Insulin-loaded mucoadhesive nanoparticles based on mucin-chitosan complexes for oral delivery and diabetes treatment

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Abstract

In this study, insulin-loaded nanoparticles (NPs) were prepared via self-gelation method using chitosan and aqueous soluble snail mucin as natural polymers. Herein, mucins were ionically interacted with chitosan at different concentrations to obtained insulin-loaded NPs, labelled as A1 (1:1) (i.e., chitosan 2 % w/v + mucin 2 % w/v) and A2 (2:1) (chitosan 4 % w/v + mucin 2 % w/v), using poloxamer and poly vinyl alcohol as solid surfactant. Such formulation was selected to provide the necessary dynamics for the formation of the nanoparticles while maintaining the surface properties that will favor the encapsulation of insulin. Each system was characterized in terms of their particle size distribution, morphology, zeta potential, and polydispersity index. *In vitro* release of insulin was evaluated in acidic solution (pH 1.2) and phosphate buffer solution (pH 7.4), and the hypoglycaemic activity was evaluated in diabetes rats. The prepared insulin-loaded NPs displayed particles with relatively smooth surfaces and an average particle size of 479.6 and 504.1 nm for A1 and A2, respectively. Zeta potential and polydispersity index, ranged from 22.1–31.2 mV and 0.155–0.185, respectively. The encapsulating efficiency for the systems A1 and A2 were 88.6 and 92.5, respectively, and a self-sustained release of encapsulated insulin was observed for over a period of 8 h. *In vivo* studies revealed a pronounced hypoglycaemic effect in diabetic rats after peroral administration of the insulin-loaded NPs compared to the effect caused by free oral insulin

solution. In addition, both the pharmacokinetic and toxicity results showed low plasma clearance of insulin and no signs of toxicity on the liver enzyme and cell viability, which suggested good biocompatibility of the NPs formulations. Overall, the formation of NPs of insulin with chitosan and snail mucin represents a potentially safe and promising approach to protect insulin and enhance its peroral delivery.

Keywords

Insulin, nanoparticles, mucin, chitosan, oral delivery

1. Introduction

In general, diabetes patients rely on exogenous insulin to maintain blood glucose homeostasis, although there are other oral hypoglycaemic agents used to reduce high blood glucose levels. Currently, insulin therapy is considered as the main treatment for diabetes, which is always administered subcutaneously. This route is considered highly invasive with many associated risks (Zambanini *et al.,* 1999; Makhlofa *et al.,* 2011). Additionally, suboptimal control of blood glucose levels and poor compliance among patients using subcutaneous administration have been reported (Owens *et al.,* 2003; Khafagy *et al.,* 2007; Lin *et al.,* 2008). To date, diabetes has no permanent cure and initiation and management are individualized, based on glycemic control, long-term benefit and tolerability (Garg *et al.,* 2005; Becker and Frick, 2008). However, in long-term management, non-compliance of patients with injection and poor tolerability have been documented, resulting in poor glycemic control (Khafagy *et al.,* 2007).

Oral delivery of insulin could be a preferred drug administration route for diabetics, as it would be easily administered, mimic normal physiological insulin release, improve glucose homeostasis and avoid the inconvenience of regular injection of insulin (Zambanini *et al.,* 1999). However, the bioavailability and therapeutic effect of oral insulin are hindered by poor absorption, which is due to its high molecular weight, acidic environment and enzymatic degradation in the stomach (Wei *et al.,* 2015; Lei *et al.,* 2017). In order to address these problems, several studies on the possibility of oral insulin delivery have been previously described (Hosseininasab *et al.,* 2014; Pereira *et al.,* 2016;

Sheng *et al.,* 2016). Despite a number of studies to overcome the barriers to oral delivery of insulin, based on our knowledge, none have hitherto progressed beyond the laboratory bench or achieved market application (Abdallah *et al,* 2011; Lei *et al.,* 2017). Therefore, the race continues to achieve oral delivery of insulin, through development of polymeric carriers or other new formulations of market value.

Materials such as synthetic polymeric nanoparticles (NPs) (Zhi *et al*., 2012) and inorganic-organic hybrid nanocomposites have also been used for the delivery of insulin (Susanta *et al*., 2017). Moreover, fluorescent polymeric nanoparticles have been exploited for biomedical applications (Sarmento *et al*., 2007). Our work focuses on the use of natural polymers motivated by their poential advantages as drug carrier. For instance, natural polymer such as mucin (polyanion) and chitosan (polycation) used as nanocarriers may represent a closer approach to reach a successful oral insulin delivery nanosystem because they offer good mucoadhesive, drug protection and facilitate drug absorption through the intestinal mucosa. Additionally, these biopolymers have been proved to play an important role regarding insulin oral bioavailability (Builders *et al*., 2008; Bin *et al*., 2010). We believe that a system based on chitosan and mucin could be a carrier for drug delivery due to their low toxicity, low immunogenicity and biodegradability, which constitute key parameters for the formulation of delivery systems (Keiuke et al., 2006; Builders *et al*., 2008; Abdallah *et al*., 2011).

In this work, we hypothesize that the hybridization of chitosan and mucin, utilizing their complementary surface charges, might improve or provide unique properties as nanocarrier. Indeed, NPs using mucoadhesive natural polymers are at the frontier of research efforts in the field of drug delivery. In this case they are aimed at circumventing the poor absorption and gastrointestinal degradation of peptide drugs, to pave way for enhanced therapeutic improvement, considering safety, biodegradability, non-immunogenicity and cost effectiveness (Lei *et al.,* 2017; Weijiang *et al.,* 2017). The reviewed literature has shown that NPs or nanodispersions of drugs using biopolymers via self-gelation have many advantages over other technologies. Many of these systems are stable in the gastrointestinal (GI) environment and can safeguard the encapsulated drug from the pH environment, drug efflux pump and enzyme degradation (Alessandra *et al.,* 2012; Bin

89 *et al.,* 2017). It is also worth mentioning that dopamine self-polymerization is an important tool for
90 functionalizing NP surfaces, which has been exploited in the control of NP interactions with cells
91 and proteins (Joonyoung *et al*., 2014). This method involves a brief incubation of the preformed
92 NPs in a weak alkaline solution of dopamine, followed by secondary incubation with desired
93 ligands. So-modified NPs have shown expected cellular interactions with no cytotoxicity or residual
94 bioactivity of dopamine and improved drug loading.

95 From a chemical point of view, chitosan is a natural polycationic and copolymer that has gained
96 tremendous acceptance among the players in the field of pharmaceutical and cosmetic formulation,
97 due to its good biocompatibility, biodegradability and mucoadhesive nature (Abdallah *et al.,* 2011).
98 Its suitability as a substrate for graft copolymerization of polymers for pH-sensitive biomaterial in
99 drug delivery has also been reported (Oak and Singh, 2012). The use of chitosan in NPs is a
100 promising strategy for improving oral insulin absorption and bioavailability. Chitosan-based NPs
101 have demonstrated significant improvements in transient opening tight junctions and targeting
102 receptors, which allow the absorption of insulin (George and Abraham, 2006; Oak and Singh, 2012).
103 However, a disadvantage of chitosan-based NPs for oral delivery of insulin is the ease of
104 protonation of chitosan in acidic regions, which results in insulin release and degradation before
105 reaching the absorption site (Ahmed *et al.,* 2016). Therefore, addition of a mucoaldhesive polymer
106 such as mucin, sodium alginate, or Eudragits RS among others can form a network of complexes
107 with chitosan for effective drug delivery (Tai and Gao, 2016).

108 Mucin is a component of the rich mucus that covers the surface of snails; it is rich in glycosylated
109 proteins and is highly viscous, which contributes to the gel-like nature of mucus. Recently, mucins are
110 well known as a substrate for polymer attachment, forming the basis for their use in drug delivery
111 (Adikwu *et al.,* 2005; Builders *et al.,* 2008). Pharmaceutically, mucins are used as excipients in drug
112 delivery, they have shown substantial antimicrobial effects in wound-infected mice (Builders *et al.,*
113 2008). Mucins have become an important ingredient in daily diets and play a vital role in preventing
114 diseases such as diabetes and high blood pressure (Adikwu *et al.,* 2005).

115 Considerable work on the polyeletrolyte complexes of chitosan NPs for insulin delivery has been
116 published (Andreani *et al.,* 2015). However, to our knowledge, there is as yet no documentation or

previous report on the combination of chitosan and aqueous soluble snail mucin, taking into consideration the advantages of individual polymers as carriers in drug delivery, while avoiding their specific demerits. Some instances are the ease of protonation by chitosan in an acidic environment (Ahmed *et al.*, 2016), and the poor drug loading capacity associated with mucin, both of which could be avoided by carefully engineering the compositions of the polymers for improved insulin delivery.

In this study, we focused on the preparation of chitosan-mucin NPs for oral insulin delivery, fabricated via a polyelectrolyte self-gelation method. Herein, we use chitosan and soluble snail mucin, which are biomedical materials that have individually attracted advanced frontline research into pharmaceutical applications for drug delivery.

Very interestingly, aside from the mucoaldhesive properties of chitosan and mucin, chitosan NPs can be easily synthesized or produced spontaneously through interaction with negatively-charged mucin, followed by inter- and intra-molecular cross-linking, in contrast to chemical cross-linking. They thereby avoid hazards to protein stability, arising from cross-linking chemicals during production. Furthermore, their mucoadhesive properties and the transient opening of tight junctions by chitosan could constitute a synergy for NPs to serve as vehicles for oral delivery of insulin-bypass GIT enzymes and allow prolonged release of insulin at the absorption site. Thus, the purpose of our study is the development of insulin-loaded NPs, potentially applied to oral delivery. We evaluated the effect of the chitosan to mucin concentration ratio on particle size characterization. The *in vitro* and *in vivo* behavior of insulin-loaded NPs was also assessed. Additionally, the toxicity of the NPs against liver enzymes and cell viability were similarly studied.

2. Experimental

2.1. Materials

The following materials were purchased from commercial suppliers: Chitosan (200-300 kDa, deacetylation degree 85 %) (Sigma, USA), Humulin (Eli Lily and Co. Indianapolis, USA), polyvinyl alcohol (PVA) (Jochem Chemical Co., Ltd, Nigeria), oleic acid (Wako Chem. Co. Ltd, Japan). Double deionized water was obtained from Lion Water, University of Nigeria. All other chemicals and solvents used in the study were of analytical or HPLC grades and were obtained from

147 the manufacturers without further purification. Snail mucin (also refers as sialomucin due to its
148 content in sialic acid) was obtained as previously reported with slight modification (Adikwu *et al.,*
149 2005). Briefly, the mucus secretion of snail was extracted by gently pressing the snail after it has
150 been removed from its shell until no mucus was secreted. The slimes collected from different snails
151 were pooled together in a container and macerated in water for 24 h at 20 °C to get a viscous mucus,
152 which was filtered through a muslin cloth to remove unwanted material. The resultant viscous
153 mucus was then dried using a lyophilizer (Christ-Alpha 1-2 LD Plus SCIQULP, Germany) at –40 °C.
154 The extracted mucins (aqueous soluble mucin) were collected and pulverized using an end-runner
155 mill (Pascal Engineering Co Ltd, England). The pulverized soluble mucins were collected and kept
156 in airtight container for further use (Mumuni *et al.,* 2019).
157
158 *2.2. Preparation of insulin-loaded NPs*
159
160 Insulin-loaded NPs were prepared via self-gelation as previously reported with slight modifications
161 (Sheng *et al.,* 2016). Briefly, 10 mL of chitosan 2 % w/v in acetic acid 1 % v/v was added to 20 mL
162 of mucin 2 % w/v as the aqueous phase. The mixture was mixed using high shear homogenization
163 for 10 min at 12,000 rpm (Ultra-Turrax® - IKA, T25 Germany). Then, 5 mL of insulin solution (100
164 IU/mL) was added to the mixture, which was mixed gently using a magnetic stirrer for 3 min at 200
165 rpm. Thereafter the aqueous phase containing a mixture of 5 mL of Poloxamer-188 2 % w/v and 5
166 mL of PVA 1 % w/v was added into the phase containing the insulin and chitosan and
167 homogenized for 5 min at 12,000 rpm. The preparation was further subjected to sonication (AT 500,
168 India) at 80W for 2 min in ice bath. Finally, the aliquots of NPs were washed twice with deionized
169 water by centrifugation (10,000 rpm, 10 °C, 60 min) and thereafter freeze-dried to obtain
170 insulin-loaded NPs. The obtained batch was labelled as A1. The same procedure was repeated using
171 chitosan solution 4 % affording the batch labelled as A2. Finally, a batch labelled as A3
172 corresponding to the unloaded NPs (no insulin added) was similarly prepared. All preparations were
173 carried out in an ice bath to avoid degradation of insulin and mucin by heat especially during
174 sonication. Note: Chitosan solution in acetic acid (1 %) was prepared as following: 1 mL of acetic

was diluted to 100 mL with deionised water. The desired weight of chitosan was added into 50 mL of the acetic acid solution (1 %) and magnetically stirred for 24 h. The resulting solution was finally diluted to the required volume using double distilled water.

2.3. Characterization of insulin-loaded NPs

2.3.1. Recovery values of insulin-loaded NPs.

The amount of the NPs recovered from the formulation was calculated using the equation (1):

$$\% \text{ Recovery} = \frac{W_1}{W_2 + W_3} \times 100 \qquad \text{Eq. 1}$$

where W_1 is the weight of the NPs (g), W_2 is the amount of insulin (g) and W_3 is the amount of carrier and additives (g).

2.3.2. Differential scanning calorimetry (DSC) *and Fourier transform infrared (FT-IR) analysis.*

Thermal analyses of insulin-loaded NPs and control samples were carried using differential scanning calorimetry (DSC) (DSC-60, Shimadzu Co., Ltd., Japan). In brief, 3–5 mg of the corresponding sample were weighed and placed in an aluminium pan and hermetically sealed under inert atmosphere (N_2). The specimen and reference sample were placed in the corresponding sample holder. Measurements were done in the temperature range from 20–220 °C, with a heating rate of 10 °C/min. The measurements were performed under nitrogen flow at a rate of 20 mL/min. All thermograms were baseline corrected using an empty pan.

FT-IR spectra of insulin-loaded NPs and control samples were recorded using a Shimadzu FT-IR 800 Spectrophotometer (Shimadzu, Tokyo, Japan). Herein, 250 mg of each the test sample was mixed with KBr and pressed into a KBr disk. The spectrum of the prepared KBr disk was recorded in the wavelength region of 400 to 4000 cm^{-1} (threshold value = 1.303 cm^{-1}; resolution = 2 cm^{-1}).

201

202 *2.3.3. Morphology, particle size characteristics and surface charges of insulin-loaded NPs.*

203

204 Morphological characterization of insulin-loaded NPs was carried out using a scanning electron
205 microscope (SEM 1000, Miniscope, Japan). Samples were collected and mounted on the sample
206 holder using a double-sided adhesive tape and covered by a gold layer (5-10 nm). Thereafter, the
207 morphology was visualized at 100 kV acceleration voltage.

208 The average particle size (z-ave), polydispersity index (PDI) and zeta potential of the
209 insulin-loaded NPs were estimated using dynamic light scattering (DLS) (Malvern Instruments,
210 Japan). In each case, samples were diluted with distilled water and measured at a temperature of
211 25 °C. All measurements were performed in triplicate and averaged.

212

213 *2.3.4. Encapsulation efficiency and drug loading capacity.*

214

215 The encapsulation efficiency (EE) and drug loading capacity (DLC) of the carrier were determined
216 using previously reported methods (Franklin *et al.*, 2018) with slight modifications. Herein, a
217 sample of freshly prepared insulin-loaded NPs (20 mg) was dispersed in 5 mL of phosphate buffer
218 solution (PBS, pH 7.4) and shaken for 5 min to dissolve the free drug. The resulting dispersion was
219 centrifuged for 30 min at 20,000 rpm (Kubota Co, Japan). The amount of insulin in the supernatant
220 after centrifugation was measured by HPLC analysis as previously reported (Mumuni *et al*., 2015)
221 with slight modifications. Briefly, the chromatographic system consisted of a pump (PU-2089,
222 Jasco, Japan), a UV–Vis detector (UV-2075, Jasco, Japan), an auto sampler (AS-206, Japan) and a
223 column C18 (250 mm x 4.6 mm; particle diameter 5 µm) (Shimadzu, Japan). A mixture of acetonitrite
224 and phosphate buffer (70:30, pH 7.4) was employed as the mobile phase and the flow rate was
225 adjusted to 1 mL/min. The mobile phase was filtered through 0.2 µm cellulose acetate membrane
226 filter (Advantec, Japan) The volume of injection was 20 µL and the total run time was 8 min. The
227 detection wavelength was 227 nm and the column temperature was maintained at 25 °C.

228

229 *2.4. In vitro release study*

230

231 *In vitro* release of insulin from the NPs was evaluated using dialysis bag membrane method with
232 slight modifications. Herein, 10 mg of insulin-loaded NPs (freeze-dried) suspended in 5 mL of
233 deionised water was placed inside a pre-treated polycarbonate dialysis membrane bag (20 kDa,
234 molecular weight cut off). The bag was incubated in 200 mL of release medium (acidic medium, pH
235 1.2 and PBS, pH 7.4) at 37 °C under 100 rpm. At predetermined time intervals, 2 mL samples were
236 collected and replaced with fresh medium after each withdrawal to maintain sink conditions. The
237 withdrawn samples were filtered and thereafter analyzed by HPLC as described in section 2.3.4.

238

239 *2.5. In vivo bioactivity and pharmacokinetics studies*

240

241 Wistar rats of either sex and an average body weight of 125 ± 2.11 g were used to study the
242 pharmacological effects of the prepared insulin-loaded NPs and the reference samples. After two
243 weeks of acclimatization in the Animal House of the Department of Pharmacology (University of
244 Nigeria, Nsukka), the rats were made diabetic by administering intraperitoneally (i.p.) 1 mL of
245 alloxan monohydrate 120 mg/kg solution dissolved in phosphate buffer (pH 4.5) as previously
246 described (Nnamami *et al*., 2010). After alloxan administration, the blood glucose levels of the rats
247 were monitored using a glucometer (Accu-Check, Roche, USA) until the diabetic state was reached
248 (i.e., persistent blood glucose levels above 23 mmol/L along with signs of diabetes were considered
249 diabetic). A total of 45 diabetic rats were divided into five groups of nine rats each. Laboratory
250 animal experimentation was obtained from the Departmental Ethical Committee in compliance with
251 the internationally approved standards for laboratory animal use.

252

253 *2.5.1. Oral administration of insulin-loaded NPs.*

254

255 Administration of insulin-loaded NPs was carried out as follows: Oral administration of
256 insulin-loaded NPs batches A1 and A2 (insulin dose 50 IU/kg) were administered to rats in groups I

and II, respectively. Free insulin solution (50 IU/kg) was administered orally to group III. Group IV received free insulin solution (5 IU/kg) subcutaneously and group V was administered with unloaded NPs (batch A3). Rats were fasted for 24 h prior to the experiments and were allowed to access drinking water. The blood samples were collected from the tail vein before the administration of tests agents and at defined time intervals after dosing. The blood glucose level was evaluated using a glucometer (Accu-Check, Roche, USA). The change in blood glucose level was represented as the percentage of the initial blood glucose value before the oral administration.

For the pharmacokinetics analysis, male Wistar rats were made diabetics as described in section 2.5, randomly divided into four groups of five rats each. Group I was injected subcutaneously insulin (5 IU/kg). Groups II, III and IV received orally insulin solution (50 IU/kg), insulin-loaded NPs A1 (50 IU/kg) and insulin-loaded NPs A2 (50 IU/kg), respectively. The blood samples (500 µL) were collected from the tail of each group at a specific time intervals, centrifuged (4500 rpm, 10 min) and subsequently analysed for insulin content using the HPLC method described in section 2.3.4.

2.6. Oral glucose tolerance test of insulin-loaded NPs

Albino Wister rats were made diabetics as previously described in section 2.5. Fifteen (15) diabetes rats were randomly divided into 3 groups of 5 rats per group. The rats were fasted overnight before the experiments and were allowed to access drinking water. Rats in groups I and II received insulin-loaded NPs contain 2 (batch A1) and 4 % (batch A2) of chitosan in a dose of 50 IU/kg. Unloaded-NPs (without insulin) as control sample administered to group III. All administrations were done orally. After 1 h the rats were given glucose solution at a dose of 2.5 g/kg body weight. Thereafter, the changes in blood glucose were measured using a glucometer (Accu-Check, Roche, USA) at time intervals of 12 h. All measurements were done in triplicate and averaged.

2.7. Toxicity tests

In order to test the cytotoxicity of insulin-loaded NPs, we evaluated the effects of the formulations on liver enzymes and cells viability using liver enzyme kit (Reflotron-Plus machine, model SN747461, Germany) and 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay, respectively. For liver enzyme test, 25 rats were purchased and acclimatized in the Animal House as discussed in section 2.5. The rats were divided into 5 groups of 5 rats per group. Rats in group I received water 5 mL (served as control), while groups II and III received batch A1 (50 IU/kg) and batch A2 (50 IU/kg) orally, respectively. Animals in group IV and V received 5.0 IU/kg and 50 IU/kg of insulin solution subcutaneously and insulin solution orally, respectively. Tests agents were administered once daily for three days. Blood samples were collected 77 h after the last dose as described in section 2.5, and were analysed for changes in the liver enzyme using a Reflotron-Plus machine (Model SN747461, Germany). All tests were carried out in triplicate and averaged.

Cell viability study was carried out following the protocol described by Zhang and co-workers (Zhang *et al.*, 2013) with slight modifications. In brief, the HT-29 cells were seeded in 96-well plates (1×10^4 cells/well) with 200 µL of growth medium including 10 % fetal bovine serum (FBS) and placed in a humidified incubator with CO_2 5% for 24 h at 37 °C. The resulted monoclonal was seeded with 250 µL of insulin-loaded NPs. The unloaded-NPs (control) was similarly treated with varying concentration from 0–500 µg/mL. Thereafter, MTT (15 µL from a 2.5 mg/mL solution) was carefully added dropwise to the well and incubated under the same conditions for 4–6 h. Thereafter, dimethyl sulfoxide (DMSO) was added to dissolve the formazan crystals and the cell viability was determined by measurement of absorbance at 505 nm using a microplate spectrophotometer (Model 20137, BioTek, USA). Data were expressed as the percentage with reference to control groups.

2.8. Stability studies of insulin-loaded NPs

Storage stabilities of freeze-dried insulin-loaded NPs (batches A1 and A2) were evaluated for any change in the physical properties of the formulations. The lyophilized insulin-loaded NPs were stored in an airtight container under controlled temperature that was maintained with humidifier

(BottleORB Model 7098, Topland Co. Japan) at 28 °C for a period of 6 months. After this time, the samples were re-dispersed in distilled water and the stability of the insulin-loaded NPs was evaluated on the basis of mean particle size, PDI, zeta potential and encapsulation efficiency (EE) as described in the previous sections.

2.9. Statistical analysis

All experimental data from this study were expressed as mean ± standard error of the mean (SEM). One-way ANOVA and student's t-test are performed on the data sets generated using Statistical Package for Social Sciences (SPSS) software, (SPSS) version 13.0; SPSS, Inc., Chicago, IL). Differences were considered significant at a level of p-value ≤ 0.05. Each experiment was performed at least three times.

3. Results and Discussion

NPs based on polymeric material have been used in trials for the delivery of drug molecules such as insulin owing to superior advantages, such as biodegradability, biocompatibility, high encapsulation efficiency, cost effectiveness, high stability and prolonged release effects (Wong *et al.,* 2016). NPs have been shown to be formed through electrostatic interaction between cationic chitosan and negatively charged polymers, with applications in the loading of proteins and peptides such as insulin (Sarmento *et al.,* 2006; Tai and Gao, 2016). The insulin-loaded NPs were prepared via the self-gelation method using chitosan and soluble mucin obtained from African giant snail, via aqueous extraction as carrier polymers. The prepared NPs were obtained by electrostatic interaction of cationic chitosan with negatively charged mucin, which resulted in a new polymer entity with superior properties compared to the individual polymers. The insulin-loaded NPs were prepared with varying concentrations of chitosan (2% and 4 % w/v) as illustrated in Figure 1.

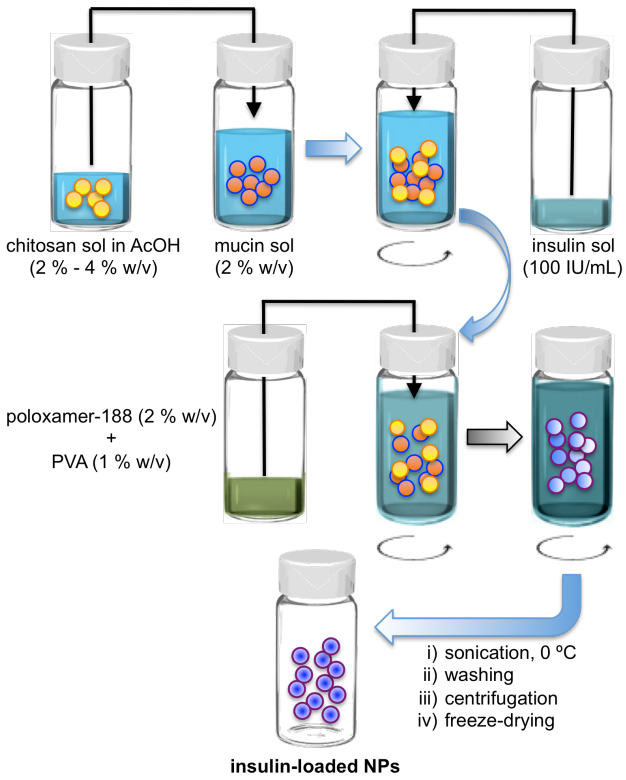

Figure 1. Schematic preparation of insulin-loaded NPs via double emulsion technique as described in the Experimental Section.

3.1. DSC and FT-IR spectra analysis

DSC was used to analyze the degree of crystallinity of the insulin-loaded NPs. The thermal behavior of a polymer was slightly affected by the presence of drug molecules, and the changes in the properties depend on the type of interaction that occurred. The DSC thermograms (Figure S1) of the NPs prepared with 2 % and 4 % chitosan presented peak melting transitions at 154.5 ± 0.3 and 124.7 ± 0.2 °C (n = 3) for A1 and A2, respectively. The enthalpies were –10.71 ± 0.42 and –22.10 ± 0.27 mw/mg respectively. The DSC thermograms of insulin and mucin presented peaks at 136.1 and 254.9, with corresponding enthalpies of 32.57 and –1.02, respectively (Figure S1). However, the transition enthalpies of chitosan could not be obtained because of the low heat of transition and unsteady baselines. Results indicate that all the NPs prepared had low enthalpy change which suggest system of low crystalinity. The DSC results also show that there were no strong chemical interaction between

356 the drug and the excipients.

357 There were on chemical interaction between the drug and the nanocarrier as confirmed by FT-IR
358 spectra analysis (Figure S2). Insulin alone showed a characteristic peak at ca. 3540 cm^{-1} and 3447
359 cm^{-1} (O-H stretching and N-H stretching), 957 cm^{-1} and 2864 cm^{-1} (C-H stretching), 2640 cm^{-1} (O-H
360 stretch of carboxylic acid), 2088 cm^{-1} (C=C stretching), 1586 cm^{-1} (C=O stretching) and 1455 cm^{-1}
361 (aromatic C=C stretching). The FT-IR spectrum of insulin-loaded NPs for batch A1 (containing
362 chitosan 2 %) showed principal characteristic absorption peaks of insulin at ca. 3590 cm^{-1} and 3292
363 cm^{-1} (O-H stretch of alcohol), 3381 cm^{-1} and 3292 cm^{-1} (N-H stretch of amine), 2926 cm^{-1} (C-H
364 stretching), 2613 cm^{-1} and 520 cm^{-1} (O-H stretch of carboxylic acid), 2061 cm^{-1} (C=C stretching) and
365 1563 cm^{-1} (C=O stretching). Batch A2 (containing chitosan 4 %) showed principal characteristic
366 absorption peaks at ca. 3566 cm^{-1} and 3509 cm^{-1} (O-H stretch of alcohol), 3261 cm^{-1} (N-H stretch of
367 amine), 2895 cm^{-1} (C-H stretching), 2895 cm^{-1} (O-H stretch of carboxylic acid), 2242 cm^{-1} and 2088
368 cm^{-1} (C=C stretching), 1316 cm^{-1} (C=O stretching) and 1474 cm^{-1} (aromatic C=C). The foregoing
369 spectroscopic data suggest that there is no incompatibility between the drug and polymers used for the
370 preparation of the NPs.

371

372 *3.2. Morphology, size and surface charge of NPs*

373 Figure 2 shows the micrograph of the insulin-loaded NPs, as characterized by scanning electron
374 microscope (SEM). The prepared sample was smooth, spherical in shape and had free-flowing
375 particles without aggregation. The average particle sizes of the insulin-loaded NPs were 479.6 ± 0.21
376 and 504.1 ± 0.50 nm for A1 and A2, respectively, while the unloaded sample (A3) shows a particle
377 size of 421 ± 2.10 nm. It is noted that there is significant increase in particle size as the concentration
378 of insulin increased. For instance, A2 has larger particle sizes compared to A1. Other studies show that
379 NPs less < 1000 nm in size favor a prolonged circulation period with improved pharmacological
380 effects (Xue *et al.,* 2013).

381

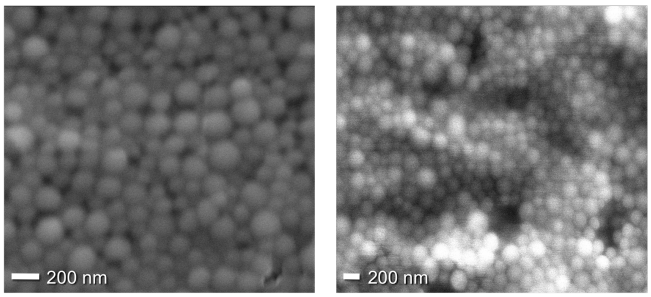

Figure 2. SEM images of samples A1 (*left*) and A2 (*right*) consisting of 2 and 4 % w/v of chitosan solution.

As shown in Table 1, the polydispersity index (PDI) and zeta potential values of sample A1 were 0.185 and 28.5 mV, respectively, and those of A2 were 0.175 and 31.2 mV, respectively. Both batches of the loaded preparation had high zeta potentials (> 25 mV), indicating good stability of the insulin-loaded NPs. A similar result was obtained for the unloaded batch of the preparation. However, the PDI (0.155) and zeta potential (22.1 mV) of the unloaded sample were lower than for the loaded batches. The positive surface charge of NPs could be ascribed to the prevalence of the chitosan charge over snail mucin, implying that the chitosan was adsorbed onto the surface of the NPs through ionic interaction with mucin and encapsulating the insulin in the core of the particles. The advantage is that positive surface charges can aid in easily transporting NPs across cell membranes, unlike neutral or negatively charged particles. Additionally, the interactions of these positively charged particles with the negatively charged mucosa walls of the gastrointestinal tract may improve the binding of the particles to the wall, and thus prolong drug release for improved absorption (Moschakis *et al.,* 2010; Murugan *et al.,* 2015). All samples showed PDIs < 1.0, indicating a unimodal particle size distribution (Figure S3). There was no significant difference in PDI between samples A1 and A2.

3.3. Encapsulation efficiency and the loading capacity

The encapsulation efficiency (EE %) and loading capacity (DLC) of the insulin-loaded NPs were very good, implying that the formulation procedure and selection of the polymer were appropriate for the formulation (Table 1). Additionally, high insulin entrapment in the NPs is presumably attributable to the high mucin content that entangles with chitosan through ionic interaction, either as a result of

hydrogen bonding or hydrophobic interaction. This could result in higher loading efficiency of insulin (Adikwu *et al.,* 2005). Similarly, Abdallah and co-workers (2011), reported that ionic interaction of chitosan NPs with hydroxypropyl methylcellulose phthalate (HPMCP) has direct link with drug encapsulation efficiency. Reports have shown that high EE % is a pointer to good drug delivery system as it also enhances its pharmacological activity (Frankline *et al.,* 2018).

Table 1

Particle size, polydispersity index (PDI), zeta potential (ZP), encapsulation efficiency (EE) and drug-loading capacity (DLC) of insulin-loaded NPs (n = 5).

Samples	PS (z-Ave, nm)	PDI	ZP (mV)	EE %	DLC %
A1	479.6 ± 0.21	0.185 ± 0.11	28.5 ± 0.02	88.6 ± 0.31	23.5 ± 1.22
A2	504.1 ± 0.50	0.175 ± 0.13	31.2 ± 0.61	92.5 ± 0.23	21.4 ± 0.23
A3	421.1 ± 2.10	0.155 ± 0.24	22.1 ± 0.01	-	-

3.4. In vitro release

In vitro release from insulin-loaded NPs followed a slow release system that lasted 10 h. As shown in Figure 3a, there was an initial relatively fast release up to 20 % within the first hour, thereafter it maintained a steady increase to a maximum of > 80 % at 10 h in phosphate buffer of pH 7.4. Figure 3b shows the amounts of insulin released through dialysis membrane with different test samples as a function of time at pH 1.2. The amount of insulin released at low pH 1.2 was found to be negligible. On the other hand, there was a significantly ($P < 0.05$) higher amount of insulin released at high pH 7.4. However, this observation was pH-dependent and the amount of insulin released significantly decreased when decreasing the pH. The maximum values of insulin release for NPs at pH 1.2 and 7.4 within the first 2 h of the evaluation were 11 and 30 %, respectively. These results could be attributed to the pH stability profile of the prepared insulin-loaded NPs due to activity of the mucin, which form part of the nanocarrier. Previous studies have shown that protonation of the amine groups of chitosan at low pH reduce its effective utilization and it is considered a major setback in using chitosan alone as oral delivery of peptide drug such as insulin (Abdallah et al., 2011; Bin et al., 2017). Interestingly,

433 the manner of release observed in this study is related to the pH responsiveness of mucin. The
434 mucoadhessiveness and decrease in the swelling capacity of mucin prevent the structural deformation
435 of the nanoparticles thereby decrease the chance of exposed the inner matrix to the dissolution fluid at
436 low pH.

437 Importantly, the release profiles of the two batches (A1 and A2) were identical and there was no
438 significant difference in the amount of drug released from the two batches of the formulation. The
439 initial fast release could be due to the unencapsulated drug, which adhered to or near the NP surface
440 that quickly detached or succumbed to pressure from the medium (Momoh *et al.,* 2015; Sharma *et al.,*
441 2016). Clinically, the initial release could be of immense medical advantage in treatment or
442 management of disease, as it constitutes the loading dose of the drug and the subsequent release could
443 serve as the maintenance dose. However, the slight increase in insulin release detected in batch A2
444 could be a result of the high concentration of insulin loaded in the formulation, and was found to be
445 not statistically significant, at $p<0.05$. The sustained release effect observed in the formulation may be
446 due to the ionic interaction between the mucin and the chitosan. This can form a multidimensional
447 entanglement network around the encapsulated drug, leading to gradual diffusion of the drug out of
448 the release medium. Such a delivery compares well with the poor sustained release in previous trials,
449 where chitosan alone was used to encapsulate insulin (Ma *et al.,* 2005; Abdallah *et al.,* 2011). The
450 result of our study shows the benefits of hybridization of polymers that would complement each other
451 as seen in our selection. The nanocarriers could be further investigation for possible pharmaceutical
452 application in an oral delivery of insulin.
453

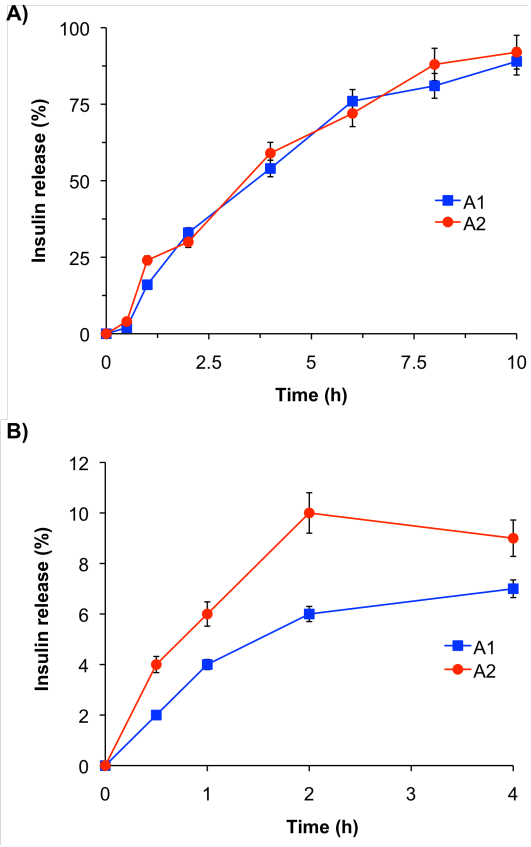

Figure 3. Release profiles of insulin from insulin-loaded NPs (systems A1 and A2) in A) phosphate buffer pH 7.4, and B) in acidic pH 1.2.

3.5. In vivo bioactivity and pharmacokinetics effect

In the present study, insulin administered subcutaneously (ins-sc) and its NPs produced significant anti-hyperglycemic effects in alloxan-induced diabetic rats, as shown in Figure 4a. There was a fast onset of action with a strong observable reduction in blood glucose levels of rats administered with subcutaneous insulin used as positive control; it reaches a maximum of 68 % within 4 h of the administration. However, the hypoglycemic effect does not last long, as the glucose levels increased within 6–8 h to nearly 100 % of initial level. There was no decrease in blood glucose levels in rats dosed with normal saline solution as negative control. This indicates that saline itself possesses no net hypoglycemic effects. However, rats in this group continued to have elevated blood glucose levels and some died as a result of this. Rats administered insulin solution orally showed no significant effects (P

> 0.05) on their blood glucose levels. On the contrary, rats in groups treated with batch A1 and A2 of insulin-loaded NPs exhibited a varying degree of blood glucose level reduction. Nevertheless, the effect observed in blood glucose levels after oral administration of insulin-loaded NPs was lower than in rats administered subcutaneous insulin solution. Interestingly, the blood glucose lowering effect was slow within the first 1–2 h in the rats treated with the prepared oral insulin-loaded NPs but the effect was sustained much longer, up to 12 h compared to 4 h duration after subcutaneous administration. In comparison, there was a significant difference in the blood glucose levels of rats treated with batches A1 and A2. It was observed that the decrease in the blood glucose levels dependent on the concentration of chitosan composition in the preparation. It was found that batch A2 with a higher concentration of chitosan 4 % w/v showed a greater effect than batch A1 (chitosan 2 % w/v), indicating that the pharmacological effect depended on the concentration of chitosan.

The high pharmacological efficacy of the insulin-loaded NPs in alloxan-induced diabetic rats can be attributed to stimulation of either more insulin secretion from the system or mopping up excess glucose from the blood by the tissues. Furthermore, insulin-loaded NPs have caused increases in insulin absorption and reduction of glucose level after oral administration, which could be due to the combined effect of the mucoadhesion of the carrier to the mucosal wall at the absorption site (Fei *et al.,* 2015; Wang *et al.,* 2016). Previous studies show that NP formulations serve as an insulin protection medium in the harsh environment inside the stomach, and that NPs facilitate increased cellular permeability to the insulin drug, improving cellular uptake and intracellular delivery (Raffaele *et al.,* 2014). Additionally, chitosan-based NPs have been reported to transiently open the tight junctions of cell and targeting receptors, thereby allowing insulin absorption (Mei-Chin *et al.,* 2011; Fei *et al.,* 2015; Wei *et al.,* 2016). Even more so, chitosan and mucin together exhibit synergic dual mucoadhesive properties, as well as the ability to protect the loaded insulin from proteolytic enzyme activity. The electrostatic interactions of cationic chitosan or positively charged insulin-loaded NPs with negatively charged mucin on the intestinal wall increases the residence time, favoring specific localization of the formulation at the site of drug absorption to increase the drug concentration gradient (Kean and Thanou, 2010; Depeng *et al.,* 2017).

Figure 4b depicts the pharmacokinetics of insulin levels in the diabetic rats. The plasma insulin

level increased to its maximum level for 1 h after subcutaneous injection of insulin and then decreased exponentially over a 4 h period, indicating a rapid clearance of insulin from the system. However, after oral administration of insulin-loaded NPs there was no initial increase in the plasma level of insulin, but rather a gradual increase in plasma insulin within the first 6–8 h, followed by a slow decrease over 12 h. This indicates that NPs maintain a prolonged supply of insulin compared to insulin administered subcutaneously, attributable to the polymers used in their preparation as carriers. However, there was no significant difference between the plasma insulin levels of rats dosed with batch A1 and A2 of insulin loaded NPs.

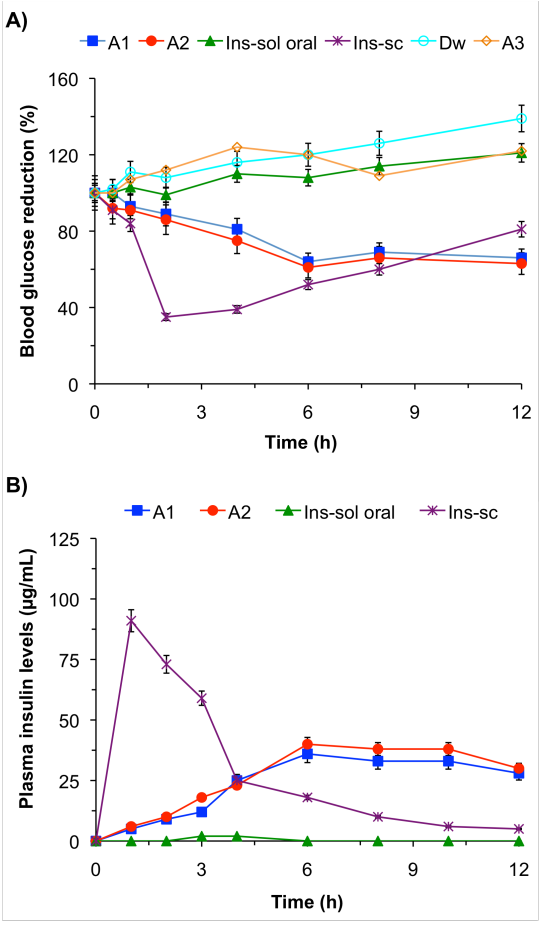

Figure 4. A) Percentage blood glucose levels in diabetic rats after orally administered test agents. B) Plasma insulin level after orally administered formulations. Abbreviations: ins-sol oral = insulin-solution; A1 = insulin-loaded NPs containing chitosan 2 % w/v; A2 = insulin-loaded NPs

containing chitosan 2 % w/v; A3 = unloaded NPs (no insulin added); ins-sc = insulin administered subcutaneously; Dw = dextrose water (negative control). Data are presented as the mean ± standard deviation (n = 5).

Table 2 shows the pharmacokinetic parameters such as: areas under the curve (AUC), time for maximum concentration (T_{max}), and maximum concentration (C_{max}) after oral administration of batch A1 and A2 of insulin-loaded NPs or insulin solution, and subcutaneous injection of insulin solution. The AUC for orally administered insulin-loaded NPs are 467.81 ± 32.26 and 478.61 ± 11.21 for A1 and A2 respectively, which are slightly higher than that of the subcutaneous injection of insulin solution, 452.21 ± 10.81. It indicates that the polymers used in the NPs have the capacity to facilitate insulin encapsulation and improve insulin absorption after oral administration.

Table 2

Pharmacokinetics values expressed as mean ± standard error (SE) after a single oral administration of insulin solution sc (5 IU/kg), insulin solution oral (50 IU/kg), insulin NPs A1 and A2 each (50 IU/kg) to rats.

Samples	AUC (µg h/mL)	C_{max} (µg/mL)	T_{max} (h)
A1	467.81 ± 32.26	36 ± 1.41	6.0
A2	478.61 ± 11.21	40 ± 1.61	6.0
Ins-sc	452.21 ± 10.80	91 ± 1.50	1.0
Ins-sol oral	96.24 ± 10.11	2 ± 2.12	4.0

3.6. Oral glucose tolerance (OGTT)

Figure 5 shows that the reduction in blood glucose level was significantly higher than after the distilled water used as negative control. However, there was a greater reduction in the postprandial blood glucose level of rats administered batch A2 (chitosan 4 %) compared to batch A1 (chitosan 2 %). This indicates that the glucose level reductions were directly determined by those chitosan concentrations. In all cases, the decrease in blood glucose levels to 64 % and 57 % for batches A1 and A2, respectively, was maximal at 6 h, and was sustained for 10 h. This study indicates that the

insulin-loaded NPs have the capacity to significantly decrease the postprandial blood glucose level for a usefully long time in diabetes-induced rats. It is worth mentioning that previous studies (Kiran *et al*., 2010) have demonstrated that unprotected insulin has little or no effect on blood glucose level after oral administration.

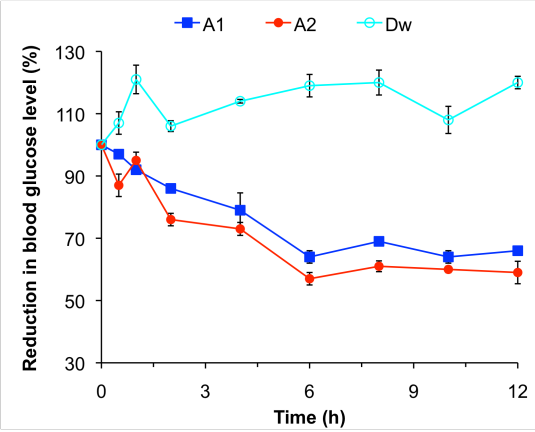

Figure 5. OGTT of insulin-loaded NPs (batches A1 and A2). Dextrose water (DW) was used as negative control in diabetic rats (n = 5).

3.7. Toxicity tests

Drug toxicology is very important in pharmaceutical formulation, especially in nano-drug delivery. Hence, insulin-loaded NPs were evaluated for their effect on the activities of liver enzymes such as: alanine aminotransferase (ALT), aspartate aminotransferase (SGOT) and alkaline phosphatase (ALP). In our studies, we assessed their cytotoxic potential against liver enzymes in normal rats. This cytotoxicity study showed that NP batches A1 and A2 caused no significant change in the tested liver enzymes compared to control, as depicted in Figure 6a. The results clearly indicate that the polymers (chitosan and snail mucin) have no negative effects on the liver cells.

As shown in Figure 6b, for the cell viability study the insulin-loaded NPs and control samples show similar results to cell viability: > 98 % after 24 h at the highest concentration used in this study. This confirms that these NPs do not affect cell viability within these experimental conditions. This

result supports the results obtained in the liver function tests. Chitosan (Abdallah *et al.*, 2011; Carino and Mathiowitz, 1999) and mucin (Adikwu *et al.*, 2005), have been previously individually evaluated and there were no reports of any forms of toxicity, such as inhibition of cell viability. Our results strongly suggest that these polymers are suitable for use in drug delivery, with a potential for *in vivo* administration

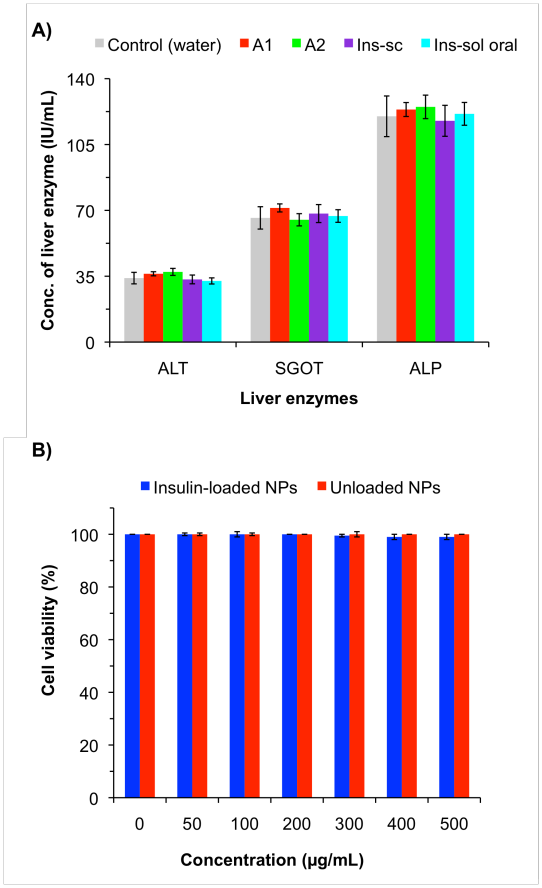

Figure 6. A) Effect of the test samples on the activities of liver enzymes. B) Cell viability study at different concentrations of insulin-loaded chitosan 4 % w/v) and unloaded NPs. Abbreviations: ins-sol oral = insulin-solution; A1 = insulin-loaded NPs containing chitosan 2 % w/v; A2 = insulin-loaded NPs containing chitosan 2 % w/v; ins-sc = insulin administered subcutaneously; Dw = dextrose water (negative control). Data are presented as the mean ± standard deviation (n = 5).

3.8. Stability study

The successful stability study results are depicted in Table 3. Six months of storage caused no significance changes in any of the parameters evaluated. These insulin-loaded NPs stably retained their original physiochemical properties for this standard period.

In general, the performance of this novel [chitosan-mucin]-based formulation is in good agreement with other synthetic polymeric vehicles (Ma *et al.,* 2005; Abdallah *et al.,* 2011; Mumuni *et al.*, 2019)

Table 3

Particle size, polydispersity index (PDI), zeta potential (ZP), encapsulation efficiency (EE) and drug-loading capacity (DLC) of insulin-loaded NPs (n = 5) after six months of storage.

Samples	PS (z-Ave, nm)	PDI	ZP (mV)	EE %	DLC %
A1	488.2 ± 0.11	0.165 ± 0.15	32.0 ± 0.05	82.1 ± 0.01	21.2 ± 0.50
A2	524.1 ± 0.01	0.170 ± 0.10	30.2 ± 0.21	89.0 ± 0.02	22.1 ± 0.20
A3	415.5 ± 0.10	0.171 ± 0.15	17.9 ± 0.50	-	-

4. Conclusion

Given the beneficial effects of biopolymer-based nanoparticles such as biodegradability, biocompoatibility, cost-effectiveness, high stability, high drug encapsulation efficiency and prolonged drug release effects, insulin-loaded NPs with high encapsulation efficiency (89-93 %) were prepared via self-gelation using chitosan (2-4 % w/v) and snail mucin as natural polymers. So-prepared insulin-loaded NPs were characterized by DSC, FT-IR, SEM, DLS and zeta potential measurements, that together with toxicity and stability tests supported the safety of the formulation. In particular, *in vitro* and *in vivo* evaluations showed a pronounced hypoglycaemic effect in diabetic rats after peroral administration compared to the effect of free oral insulin solution. A self-sustained release profile of encapsulated insulin was observed over a period of 8 h. The observed reduction of the blood glucose levels was lower than the effects observed in rats treated with subcutaneously administered insulin solution. In addition, both the pharmacokinetic and toxicity studies of the formulations showed low plasma clearance of insulin and no signs of toxicity on the liver enzyme and cell viability suggesting good biocompatibility. Overall, the foregoing results suggest that the formation of NPs of insulin with

chitosan and snail mucin is a potentially safe and promising approach to protect insulin and enhance its peroral delivery. Further efforts towards clinical studies will be carried out in our laboratories.

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