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Research article

Microemulsion-based approach for oral delivery of insulin: formulation design and characterization

Mumuni A. Momoh [a,*], Kenechukwu C. Franklin [a], Chinazom P. Agbo [a], Calister E. Ugwu [b], Musiliu O. Adedokun [c], Ofomata C. Anthony [d], Omeje E. Chidozie [a], Augustine N. Okorie [e]

[a] Drug Delivery Research Unit, Department of Pharmaceutics, Faculty of Pharmaceutical Sciences University of Nigeria Nsukka, Enugu State, Nigeria
[b] Department of Pharmaceutical Technology and Industrial Pharmacy, University of Nigeria Nsukka, Enugu State, Nigeria
[c] Department of Pharmaceutics and Pharmaceutical Technology, Faculty of Pharmacy University of Uyo, Akwa-Ibom State, Nigeria
[d] National Centre for Energy Research and Development, University of Nigeria Nsukka, Nigeria
[e] Department of Pharmacology and Toxicology University of University of Nigeria Nsukka, Enugu State, Nigeria

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ABSTRACT

Oral delivery of insulin provides a good alternative because it is non-invasive and patient-friendly. However, multiple challenges affected this route. To overcome barriers for oral delivery of insulin, we aimed to develop a novel insulin-loaded microemulsion system based on snail mucin for oral administration. The strategy in the novel system of using mucin loading insulin into the inner core of prepared water in oil microemulsion to provide sustained released, increased in vivo stability and enhanced drug absorption in the gastrointestinal tract. We report how microemulsion composed of varying ratios of snail mucin and Tween® 80 (1:9–9:1) using oil/water emulsion preparation method influenced insulin performance after oral administration. The results obtained include an encapsulation efficiency of above 70 %; in vitro release was sustained over 10 h and *in vivo* evaluations in diabetic rat model shows that insulin-loaded microencapsulation effectively reduced blood glucose levels over a period >8 h after oral administration. Therefore, we suggest that the developed formulation for oral insulin can be a promising alternative dosage form for oral protein delivery.

1. Introduction

Subcutaneous injection of insulin has been the main route for the treatment of insulin-dependent diabetic patients (Amidi et al., 2010; Babiker and Datta, 2011). However, this means of insulin administration usually results in suboptimal control, fluctuation in blood glucose levels with poor patient compliance due to the associated pain and side effects (Jin et al., 2012). The availability of orally administered insulin would not only alleviate pain, but will also presents the normal physiological cascade of insulin and might provide a better glucose homeostasis (Yu et al., 2015). Interestingly, orally administered insulin is absorbed by the intestinal epithelium and reaches the liver through the portal vein and can directly inhibit hepatic glucose output, as opposed to conventional subcutaneous (sc) insulin, which can lead to peripheral hyperinsulinemia and its associated complications (Momoh et al., 2019; Mukhopadhyay et al., 2015). Nonetheless, orally administered insulin is faced with many difficulties, as it has to confront various barriers in the gastrointestinal (GI) tract (Kean and Thanou, 2010; Ma et al., 2005).

Several strategies such as the use of permeation enhancers, mucoadhesives, protease inhibitors, the utilization of multifunctional polymers, and micro-/nano particulate drug delivery systems and gelatin as carriers have been studied to improve oral delivery of protein therapeutics. But none of these strategies have made it to clinical evaluation (Al-Salami et al., 2012; Mukhopadhyay et al., 2015). Formulation scientists have researched on the use of nanoparticles (NPs) as carrier for peroral insulin delivery. This method has utilised ionic crosslinking, emulsion-evaporation, co-precipitation and self-assembled technique, among others. Despite the impressive findings, the cytotoxicity of the nanocarreirs is of much concerns, hence the need for safer alternatives with proven therapeutic efficacy (Erel et al., 2016).

Similarly, synthetic or natural polymers have been developed as insulin carrier for its oral delivery (Gao et al., 2014). However, the major challenges are the toxicity associated with many synthetic polymers,

* Corresponding author.
E-mail address: audu.momoh@unn.edu.ng (M.A. Momoh).

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these has restricts their effective application as oral carriers of insulin. Interestingly, natural polymers, such as alginate, dextran, cellulose, j-carrageenan, chitosan and mucin, has received much attention as the focus point for effective delivery of oral insulin. For instance, natural biomaterials such as chitosan have been widely favoured in the area of oral insulin delivery due to its positive surface charge, mucoadhesive properties and enhanced permeation properties (Liu et al., 2016; Paul et al., 2017). Unfortunately, the ease of protonation associated with chitosan formulation in acidic environment has reduced its usage in biomedical and pharmaceutical applications.

Mucins are high molecular weight glycosylated proteins, which form the structure-forming component of mucus and are responsible for it bioadhessiveness (Arhewoh et al., 2014; Adikwu et al., 2005; Builders et al., 2008). Additionally, mucin serves as protectants, lubricants and a source of attachment point for mucoadhesive polymers. Mucin is considered to be resistant to proteolytic enzymes and acid pH degradation in the GIT, thus its use in biomedical and pharmaceutical applications has gain more attention among researchers in protein drug delivery (Mortazavi et al., 1992; Adikwu et al., 2005; Builders et al., 2008; Georgia and Thomas, 2018).

Microemulsion is a delivery system that consist of dispersed system involving surfactant, cosurfactant, oily phase and an aqueous phase in a required ratios. The system is considered thermodynamically stable and aids the delivery of some sensitive molecules (Gasco, 1997). Interestingly, microemeulsions, have been shown to have advantages in the area of drug solubilisation. They prevent degradation of sensitive molecules, have ease of preparation, low viscosity, high encapsulation efficiency among others. More so, water-in-oil type microemulsions are especially attractive for oral protein delivery due to their protection ability, augmentation of oral bioavailability and increase of protein absorption through the gastrointestinal tract (Gülşah et al., 2016).

In this study, we evaluated the mucin obtained from the African giant snail (*Archachatina marginata*) which has a comparative advantage than mucin obtained from porcin and bovine (Sylvester et al., 2016) in the formulation of a novel insulin-loaded microemulsion with the aim of protecting the insulin from degradation after oral administration. The concept behind this formulation is that the system can protect insulin from gastric degradation, enhance protein transfer from intestine and provide long lasting reduction of blood glucose level. Based on this, we utilised the mucoadhessive properties of mucin and in combination with tween 80 to enhance the payload of insulin in the inner core of the polymer to obtain more stable system, with a higher protective function against acidic and enzymatic degradation in the stomach and better mucoadhesive properties throughout the intestinal system. Furthermore, microemulsion has been found to have an excellent way of delivering pH sensitive molecule and its capability to protect pH-sensitive molecule has been established by many researchers (Singh et al., 2010; Zhang et al., 2003; Gülşah et al., 2016bib_citation_to_be_resolved). However, to the best of our knowledge there is no report in literature on the utilization of snail mucin based microemulsion system for oral insulin delivery. It is our belief that mucin based microemulsion for oral insulin is a novel drug carrier system that could protect insulin from degradation, increase it payload and improve it residence time at the site of drug absorption for increased bioavailability of insulin. The insulin-loaded microemulsions (MEs) were prepared by double emulsion method and characterised based on particle size, encapsulation efficiency and *in vitro* insulin release profiles. Furthermore, the *in vivo* study and pharmacokinetic activities after oral administration were evaluated in diabetic rat model.

2. Material and methods

2.1. Materials

The materials used for the formulation include: citric acid, sodium hydroxide (Merck, Germany), sodium chloride, acetone, concentrated HCl (BDH, England), alloxan (Sigma Chemical company, USA), potassium dihydrogen phosphate (May & Baker Ltd., Dagenham, England), sodium hydroxide (BDH, England), streptozotocin (STZ, $\geq$ 98%) (Sigma Aldrich, USA), ACCU-CHECK machine (USA) and insulin (Eli Lilly, Nigeria). They were used as procured without further treatment. Mucin was prepared in the sterile laboratory of the Department of Pharmaceutics, University of Nigeria Nsukka. Other reagents and solvents were of analytical grade and were used as supplied. Distilled water was obtained from our laboratory and was used throughout the research.

2.2. Extraction of snail mucin

African giant snails were procured from three local markets in Enugu and Benue states of Nigeria. The snails were kept in laboratory condition for a week before the processing. The extraction of mucin from snail was based on the previously reported procedure (Adikwu et al., 2005), with slight modifications. The shells of the giant African land snails were knocked open at the apex and a spirally coiled rod inserted to remove the fleshy body from where the excretory parts were extracted. The fleshy parts were then placed in 250 ml of water and washed several times until the slimy mucin was completely washed off. These washings were pooled together in an aluminum bucket, precipitated with chilled acetone and then dried by lyophilization.

2.3. Preparation of insulin-loaded microemulsion

Oil-in-water (o/w) emulsions were prepared using light liquid paraffin as the oily phase and various combinations of Tween® 80 and snail mucin powder as shown in Table 1. A 2g quantity of the mucin and other ingredients as shown in Table 1 were weighed and distributed in the oil phase contained in 100-mL beaker. The calculated quantity of Tween® 80 and 1.0 mL of 100 IU/mL of insulin were accurately measured and mixed by vortex. The final solution was added to the oil phase as the aqueous phase, and the volume was made to 100 mL with double distilled water. The final emulsion contained 30 % liquid paraffin.

Table 1. Formulation composition of the emulsion.

Formulation codes	Mucin:Tween-80	Mucin (g)	Tween® 80 (g)	Span® 60 (%)	Insulin (100 IU/ml)
A1	1:9	0.33	2.97	0.20	10.00
A2	2:8	0.66	2.64	0.20	10.00
A3	3:7	0.99	2.31	0.20	10.00
A4	4:6	1.32	1.98	0.20	10.00
A5	5:5	1.65	1.65	0.20	10.00
A6	6:4	1.98	1.32	0.20	10.00
A7	7:3	2.31	0.99	0.20	10.00
A8	8:2	2.64	0.66	0.20	10.00
A9	9:1	2.97	0.33	0.20	10.00

A1-A9, are insulin-loaded emulsions containing various ratios or quantities of mucin to Tween 80 (1:9 to 9:1, respectively).

The above mixture was emulsified by mixing at 4000 rpm for 5 min using Ultra-Turrax homogenizer (T25, IKA, Germany). These procedures were used in all the preparations.

2.4. Characterization of the formulation

2.4.1. Physical appearance of the insulin-loaded microemulsions

Visual observation of the physical appearance of the formulation such as changes in colour, odour and aesthetics value throughout the study period were evaluated.

2.4.2. Time dependent pH evaluation

The pH values of the prepared emulsions were measured using a digital pH meter (Horibal pH Meter, Laqua Japan). This evaluation was made at 0, 8 and 16 weeks. All the readings were determined in triplicates and the averages of the measurements were recorded.

2.4.3. Morphological and particle size analysis

The morphological analysis and particle size of the formulated emulsion was carried out by optical digital light microscope (DLM) (Leica Diestar, Germany) and images captured with digital moticam 3.0 camera. Briefly, a drop of the insulin-loaded emulsion was placed on a microscopic slide (Marinfield, Germany) using a micropipette, covered with a cover slip and viewed under a digital light microscope (Lieca, Germany) attached to a Motic image analyzer (Moticam, China). With the aid of Motic Images Plus 2.0 software in the microscope, sizes of the particles were measured and the averages were taken. The same procedure was used in all the batches of the preparation. All measurements were made in triplicates.

2.4.4. Rheological properties of the microemulsion

The flow property of the emulsion was evaluated using an Ostwald viscometer. The time of flow, density, relative density and viscosity of the various microemulsion batches were determined using Eq. (2).

$$\eta rel = \frac{\eta}{\eta_0} = \frac{\rho t}{\rho_0 t_0} \tag{1}$$

where:

η_{rel} = relative density, ρ = density of sample, t = time of outflow of sample

ρ_0 = density of water, t_0 = time of outflow of water.

2.4.5. Encapsulation efficiency (EE %) and drug loading (DL)

Briefly, a 10 mL quantity of insulin-loaded emulsion was measured and placed in a 20 mL test tube and vortexed for 10 min and further subjected to centrifugation at 1500 rpm for 5 min. The amount of insulin contained in the samples was determined using high performance liquid chromatography (HPLC) method as described below (section 2.10). The EE and DL were evaluated using Eqs. (2) and (3), respectively.

$$\text{EE } (\%) = \frac{\text{Amount of insulin added} - \text{Amount insulin in suspension}}{\text{Amount of insulin added in the emulsion}} \text{ x } 100 \tag{2}$$

$$\text{Loading capacity} = \frac{\text{amount of insulin encapsulated}}{\text{weight of the carrier}} \text{ X } 100 \tag{3}$$

2.4.6. Description of HPLC method

Briefly, the HPLC system consisted of pump (PU- 2089, Jasco, Japan), UV–Vis detector (UV-2075, Jasco, Japan), auto sampler (AS- 206, Japan) and 250 mm × 4.6 mm column C18 particle diameter 5 μm (Shimadzu, Japan). The solution used as the mobile phase is a mixture of acetonitrite and phosphate buffer (70: 30, pH 7.4), and the flow rate was adjusted to 1 ml/min. The mobile phase was filtered through 0.2 μm cellulose acetate membrane filter (Advantec, Japan). The volume of injection was 20 μl and the total run time was 8 min. The detection wavelength was 227 nm and the column temperature was maintained at 25 °C.

2.4.7. In vitro release of insulin from the microemulsion

The *in vitro* release of insulin from different formulations through dialysis membranes (MWCO: 10,000–16,000) was performed in phosphate buffer for 12 h at 37 ± 1 °C using a dialysis membrane as barrier and magnetic stirrer plate assembly method. Briefly, the polycarbonate dialysis membrane (pore size 0.22 mm) was soaked in the dissolution medium for 24 h prior to the commencement of each release experiment. In each case, approximately 3–5.0 mL emulsions were added into the dialysis membrane containing 2.0 mL of the release medium. Thereafter, it was suspended in a 250-mL beaker containing 150 mL of dissolution medium and agitation was provided magnetically using a magnetic stirrer at 100 rpm (Remi Instruments, Mumbai, India). At predetermined time intervals, 5.0 mL sample was withdrawn using a 5 mL-needle and syringe. The same volume of medium was replaced immediately in order to maintain sink condition. The amount of insulin in the withdrawn samples was determined using a HPLC quantitative method as described earlier and the release profiles plotted as percentage drug released against time.

2.4.8. In vivo pharmacodynamics evaluation

Albino Wister rats weighing 140–150 g were obtained from the Central Animal House of the Department of Pharmacology, University of Nigeria. The rats were kept in standard animal cages and left for one week to acclimatize to the new laboratory environmental condition at a room temperature and relative humidity of 22 ± 5 °C and 50 %, respectively. The rats had free access to food and water, and temperature and light were controlled to mimic their natural habitat.

Prior to the time of the experiment, Animal Ethics Committee at University of Nigeria Nsukka approved the research protocol for the use of laboratory animals. All experiments were performed according to the International Code of Practice for the care and use of animals for scientific purposes.

Diabetes was induced in rats by a single intraperitoneal injection of streptozocin (50 mg/mL in a 10 mM citrate buffer pH 4.5) at 50 mg/kg. Seven-ten days post administration; the blood glucose level of the rats were periodically examined using Accu-check glucometer. Rats with blood glucose level above 250 mg/dL along with other signs and symptom of diabetes were considered diabetic and were used in the investigations.

2.4.8.1. Antidiabetics evaluation. The diabetic rats were fasted for 12 h before being used for the experimental procedures and remained fasted for 24 h during the experiment, but had free access to water ad libitum. Forty (40) rats were randomly divided into eight groups of five rats per group and were housed in a separate cage. Insulin-loaded microemulsion (batches A1, A3, A5, A6 and A9) were administered by intragastric gavage to rats in group 1–5, respectively at insulin dose of 50 IU/kg. Rats in group 6: intragastric administration of insulin oral solution (50 IU/kg, 2.0 ml), rats in group 7: intragastric administration distilled water (2.0 ml) and rats in group 8: subcutaneous (sc) administration of insulin (5.0 IU/kg). All rats were dosed once, and the effects on blood glucose levels were evaluated thereafter.

Blood samples were collected from the tip of the tail vein before and at predetermined time intervals after administration. Blood glucose levels were measured using glucometer (Accu-Check, Roche, USA). The changes in blood glucose levels represented in percentage to the initial blood level before the administration of test sample were plotted against time.

2.4.9. Pharmacokinetic study

For the pharmacokinetic study, batch (A9) of the formulation with highest blood glucose levels reduction was selected and analyzed for plasma insulin levels. Blood samples were collected at predetermined time intervals, centrifuged at 10000 rpm at 10 °C for 10 min and the

insulin was subsequently quantified using HPLC as described earlier. The area under the curve (AUC) of the blood glucose levels of the plasma insulin concentration were calculated using the trapezoidal method. Other pharmacokinetics parameters such as plasma concentration, time for peak concentration, are calculated directly from the concentration-time graph.

2.4.10. Stability study

For stability study, the optimized insulin-loaded microemulsions were evaluated every 6 months for a period of 12 months under different temperature conditions. A 20 mL sample of the optimized emulsion was kept in an aluminum capped glass vials and stored at 25 °C and 4–6 °C out from direct light. Aliquot sample was withdrawn every 6 months and the pH, particle size, encapsulation efficiency and loading capacity were evaluated as described previously.

2.4.11. Data and statistical analysis

All experiments were performed in replicates ($n = 3$) for validity of statistical analysis. Results were expressed as mean ± SD. ANOVA and Student *t*-tests were performed on the data sets generated using Origin® for Windows and SigmaPlot® 11. Differences were considered significant for p values <0.05.

3. Results and discussion

3.1. Characterization of insulin-loaded microemulsion

3.1.1. Physical appearance, morphology and particle sizes of insulin-loaded microemulsion

Figure 1 shows the photomicrographs of the insulin-loaded microemulsions. It was observed that one month post-formulation the micrographs of the microemulsion showed globules that were spherical in shape and smooth, and were not different from what was observed (Figure not shown) within the 24 h of the preparation. From visual examination (Figure 2) it was observed that there was no separation of phases in the microemulsion; this implies that the surfactant concentrations as well as all other excipients employed in the formulation of the insulin-loaded microemulsion were appropriate to give a homogenous and stable formulation. The particle sizes were in the range of 36 ± 0.1 to 66.10 ± 0.3 μm, with batch A1 and A9 having the smallest and largest sizes respectively (as presented in Table 2). The particles sizes of the microemulsions depended largely on the quantity of mucin used in the formulation. It was observed that as the concentration of mucin increased there was a corresponding increase in the particle size. The microemulsion shows positive zeta potential which is important for achieving interaction between the sailic component of the system as well as the mucin layer of the gastrointestinal tract (GIT) at the absorption site for effective and prolonged delivery of the incorporated insulin (see Table 3).

3.1.2. Time dependent pH analyses

The pH of the different batches of insulin-loaded microemulsion were measured 24 h, 8 week, and 16 weeks after preparation to ascertain the variation of pH with time, which could be a function of degradation of the insulin or the carriers/excipients used in the preparation (Table 2). There was a slight decrease in the pH of the formulations within the storage period. . The decrease in the pH of the formulation in some of the batches could be an evidence of probably the degradation of the mucin components of the formulation and release of more of the amino component of the mucin. However, the decrease or slight increase in the pH of the formulation is not significant ($p > 0.05$) enough to affect the pharmacodynamics and pharmacokinetics activities of the formulation.

3.1.3. Encapsulation efficiency (EE %) and loading capacity (LC)

The role of the formulated microemulsion is to deliver protect and deliver the incorporated insulin molecule intact to the site where absorption would take place. Thus, the ability of the delivery system to accommodate active molecules is an important factor to be considered in the choice of the material as carrier or excipients. These can be expressed by the entrapment efficiency (EE %) and loading capacity. EE% defines the ratio between the weight of entrapped active pharmaceutical ingredient (API) and the total weight of API added to the dispersion, while LC

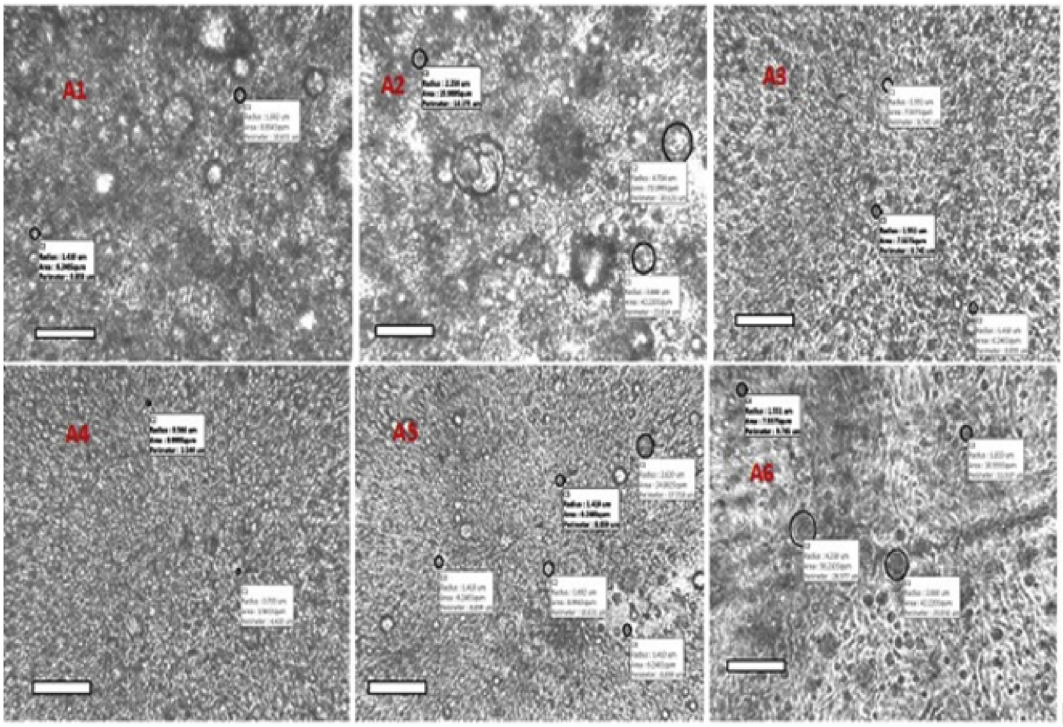

Figure 1. Representative of photomicrographs of (A1) Mucin:Tween-80 1:9, (A2) Mucin:Tween-80 2:8, (A3) Mucin:Tween-80 3:7, (A4) Mucin:Tween-80 4:6, (A5) Mucin:Tween-80 5:5, (A6) Mucin:Tween-80 6:4. Bar represents 70 μm.

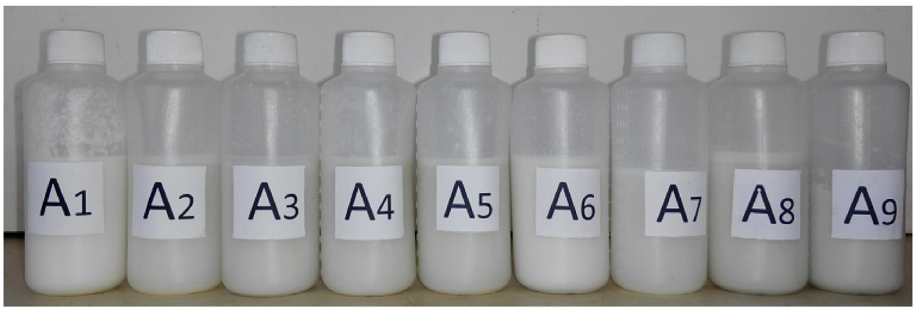

Figure 2. Representative picture of prepared samples of insulin-loaded microemulsion.

expresses the ratio between the entrapped API and the total weight of the carriers. Table 2 shows the EE % and the LC obtained for the various batches of insulin-microemulsion after 4 weeks of storage. The EE % ranged from 54.4 to 74.9 % and LC ranged from 23.1 to 37.1 as shown in Table 2. Insulin being an aqueous soluble drug, the low value obtained from the EE % is expected and also since the formulation was not completely lyophilized. Very significant amount of the insulin was not completely encapsulated in the microparticles. All the same the amount of insulin encapsulated in the microparticle was significant enough to induce a decrease in the blood glucose lowering effect as seen in the *in vivo* study.

3.1.4. *In vitro release of insulin from microemulsion*

The *in vitro* release profiles of insulin in phosphate buffer at pH 6.4 indicates very significant release of insulin from all batches of the formulation as shown in Figure 3. The prepared insulin-loaded microemulsion formulations were characterized by an early burst effect during which insulin showed biphasic release i.e a fast onset of release within the first 1 h followed by a gradual or a slow release of the insulin over a prolonged period of time. Interestingly, the formulation show a sustained release pattern, indicating the possibility of getting a once daily formulation which will be patient friendly and convenient. Earlier works on protein delivery have shown that when microemulsions are prepared by w/o/w method, especially with water-soluble drugs such as peptide and proteins, there is always a significant tendency for the API to migrate to the aqueous dissolution medium, thereby concentrating at the surface of the particles and subsequently resulting in an initial burst release (Jameela et al., 1997; Zhang et al., 2012). The early burst release in formulations of this kind involving insulin is highly desired in clinical practice where loading dose or fast onset of action of the drug is required. This is usually followed by slow release which would serve as maintenance dose. Comparing the percentage release in all batches of the formulations that was evaluated revealed that maximum release was obtained in batch A9 (82 %), while batch A1 (60 %) gave the least release after 16 h. This indicated that insulin release from the emulsions increased with decrease in particle sizes of the emulsions, which is consistent.with earlier report (Zhang et al., 2012).

3.1.5. *In vivo bioactivities of insulin-loaded microemulsion*

Insulin-loaded microemulsions were administered orally to overnight fasted diabetic rats. The reduction of the initial glucose levels versus time after intragastric administration of selected prepared insulin-loaded samples, insulin solution, distilled water and subcutaneous administration of insulin is presented in Figure 4a. As expected, no significant hypoglycemic effect (<5 %) was observed after oral administration of the insulin-solution, this indicates poor oral absorption of insulin solution in gastrointestinal system. However, the slight reduction in blood glucose levels as observed in Figure 4a, could be attributed to the absorption of a small fraction of insulin prior to its degradation. This observation is in agreement with an earlier works (Elghazi et al., 2006; Kim et al., 2012). As expected there was observed no significant hypoglycemic effects after oral administration of distilled water which is used as a negative control. Instead there was a continued increase in blood glucose levels throughout the 16 h study period and some of the rats died in the process. Pharmacologically, subcutaneous (SC) injection of the insulin solution produced a faster onset of action by lowering the blood glucose levels from 100-67 % with nadil effect of 33 % within 2–4 h after administration. However, the effect did not last as there was a sharp increase in blood glucose levels after 5–6 h and this continued and is close to the initial basal levels within 10 h.

Encouragingly, oral administration of the insulin-loaded microemulsion produced a slower rate of blood glucose reduction levels, but this effect was sustained over a longer period of time when compared with the SC administration of insulin. In other words, oral administration of the prepared microemulsion showed more prolonged hypoglycemic activity without initial hypoglycemia, when compared with the s.c. administration of insulin. Maximum glucose reduction of 52 % was attained in batch A9 at 16 h. The decrease in blood glucose levels after oral administration of batch A1, A3, A5 and A6 were not as significant as that produced by batch A9. This observation could be attributed to the

Table 2. Physicochemical properties of a insulin-loaded microemulsion prepared with different blends of mucin and Tween®80. Data are shown as mean ± SD ($n = 3$).

Code	1. Formulation characteristics				pH values		
	PS (μm)	Code	PS (μm)	Code	PS (μm)	Code	PS (μm)
A1	36 ± 01.0	A1	36 ± 01.0	A1	36 ± 01.0	A1	36 ± 01.0
A2	41 ± 33.0	A2	41 ± 33.0	A2	41 ± 33.0	A2	41 ± 33.0
A3	36 ± 31.0	A3	36 ± 31.0	A3	36 ± 31.0	A3	36 ± 31.0
A4	48 ± 03.0	A4	48 ± 03.0	A4	48 ± 03.0	A4	48 ± 03.0
A5	49 ± 11.0	A5	49 ± 11.0	A5	49 ± 11.0	A5	49 ± 11.0
A6	51 ± 04.0	A6	51 ± 04.0	A6	51 ± 04.0	A6	51 ± 04.0
A7	56 ± 15.0	A7	56 ± 15.0	A7	56 ± 15.0	A7	56 ± 15.0
A8	68 ± 23.0	A8	68 ± 23.0	A8	68 ± 23.0	A8	68 ± 23.0
A9	66 ± 21.0	A9	66 ± 21.0	A9	66 ± 21.0	A9	66 ± 21.0

PS = Particles size, EE = encapsulation efficiency and LC = Loading capacity. Note: A1- A9 were the insulin-loaded microemulsion.

Table 3. Rheological properties of the microemulsions; mean ± SD (n = 3).

Code	Time flow (s)	Density (g/cm3)	Relative density	Viscosity (cp)
A1	60 ± 1.21	0.65 ± 1.21	0.65 ± 1.21	0.89 ± 1.21
A2	56 ± 1.21	0.70 ± 1.21	0.70 ± 0.21	0.90 ± 1.11
A3	50 ± 31.0	0.78 ± 0.34	0.78 ± 0.14	0.89 ± 1.21
A4	52 ± 03.0	0.75 ± 2.30	0.75 ± 2.01	0.89 ± 1.21
A5	50 ± 11.0	0.78 ± 0.30	0.78 ± 1.30	0.93 ± 0.41
A6	49 ± 04.0	0.80 ± 1.21	0.80 ± 1.11	0.90 ± 0.42
A7	45 ± 15.0	0.87 ± 0.34	0.87 ± 0.11	0.89 ± 0.33
A8	44 ± 23.0	0.89 ± 0.30	0.89 ± 0.10	0.89 ± 0.21
A9	43 ± 21.0	0.91 ± 0.34	0.91 ± 0.21	0.89 ± 0.62

ratio of mucin to Tween®80 in the formulation. Our result show higher decrease in blood glucose as compared with the previous study (Gülşah et al., 2016), where the author developed chitosan based microemeulsion with which resulted in insignificant decrease in glucose levels after oral administration. The author attributed the poor decrease in glucose levels to protonation of chitosan in acidic environment. Interestingly, as shown in Figure 4a, our work on mucin microemuslion formulation shows a significant decrease in blood glucose levels, indicating the protection, delivery and improved insulin absorption after oral administration. Previous work by Abdallah et al. (2011) on chitosan alone as a carrier for oral insulin suffered due to protonation of chitosan, a result that was over come in our study.

The decrease in blood glucose levels observed in insulin-loaded microemulsion after oral administration in diabetic rats model could be attributed to following: (a) the muco-adhesive property of mucin which served as an efficient tool for the protection of insulin in harsh acidic environment inside the stomach, (b) this also increased the contact time at the site of drug absorption, (c) the microemulsion delivery system may have increased the payload of the drug and improved the permeability of insulin, thereby enhancing its absorption into the biological system (Jin et al., 2012; Yu et al., 2015).

Previous works have shown that emulsion-based dispersions increased total interfacial area as droplet size decreased which is also an added advantage for drug absorption (Damg'e et al., 2007; Khafagy et al., 2007; Wong et al., 2016). More so, surfactants such as Tween® 80 and mucin may provide protection by preventing exposure to the oil–water interface thereby preventing degradation of insulin. This is mainly due to steric effect which occur as a result of interaction between the carrier and the loaded drug. Thus, protection of the aggregation-prone hydrophobic sites on the insulin surface (Bam et al., 1998; Builders et al., 2009; Wang et al., 2016).

Additionally, the probability of dissociation of insulin tetramers/dimers into monomers was avoided since there was no harsh chemical involved in the preparation of the microemulsion. More so, the emulsification was performed under mild conditions to preserve the biological activity of insulin as well as prevent the degradation of mucin.

3.1.6. Pharmacokinetics study

The corresponding plasma insulin concentration-time profiles and the pharmacokinetics parameters are shown in Figure 4b, the mean plasma concentration of insulin following oral administration of the insulin-loaded microemulsion (batch A9, which is the optimized batch) to rats maintained a longer circulation time than the reference sample. The peak maximum of the reference sample was at its highest within the first 1–2 h after administration than the formulated microemulsion. According to

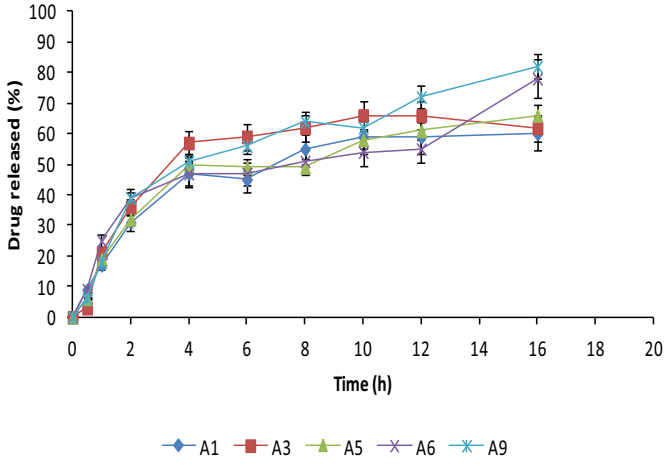

Figure 3. Insulin release from insulin-loaded microemulsion prepared with a mucin:Tween-80 in varying ratios in intestinal pH 6.4 simulated fluids at 37 °C (mean ± SD, n = 3).

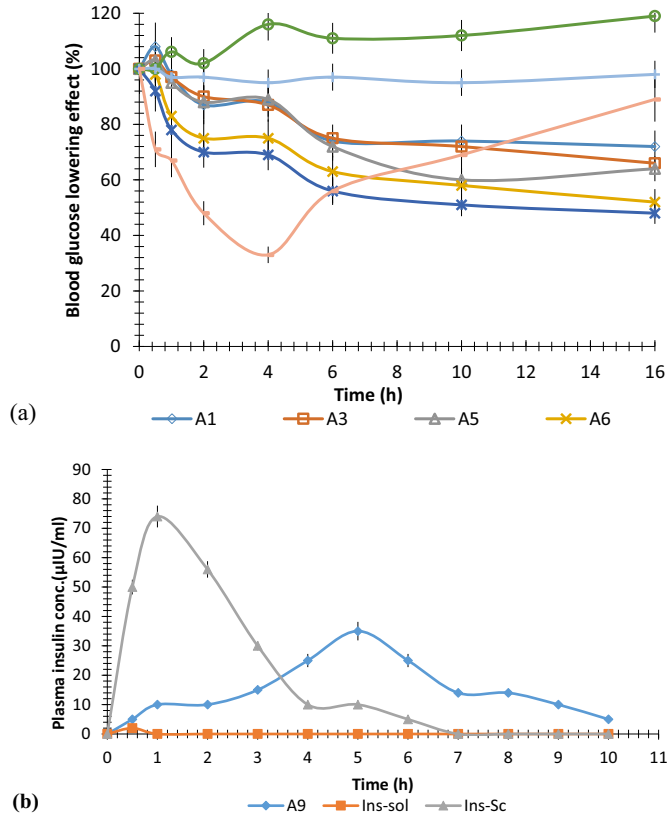

Figure 4. (a) Blood glucose levels after oral administration of different formulations of insulin and control samples. (b) Plasma insulin level vs. time profiles of the diabetic rats following the administration of insulin formulation batch A9, (mean ± SD, n = 5).

Table 4. Properties of the insulin-loaded microemulsion prepared with different blends of mucin and tween-80. After 12 months of storage mean ± SD ($n = 3$).

Code	6 months				12 months			
	PS (μm)	% EE	LC	pH	PS (μm)	% EE	LC	pH
A1	45 ± 0.10	54.3 ± 0.10	20.1 ± 0.21	4.9 ± 0.21	43 ± 0.40	53.3 ± 0.10	18.9 ± 0.10	4.1 ± 0.23
A3	41 ± 0.10	50.9 ± 0.34	27.1 ± 0.32	4.8 ± 2.01	39 ± 1.60	51.9 ± 0.34	26.1 ± 0.14	4.5 ± 1.09
A5	52 ± 0.10	58.9 ± 0.30	30.2 ± 0.40	52 ± 0.10	4.6 ± 1.11	56.9 ± 0.30	28.9 ± 0.30	4.2 ± 2.31
A8	67 ± 0.30	67.9 ± 0.30	25.6 ± 0.10	4.7 ± 1.31	66 ± 0.30	65.9 ± 0.30	23.6 ± 0.10	4.3 ± 2.00
A9	69 ± 0.10	73.9 ± 0.34	35.1 ± 0.21	4.6 ± 3.17	68 ± 0.10	72.9 ± 0.34	36.1 ± 0.21	4.0 ± 3.10

PS = Particles size, EE = encapsulation efficiency and LC = Loading capacity. Note: A1, A3, A5, A8 and A9 were the insulin-loaded microemulsion.

the results of the pharmacokinetic parameters for insulin such as: the maximum plasma concentration (C_{max}), peak time T_{max} and the area under the curve (AUC)], the T_{max} of insulin for the commercial insulin was 1.0 h, which was faster than that of the formulated microemulsion with *T*max at 5h. The maximum concentration (C_{max}) of the insulin and the reference sample were 74 IU/ml, 35 IU/ml and 2.0 IU/ml for A9, for Ins-Sc and Ins-Oral, respectively. The area under the curve (AUC) somehow deviated from the trend of the concentration (C_{max}) and the peak time (T_{max}). The AUC of the formulated insulin-microemulsion was significantly ($p < 0.05$) higher than that of reference sample. The plasma insulin level peaked 5 h after oral administration of the formulation, and then gradually declined during the 10 h period that the study lasted. The *AUC* (0–24 h) and *AUC*∞ were 488.5 and 524.6 μg mL–1 h–1, respectively, and were found to be significantly ($p < 0.05$) higher than the corresponding values (257.2 and 262.8 μg mL^{-1} h^{-1}, respectively) for the ins-Sc. In contrast, there was no obvious detectable plasma concentration of the insulin solution after oral administration.

3.1.7. Storage stability studies of the formulation

Table 4 shows the stability evaluation test on the insulin-loaded microemulsion after storage for a period of 12 months. As shown in Table 4, the samples had similar stability with regards to the particle size, encapsulation efficiency and loading capacity. The values slightly increased with increase in storage time. In other words, the particle sizes increased upon storage for 6–12 months, which may be due to partial crystallization of the mucin. The slight increase in the growth of the particles did not affect their shapes (Figure not shown). The encapsulation and loading capacity were not significantly affected.

Reports have shown that, the growth of particle size or changes in the particle size of formulation especially emulsion or oil-containing preparations is due to aggregation and subsequent growth by Ostwald ripening or sintering (Ayogu et al., 2009). It showed that the formulations regardless of the ratio of mucin to Tween® 80 did not differ much as to affect the release of the active pharmaceutical ingredient incorporated into the formulation. In other words; Ostwald ripening is a spontaneous process that occurs because larger crystals are more energetically favored than smaller crystals. It is so because the formation of many small crystals is kinetically favored (i.e. they nucleate more easily) while large crystals are thermodynamically favored. Thus, from the standpoint of kinetics, it is easier to nucleate many small crystals. However, small crystals have a larger surface area to volume ratio than large crystals. Molecules on the surface are energetically less stable than the ones already well ordered and packed in the interior. Large crystals, with their greater volume to surface area, represent a lower energy state. Thus, many small crystals tend to attain a lower energy state if transformed into large crystals and this is actually what happens with Ostwald ripening (Boistelle and Astier, 1988). The principle behind the aggregation of the particles over time may be further explained by the DLVO theory, which describes the interacting forces existing between particles to include electrical repulsion forces (VR) and the van der Waals attraction (VA); these parameters are additive (Aulton, 1999). In this context, it is very obvious that, though there were changes in particle size after drug incorporation as compared with unloaded particles, but that was not enough to affect the stability of the preparation (Table 4).

As per the pH (Table 4), it is clear that there was a gradual decrease in pH value of microemulsion containing insulin in all the ratios of the formulations. From the overall values, insulin-loaded microemulsion with Mucin:Tween-80 (Batches A1 and A9) had the highest significant ($p < 0.05$) reduction. The slight decline in the pH values from alkalinity through neutrality to acidity in most of the formulation was not attributed to insulin degradation since in our preliminary evaluation of the excipients, there was a same fall in pH in the mixture of the carriers alone without insulin from 5.5 to 4.7. Degradation of mucin or tween-80 or both may lead to the release of the oily component that could be further release of a free fatty acids may be implicated in the fall to acidic pH. Ayogu et al., 2009, argued that particle surface pH in some systems may be two-to-three units different from the pH of the bulk because of double layer effect.

4. Conclusions

Insulin-loaded microemulsion for oral delivery was developed to overcome the multiple challenges of oral delivery system for insulin with snail mucin and Tween-80 ratio mixtures. The short-term in vivo efficacy in STZ induced diabetic rats provided the proof of concept by a modest glucose reduction at a dose administered. This indicate that the emulsion based mucin was able to preserve the biological activities of insulin after oral administration.

Results show that batches A6 and A9 had better blood glucose lowering effect compared to other batches of the formulation. Furthermore, when the results were compared to the subcutaneously administered insulin, it was observed that the formulation had a better sustained blood glucose reduction effect than the positive control within the 16 h duration of the study. This finding indicates that these microemulsion formulations could improve the encapsulation efficiency of loaded insulin, stabilise the formulation and possess great potential to be applied in oral insulin delivery. Therefore, we recommended validation of this method using higher animal.

Declarations

Author contribution statement

Mumuni A. Momoh, Kenechukwu C. Franklin, Chinazom P. Agbo, Calister E. Ugwu: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Musiliu O. Adedokun, Ofomata C. Anthony, Omeje E. Chidozie, Augustine N. Okorie:Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

References

Abdallah, M., Yuichi, T., Hirofumi, T., 2011. Design and evaluation of novel pH-sensitive chitosan nanoparticles fororal insulin delivery. Eur. J. Pharmaceut. Sci. 42, 445–451.

Adikwu, M.U., Aneke, K.O., Builders, P.F., 2005. Biophysical properties of mucin and its use as a mucoadhesive agent in drug delivery: current development and future concepts. Niger. J. Pharmaceut. Res. 4, 60–69.

Al-Salami, H., et al., 2012. Probiotics decreased the bioavailability of the bile acid analog, monoketocholic acid, when coadministered with gliclazide, in healthy but not diabetic rats. Eur. J. Drug Metab. Pharmacokinet. 37 (2), 99–108.

Amidi, M.E., Mastrobattista, W., Jiskoot, W.E., 2010. Chitosan-baseddelivery systems for protein therapeutics and antigens. Adv. Drug Deliv. Rev. 62, 59–82.

Arhewoh, M.I., Eraga, S.O., Maroh, O., 2014. Transdermal delivery of bovine serum albumin using snail mucin.East and central. Afr. J. Pharmaceut. 17, 18–24.

Aulton, M.E., 1999. Pharmaceutics: the Science of Dosage Form Design. Internatio, Edinburgh, London, New York, Philadelphia, Sydney Toronto.

Ayogu, I., Ogbonna, O., Ayolugbe, C., Attama, A., 2009. Evaluation of the pharmacodynamic activity of insulin from bilosomal formulation. Curr. Drug Deliv. 6 (4), 415–418.

Babiker, A., Datta, V., 2011. Lipoatrophy with insulin analogues in type idiabetes. Arch. Dis. Child. 96, 101–102.

Bam, Narendra B., et al., 1998. Tween protects recombinant human growth hormone against agitation-induced damage via hydrophobic interactions. J. Pharmaceut. Sci. 87 (12), 1554–1559.

Boistelle, R., Astier, J.P., 1988. Crystallization mechanisms in solution. J. Cryst. Growth 90 (1–3), 14–30.

Builders, F.P., et al., 2009. Preparation and characterization of mucinated cellulose microparticles for therapeutic and drug delivery purposes. Eur. J. Pharm. Biopharm. 72, 34–41.

Builders, F.P., Kunle, O.O., Adikwu, M.U., 2008. Preparation and characterization of mucinated agarose: a mucin-agarose physical crosslink. Int. J. Pharm. 356, 174–180.

Damge, C., Maincent, P., Ubrich, N., 2007. Oral delivery of insulin associated to polymeric nanoparticles in diabetic rats. J. Control. Rel. 117, 163–170.

Elghazi, L., Balcazar, N., Bernal-Mizrachi, E., 2006. Emerging role of protein kinase B/akt signaling in pancreatic [Beta]-Cell mass and function. Int. J. Biochem. Cell Biol. 38, 157–163.

Erel, G., Kotmakc¸i, M., Akbaba, H., et al., 2016. Nanoencapsulated chitosan nanoparticles in emulsion-basedoral delivery system: in vitro and in vivo evaluation of insulin loaded formulation. J. Drug Deliv. Sci. Technol. 36, 161–167.

Gasco, M.R., 1997. Microemulsions in the pharmaceutical field: perspectives and applications. In: Industrial Applications of Microemulsions. Marcel Dekker, New York, NY, USA, pp. 97–122.

Gao, X., Cao, Y., Song, X., et al., 2014. Biodegradable, pH-responsive carboxymethyl cellulose/poly (acrylicacid) hydrogels for oral insulin delivery. Macromol. Biosci. 14, 565–575.

Georgia, P., Thomas, C., 2018. Mucins as multifunctional building blocks of biomaterials. Biomater. Sci. 6, 2282–2297.

Gülşah, E.Mustafa K., Hasan, A., Sumru, S.K., Ayşe, G.K., 2016. Nanoencapsulated chitosan nanoparticles inemulsion-based oral delivery system: in vitro and in vivo evaluation of insulin loadedformulation. J. Drug Deliv. Sci. Technol. 36, 161–167.

Jameela, S.R., Suma, N., Jayakrishnan, A., 1997. Protein release from poly(epsilon-caprolactone) microspheres prepared by melt encapsulation and solvent evaporation techniques: a comparative study. J. Biomater. Sci. Polym. Ed. 8, 457–466.

Jin, Yun, et al., 2012. Goblet cell-targeting nanoparticles for oral insulin delivery and the influence of mucus on insulin transport. Biomaterials 33, 1573–1582.

Kean, T., Thanou, M., 2010. Biodegradation, biodistribution and toxicity of chitosan. Adv. Drug Deliv. Rev. 62, 3–11.

Khafagy, E.S., Morishita, M., Onuki, Y., Takayama, K., 2007. Current challenges in non-invasive insulin delivery systems: a comparative review. Adv. Drug Deliv. Rev. 59, 1521–1546.

Kim, A., Jong, A., Ä, Christoffer, Anna, S., Kenneth, A.D., 2012. Role of cell cycle on the cellular uptake and dilution of nanoparticles in a cell population. Nat. Nanotechnol. 7, 62–68.

Liu, M., Zhang, J., Zhu, X., et al., 2016. Efficient mucus permeation and tight junction opening by dissociable"mucus-inert" agent coated trimethyl chitosan nanoparticles for oral insulin delivery. J Control. Release 222, 67–77.

Ma, Z., Lim, T.M., Lim, L.Y., 2005. Pharmacological activity of peroral chitosan–insulin nanoparticles in diabetic rats. Int. J. Pharm. 293, 271–280.

Mortazavi, S.A., Carpenter, B.G., Smart, J.D., 1992. Comparative study on the role played by mucusglycoprotein in the rheological behaviors of the mucoadhesive/mucosal interaction. Int. J. Pharm. 94, 195–201.

Momoh, M.A., Emmanuel, Ossai C., Onyeto, Azubuike C., Darlington, Youngson, et al., 2019. Preparation of snail cyst and PEG-4000 composite carriers via PEGylation for oral delivery of insulin: an in vitro and in vivo evaluation. Trop. J. Pharmaceut. Res. 18 (5), 919–926.

Mukhopadhyay, P., Chakraborty, S., Bhattacharya, S., Mishra, R., Kundu, P.P., 2015. PH-sensitive chitosan/alginate core? Shell nanoparticles for efficient and safe oral insulin delivery. Int. J. Biol. Macromol. 72, 640–648.

Paul, P.K., Nopparat, J., Nuanplub, M., et al., 2017. Improvement in insulin absorption into gastrointestinalepithelial cells by using molecularly imprinted polymer nanoparticles: microscopic evaluationand ultrastructure. Int. J. Pharm. 530, 279–290.

Singh, M.N., Hemant, K.S., Ram, M., Shivakumar, H.G., 2010. Microencapsulation: a promising technique for controlled drug delivery. Res. Pharm. Sci. 5, 65–77.

Sylvester, O.E., Prince, U.O., Emmanuel, O.O., Matthew, I.A., 2016. An investigation of the properties of mucin obtained from three sources. Pharma Innov. J 5 (12), 08-12.

Wang, J., et al., 2016. Positive/negativesurface charge of chitosan based nanogels and its potential influence on oralinsulin delivery. Carbohydr. Polym. 136, 867–874.

Wong, C.Y., Martinez, J., Dass, C.R., 2016. Oral delivery of insulin for treatmentof diabetes: status quo, challenges and opportunities. J. Pharm. Pharmacol. 68, 1093–1108.

Yu, Fei, et al., 2015. Enteric-Coated capsules filled with mono-disperse micro-particles containing PLGA-lipid-PEG nanoparticles for oral delivery of insulin. Int. J. Pharm. 484, 181–191.

Zhang, L., et al., 2003. Synthesis andevaluation of a novel _-Cyclodextrin derivative for oral insulin delivery andabsorption. Int. J. Biol. Macromol. 61, 494–500.

Zhang, L., Song, L., Zhang, C., Ren, Y., 2012. Improving intestinal insulinabsorption efficiency through coadministration of cell-penetrating peptide andhydroxypropyl-ß-cyclodextrin. Carbohydr. Polym. 87, 1822–1827.