>
<td>542 ± 12 nm 961 ± 19 nm</td>
</tr>
<tr>
<td>Order of chitosan addition</td>
<td>Addition of chitosan before crosslinker would give larger nanoparticles with high polydispersity index</td>
</tr>
</tbody>
</table>

The pH range was chosen to allow contrary charges of polyelectrolytes in order to provide the formation of nanoparticles. The decrease of pH from 5.2 to 4.7 slightly decreased the mean particle size of obtained nanoparticles, but the opposite effect was observed by decreasing the pH from 4.7 to 4.2 since the mean size of particles significantly increased when pH of aqueous solution reached values around 4. It is believed that at this pH range, alginate approaches its pKa values, the first of guluronic acid around 3.7, and a significant part of it starts precipitating and aggregating, which may contribute to increase the particle size mean value.

It was demonstrated that an alginate solution of pH 4.7 generally produces smaller particle sizes when combined with chitosan. It can be explained by the fact that as chitosan is poorly water soluble at neutral or alkaline pH, its solution is prepared under acidic conditions. Chitosan is likely to precipitate out from solution upon addition of an alginate solution with higher pH resulting in less chitosan available for nanoparticles formation.

Also as the pKa of chitosan is reported to be "6.5", an alginate solution of neutral pH, upon addition, would result in the majority of amine groups of chitosan being un-protonated and, therefore, unable to participate in ionic interactions with alginate. At pH (4.2 & 5.2), alginate and chitosan would be precipitated in the medium and formed large aggregates and polydisperse particle size distribution and caused heterogenous system.

The addition of chitosan after calcium chloride produced smaller nanoparticles (370 ± 16) nm compared to those obtained with chitosan added before calcium chloride (846 ± 28) nm. This may be due to that when calcium chloride is added as the first compound into the alginate solution, the interaction between calcium ions and alginate occurs at oligopolyguluronic sequences, and compact egg-box structures are formed. The addition of chitosan as the second compound then stabilizes this system to give small nanocapsules.

The addition of calcium chloride before chitosan formed smaller nanoparticles with negative charged nanoparticles due to interaction between polyguluronic residues with calcium cations while addition of chitosan firstly caused neutralization of both guluronic and mannuronic residues which caused increasing of zeta potential. Addition of chitosan firstly caused large nanoparticulate
Characterization of Optimum Conditions Affecting Entrapment Efficiency: From Table 2:
By increasing the insulin concentration 5 mg to 7 mg added to sodium alginate solution, the entrapment efficiency (EE) increased from (73.6 ± 19%) to (82.10 ± 11%). It can be explained that, encapsulation of proteins within nanoparticles depends on unsaturated sites to formation of hydrogen and electrostatic bonds on alginate chains. It is obvious that insulin interacts with carboxylic groups of alginate chains via electrostatic bonds.

At alginate: chitosan ratio (4.3:1), entrapment efficiency would be highest at (79 ± 12%) because chitosan would coat alginate nuclei which would penetrate the alginate porous gel network and then would prevent drug leakage. But with decreasing alginate: chitosan mass ratio from 4.3:1 to 2:1, additional chitosan concentration would be initially in contact with alginate nucleus. So, the higher is the resulting nanoparticle mass and the lower the contribution of insulin to final nanoparticles.

Zeta potential value of chitosan / alginate complex increased with decreasing alginate: chitosan ratio, at weight ratio 2:1 was further increased to (+ 12.8 ± 1.3) mV because of the accumulation of excessive positive charge from chitosan and also neutralization of negative charges on alginate molecules by the positive charges on insulin molecules. Polydispersity index increased with increasing chitosan concentration may be due increased aggregation of prepared particles.

Entrapment of insulin into alginate/chitosan nanoparticles prepared by ionotropic pre-gelation method was found to be significantly lower than those prepared by other preparation method. This may be explained upon the basis that, at higher concentrations of the components that make the bulk of the nanoparticle’s matrix, less volume is available for drug entrapment. Thus, the presence of CaCl₂ that makes the bulk of the nanoparticles and causes the particles to be smaller and denser decreased the entrapment of the drug. Thus, entrapment efficiency increased from (65 ± 11%) to...
(76 ± 18%) with increasing calcium chloride concentration from 0.18% to 0.22% \(^{32}\).

Since the isoelectric point (pI) of insulin is around 5.3, positively charged insulin would interact strongly with negatively charged alginate at pH 4.7 (pKa ∼ 3.4). Positively charged \( \text{Ca}^{+2} \) can also establish ionic bridges with carboxylic residues of insulin amino acids improving the interaction with alginate pre-gel matrix. Thus, ionic equilibrium seems to be the primary factor affecting alginate-insulin interaction. Positively charged chitosan (pKa∼6.3) then forms a complex with free negative alginate acid residues and carboxylic residues of insulin. Because of its amphoteric properties, insulin can ionically bind with both polymers at pH 4.7, improving its association with the particles. This pH is also required to preserve insulin stability since lower pH triggers fibrillation \(^{33}\).

At this pH range electrostatic interactions take place between polymers and insulin. The decrease of pH from 4.7 to 4.2 led to an increase of entrapment efficiency at \( (88 ± 15\%) \). However, when the pH of the aqueous solution was set to 4.2, the particles became much larger (>1 m). In this pH range alginate approaches its pKa value and a small part of it starts to precipitate as alginic acid. The precipitated alginate can contribute to the increased mean particle size measured by photon correlation spectroscopy. At pH (4.2 & 5.2), alginate and chitosan would be precipitated in the medium and formed large aggregates and polydisperse particle size distribution and caused heterogenous system \(^{34}\).

The introduction of dextran sulphate into alginate/chitosan nanoparticles provided a higher retention of insulin than pure nanoparticles. The increase of EE promoted by dextran sulphate could be explained by a higher network density causing physical retention of the insulin inside the nanoparticles. The electrostatic interaction between insulin and the polyanion might also favour the retention of insulin within the nanoparticles matrix. \(^{35}\)

Increasing dextran sulphate concentration from (0.01 to 0.02% w/v), entrapment efficiency would be increased from (82.7 ± 16 to 86.4 ± 20%) but there is no significant increase in EE when dextran sulphate concentration increased to 0.03% w/v. Increasing concentration of dextran sulfate decreased the zeta potential confirming the higher charge density provided by the polyanion \(^{36}\).

Increasing stirring speed from 600 to 1000 rpm, entrapment efficiency was increased from (66.8 ± 14%) to (89.6 ± 26%) while at 800 rpm entrapment efficiency was at (88.34 ± 11%). It was found that increasing of mixing intensity seems to influence and promote interaction between calcium ions and alginate G-residues at higher speed intensity, increasing alginate intermolecular contact. Entrapment efficiency was increased from (78.6 ± 10 to 84.5 ± 17%) when stirring time increased from 30 to 60 min while at 90 min; entrapment efficiency was decreased to (69.3± 21 %) \(^{37}\).

Since the alginate and \( \text{Ca}^{+2} \) used to form the pre-gel are at very low concentration, it is expected that ionic interaction is less and slower than that encountered using more conventional formulation approaches, so mixing time would promote ionic interaction and also that the interaction between insulin and polysaccharides are mainly ionic and are promoted by a longer and closer contact between opposite charges \(^{35}\). Increasing the curing time above 60 min resulted decrease in the drug entrapment efficiencies, since prolonged exposure in the crosslinking medium caused greater loss of drug through cross-linked nanoparticles \(^{35}\).

**Preparation of Insulin - Loaded Nanoparticles:**

Nanoparticles were prepared based on ionotropic pre-gelation followed by complexation of biomaterials carrying opposite charges under controlled pH conditions. A standard formulation as shown in **Fig. 5** was prepared at room temperature under magnetic stirring at 800 rpm. Ionotropic pre-gelation involves dropwise extrusion of 7.5 ml of 0.2% (w/v) calcium chloride solution into 117.5 ml of a solution at pH 4.9 containing 0.06% (w/v) alginic sodium salt, 0.02% (w/v) dextran sulphate and 7 mg of insulin (equivalent to 200 IU insulin). Complexation then involves dropwise addition of 25 ml of a solution at pH 4.6 containing 0.014% (w/v) chitosan for stabilization of pre-gel nuclei into nanoparticles. The nanoparticle suspension was then centrifuged at 4 °C in the Amicon_ Ultra-15 (Ultracel-100K) centrifuge tube with 100 kDa cut off at 15,000 rpm for 20 min to separate free polymers from nanoparticles.
Nanoparticles in the dialysis tube were evaluated for their size and zeta potential. The solution collected in the outer tube was analyzed for loading efficacy.\textsuperscript{38, 39}

**FIG. 5: TEM IMAGES OF OPTIMUM FORMULA OF NANOPARTICLES (A) UN-LOADED (B) INSULIN-LOADED**

**In-vitro Drug Release from the Prepared Nanoparticles:**

**At Acidic Medium: (pH 1.2):** From Fig. 6: At acidic pH, calcium alginate was protonated into the insoluble but swelling form, this displays properties of swelling that explains the minor drug release. The nanoparticles showed burst drug release in acidic medium (20% w/w of initial drug amount) may be due to the stability of alginate at low pH and conversion of calcium alginate to the insoluble alginic acid which tightening the gel mesh works. Therefore, the observed immediate release of insulin at pH 1.2 may be related to release from the nanoparticle surface, due to the weak interaction forces between the polyelectrolytes and the protein on the surface. However, insulin entrapped within the matrix faced an additional physical barrier. Further gastric protection against insulin release can be attributed to the more effective retention by a tight alginate network that forms at low pH.\textsuperscript{14}

**At Alkaline pH Medium:** From Fig. 6: The nanoparticles showed the maximum release of insulin about 74% of initial insulin amount. The solubility of chitosan and insulin in phosphate buffer was lower than that in acidic medium and so burst effect was not observed. Alginate polymer chain had been swollen in pH greater than 6.5 and obtains more porosity in the structure of nanoparticle resulting in more insulin release. In general, At pH 7.4 the NH\textsubscript{3}+ groups of chitosan get deprotonated to yield uncharged NH\textsubscript{2} groups and, this way, is reduced their electrostatic interactions with COO\textsuperscript{-} groups of alginate chains. Consequently, the nanoparticles begin to disintegrate along the release of the encapsulated insulin. The disintegration of alginate/chitosan nanoparticles can also be due to ion exchange between Na\textsuperscript{+} ions from the phosphate buffer and Ca\textsuperscript{+2} ions from the egg-box cavities of polyguluronate blocks of alginate.\textsuperscript{24, 39}

**Analysis of Kinetic Model of Drug Release from the Prepared Nanoparticles:** The optimum formula of insulin nanoparticles followed zero order release and Higuchi model diffusion and from Peppas plot the drug transport mechanism was found to be super case II transport as (n>0.89)\textsuperscript{38}.\textsuperscript{38}

**FIG. 6: IN-VITRO DRUG RELEASE VERSUS TIME FOR FREE INSULIN (RED SQUARES) & ENCAPSULATED INSULIN (BLUE SQUARES)**

**At Mild Acidic Medium (pH 4.5):** From Fig. 6: It was found that the insulin release was significantly prevented, in nanoparticles preparation insulin was loaded under this pH as mentioned before; when the nanoparticles experienced at this pH value, the electrostatic attraction between insulin and alginate was became stronger because of more positive charges of insulin. This pH response was absent in case of non-encapsulated (free) insulin, so free insulin was suffered from more degradation of about 63% of the initial amount of insulin.\textsuperscript{39}
Evaluation of Nanoparticles Stability after Electrolyte and Non-Electrolyte Addition: From Table 3: During the stability studies, NaCl as an electrolyte, and mannitol as a non-electrolyte similarly affected the polyelectrolyte complex (PEC) integrity. The electrolyte replaced the electrostatic interaction between the polymers causing the PEC to dissociate, which is evident in the significant reduction in the average count rate. The non-electrolyte also influenced the dissociation of the polymer assembly, but this effect was minor compared to the electrolytes. After stirring both dispersions with either NaCl or mannitol for an additional 24 h, it was observed that in the presence of mannitol the polymers were able to reassemble into complexes reaching the same concentration as before the addition of mannitol (evident from the average count rate) \(^{11}\). It was suspected that at the beginning, the non-electrolyte mannitol probably temporarily interfered in the polymer interactions reducing their affinity to associate, but after prolonged stirring of the dispersion, the polymers were able to reinstate their original state, forming the complexes irrespective of the presence of mannitol. This effect was not observed with the electrolytes, where NaCl induced a permanent dissociation of the complexes, which could not recover even after prolonged stirring \(^{11}\).

TABLE 3: EFFECT OF ELECTROLYTE (NaCl) AND NON-ELECTROLYTE (MANNITOL) ON THE STABILITY OF POLYELECTROLYTE COMPLEX

<table>
<thead>
<tr>
<th>Sample / additive / process</th>
<th>Particle size (nm)</th>
<th>Polydispersity index (PDI)</th>
<th>Average count rate (kcps)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PEC</td>
<td>365 ± 13</td>
<td>0.292 ± .01</td>
<td>282 ± 12</td>
</tr>
<tr>
<td>PEC + mannitol</td>
<td>340 ± 10</td>
<td>0.203 ± 0.06</td>
<td>224 ± 10</td>
</tr>
<tr>
<td>PEC + mannitol + stirring (24 h)</td>
<td>312 ± 16</td>
<td>0.221 ± 0.03</td>
<td>218 ± 16</td>
</tr>
<tr>
<td>PEC</td>
<td>365 ± 13</td>
<td>0.292 ± .01</td>
<td>282 ± 12</td>
</tr>
<tr>
<td>PEC + NaCl</td>
<td>470 ± 18</td>
<td>0.315 ± 0.023</td>
<td>78 ± 7.4</td>
</tr>
<tr>
<td>PEC + NaCl + stirring (24 h)</td>
<td>625 ± 20</td>
<td>0.524 ± 0.06</td>
<td>147 ± 10.8</td>
</tr>
</tbody>
</table>

In-vivo Pharmacological Activity of Insulin-Loaded Nanoparticles: The pharmacological effect of insulin-loaded nanoparticles was evaluated in diabetic rats dosed orally at loading levels of 25, 50 and 100 IU/kg. Changes in plasma glucose compared to those dosed subcutaneously at 2.5 IU/kg and with empty nanoparticles are shown in Fig. 7. After subcutaneous administration, it was found a rapid reduction of glycemia after 2 h (50% of basal value) and then a rapid increase was found after 4 h. A significant difference in plasma glucose reduction between insulin-loaded and empty nanoparticles was observed, especially 8 to 14 h after administration (P<0.05) \(^{14}\). At an insulin dose of 100 IU/kg, a faster onset of action was elicited compared with doses of 25 or 50 IU/kg. The reduction in blood glucose was less in the animals receiving the dose of 25 IU/kg compared with those dosed with 50 and 100 IU/kg. However, the overall plasma glucose levels were similar for doses of 50 and 100 IU/kg. In addition, these higher two dose levels resulted in similar values of cumulative hypoglycemic effect, and similar minimum blood glucose (55% of basal level) \(^{14}\).

A dose - response effect was observed between 25 and 50 IU/kg but absent between 50 and 100 IU/kg, which may be due to saturation of the insulin absorption sites at 50 IU/kg. The reduction in blood glucose was less for animals receiving 25 IU/kg, which could indicate that the level of absorption saturation had not been reached. The absence of hypoglycemic effect after administration of empty nanoparticles confirms that the decrease of blood glucose levels was exclusively due to the physiologic effect of insulin \(^{13, 14}\).

FIG. 7: GLYCEMIA AFTER ORAL ADMINISTRATION OF ORAL INSULIN-LOADED NANOPARTICLES AT 25 IU/kg (BLUE SQUARES), 50 IU/kg (RED SQUARES), 100 IU/kg (GREEN TRIANGLES) COMPARED TO 2.5 IU/kg DELIVERED SUBCUTANEOUSLY (BLACK SQUARES) AND EMPTY NANOPARTICLES (BLUE STARS). BASAL VALUE (> 350 mg/dl)

To investigate whether nanoparticles enabled intestinal absorption of free insulin, diabetic rats
were administered a suspension of empty nanoparticles in insulin solution (50 IU/kg), and comparisons drawn to a group administered insulin solution alone (50 IU/kg). As observed in Fig. 8, both groups showed small hypoglycemic effects between 2 and 6 h after administration. However, this effect appeared sooner and was minor compared to the hypoglycemic effect of the insulin-loaded nanoparticles. A small fraction of the insulin could be absorbed through the intestinal wall exerting a hypoglycemic effect as observed by others. In fact, a small amount of human insulin after oral solution administration was detected in the blood by ELISA, indicating that it was directly absorbed. The direct uptake of insulin has been attributed to specific insulin receptors in intestinal enterocytes and rapid internalization by the epithelial cells to the interstitial space from which it reached the blood circulation.

In addition, the extent of release within the gastrointestinal tract may be much less than observed in-vitro due to the viscous lumen and fasted animal conditions. The removal of insulin from the site of absorption may be considered to be one of the barriers against free insulin absorption. For this reason, increasing the retention time on the mucosa due to adhesive particles can improve bioavailability. It is also possible that uptake of nanoparticles by Peyer's patches in the posterior ileum can contribute to the maintenance of insulinemia and consequent hypoglycemic effect.

**CONCLUSION:** In this work, the effect of insulin entrapment into alginate/chitosan nanoparticles has been examined. Formation of alginate/chitosan nanoparticles was dependent on alginate:chitosan mass ratio and pH of production. Insulin-loaded nanoparticles were successfully produced at pH 4.7 and with alginate:chitosan mass ratio of 4.3:1 and with high insulin entrapment efficiency. It was shown by DSC, FTIR and XRD that insulin preserved its intrinsic conformation after mixing with polymers and following release in both gastric and intestinal simulated conditions and it was found that optimum formula could reduce the release in the low pH gastric environment, while maintaining a high release in the intestinal environment.

Additionally, in-vivo results clearly indicated that the insulin-loaded nanoparticles could effectively reduce the blood glucose level in a diabetic rat model. In conclusion, polyelectrolyte ionotropic pre-gelation appears to be a promising approach to produce nanoparticles containing insulin, as well as potentially other therapeutic polypeptides for oral delivery.
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CONFLICT OF INTEREST: Nil

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