Nigerian Journal of Pharmaceutical Sciences
Vol. 22, No2, 2023, ISSN: 0189-823X

HYBRIDIZATION OF SNAIL EPIPHRAGM AND CHITOSAN AS A CARRIER OF ORAL INSULIN DELIVERY IN DIABETES MANAGEMENT

[1]Alfa, J., [1]Okeke, C. I., [2]Romanus, O. C.,[3]Chidozie, O. E., [4]Hanifah, A., [5]Saliu, H. M.,[6]Okino, U. S., [3]Amadi, B., [3]Augustus, E., [3]Anih, C. V. and [*1,3,5]Mumuni, M. A

[1]Department of Pharmaceutics and Pharmaceutical Technology, Faculty of Pharmaceutical Sciences, Bingham University Karu, Nasarawa State, Nigeria.
[2]Department of Pharmaceutics and Pharmaceutical Technology, Enugu State University of Sciences and Technology, Enugu, Nigeria
[3]Drug Delivery Research Unit, Department of Pharmaceutics, Faculty of Pharmaceutical Sciences University of Nigeria Nsukka, Enugu State, Nigeria
[4]Department of Medical Laboratory Sciences, School of Medical Laboratory Sciences, Usmanu Danfodiyo University Sokoto, Nigeria
[5]Department of Microbiology, Faculty of Natural Sciences Prince Abubakar Audu University Ayingba, Kogi State, Nigeria.
[6]Department of Medical Microbiology, College of Health Sciences, Prince Abubakar Audu University Ayingba, Kogi State, Nigeria.

***Author for correspondence**: audu.momoh@unn.edu.ng; momohmumuni123@gmail.com; +2348037784357

ABSTRACT

Oral insulin delivery remains a mirage among the pharmaceutical scientists due to inability to circumvent its inherent stability in gastrointestinal tract after oral administration. In this study, insulin-loaded microparticles for oral delivery were prepared with snail epiphragm and chitosan combined at different concentrations of chitosan using a novel method based on polymer hybridization. Four insulin-loaded batches were prepared and labeled as FS1, FS2, FS3 and FS4 containing 1:1, 1:2, 1:3 and 1:4 of snail epiphragm (SE) and chitosan (CS), respectively. The unloaded (drug free) batch was similarly prepared and labeled as FS0. Characterizations such as particle size, morphology and thermal properties were evaluated. The *in vitro* release and blood glucose reduction after oral administration to diabetic rats was determined. The particles formed ranged from 121.0 ± 0.12 to 59± 1.06 µm, and were generally not spherical and varied in sizes. The loading and encapsulation efficiency were generally high. The minimum and maximum EE were 90.5 ± 0.03 and 97.7 ± 0.22 % for FS2 and FS4, respectively. The *in vitro* release of insulin from the formulations varied with the chitosan concentration, with FS1 (50 %) and FS2 (89 %). The percentage blood glucose reduction for the subcutaneously administered insulin was significantly ($p < 0.001$) higher than the formulations. *In vivo* studies revealed a pronounced hypoglycaemic (34.67±3.65 %) effect in diabetic rats after peroral administration of the insulin-loaded MPs compared to the free oral insulin solution within 12h of administration. Therefore, these findings clearly suggest that the snail epiphragm-chitosan based microparticles offer an interesting mode of insulin delivery orally for the treatment of diabetes.

Key words: Snail epiphragm, Chitosan, Hybridization, Glucose reduction.

INTRODUCTION

Excipients are generally considered as one of the cardinal factors in effective drug delivery (Mumuni *et al.,* 2020). It has been on the front burner in the field of scientific

drug discovery and formulation to modify the existing carriers to enable their full utilization as finding new excipients is very tasking and time consuming. The failure in the delivery of sensitive molecules like peptides and insulin for oral administration in the management of diabetes has been a serious issue among the health practitioners. This critical issue has been justified and recently received increased attention as to prepare a different entity with superior functions compared to the native or individual materials (Adikwu *et al.,* 2005). In this case, the modified excipients or their combinations are expected to have an improved drug delivery property.

Snail epiphragm (SE) is a natural substance that serves as protective covering of the opening of the snail shell during unfavourable condition. This cyst is said to contain some slimy features that aid in mucoadhesion. Previous reports have shown that the snail cyst possessed high mucous content and some minerals that are of health benefits (Navarro *et al.,* 2018).

Chitosan (CS) is the N-deacetylated derivative of chitin which is the most abundant natural amino polysaccharide. CHS is an important natural polymer for its combination of properties like bio-compatibility, biodegradability, and bio-activity (Abdel-Rahman *et al.,* 2016). Hence, CS is widely used in various areas, such as: biomedical products (Khan et al., 2016), food processing, cosmetics and drug delivery system (Jimtaisong *et al.,* 2013). The abundant amino groups make CS molecules can be used to surface modify MPs containing carboxyl groups. The CS modification promote MPs to be adhered and infiltrated into mucus members. Then, the intestinal epithelial cells transiently open a tight junction (Andreani *et al.,* 2015). The released insulin will penetrate through the paracellular pathway and enter bloodstream. Therefore, the CS-modified MPs improve the bio-availability of insulin, and avoid their degradation by enzymes. In recent times, snail epiphragm (SE) has gained attention in the area of drug delivery as well as the mucin obtain from the mucous content of snail slime as a result of their mucoadhesive properties (Adikwu, 2005; Mumuni *et al.,* 2020).

Insulin is a therapeutic macromolecule intensively used to improve the life of diabetics. To date, administration of insulin is by the subcutaneous route only and this is associated with unwanted side effects and poor compliance. 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). The degradation of insulin after oral administration has been the huge challenges among the formulation scientist. Carrier readjustment and modification has been an ongoing effort toward actualization of the dream of oral insulin formulation for the benefit of diabetes patients (Zambanini *et al.,*1999).

In this study insulin-loaded snail epiphragm modified with chitosan based microparticles were prepared via molecular hybridization method and characterized for *in vitro* release and *in vivo* oral delivery of insulin.

MATERIALS AND METHODS

Materials

The following chemicals were bought from their suppliers and used without further purification: Acetone (Merck, Germany), High molecular weight chitosan with 99% purity (Sigma Aldrich USA), Span 60, Humulin 70/30 (Eli lily and company,

USA), ethanol, paraffin oil (Moko pharm., Ltd Lagos, Nigeria), Terrestrial African giant snails were purchased from the Ogige local market in Nsukka, Enugu state. All other chemicals used were reagent grade.

Methods

Preparation of Snail Epiphragm (SE) from *Archachatina marginata*

The SC was obtained from snail by gently removing it from the shell cover. The whitish flakes were cleaned off of any dirt and washed in distilled water, dried and pulverized using an end-runner mill (Pascal Engineering Co. Ltd, England). The resultant powder was soaked in water for 24 h and the insoluble suspended particle was removed followed by lyophilization to obtain a smooth powder of SE.

Formulation of Microparticles

The microparticles were formulated by a combination of two polymers (chitosan and snail e**piphragm**) using w/o/w double emulsion method. The organic phase was first prepared by dissolving 1 g of chitosan in 100 mL of glacial acetic acid to produce a 1% v/v solution. The chitosan solution was sonicated at an amplitude of 80 rps for 60 s using an ultrasonic probe (Athena Technology Virginia, USA). The aqueous phase was prepared using 1 g of snail mucin, dispersed in 20 mL of distilled water using a 100 mL beaker and the solution was sonicated at an amplitude of 80 rps for 60 s. About 10 mL of 100 I.U/mL of insulin (Humulin 70/30) solution was added dropwise to the mucin dispersion and was gently mixed using a magnetic stirrer (Remi Equipment Pvt Ltd, Mumbai). A 5 mL of 0.5% span 60 was added to a mixture of ethanol and liquid paraffin in the ratio of 1:1 in a 100 mL beaker and properly stirred using a stirrer. The aqueous phase containing insulin and mucin dispersion was gradually added dropwise using a syringe into the beaker containing the liquid paraffin mixtures and was kept under magnetic stirring at 100 rpm. To the above solution, the organic phase was gradually added and allowed for continuous stirring for 3 min. Thereafter, the emulsification was further subjected to gentle sonication at 20 rps for 20 s. The prepared microparticles were divided into two portions, one portion was lyophilized and the other part was stored without lyophilisation at a temperature of 20 ^{o}C for further use. The procedure was repeated using a SE-CS ratio of 1:1, 1:2, 1:3, and 1:4 to obtain different batches of microparticle FS-1, FS-2, FS-3, and FS-4, respectively.

Determination of Percentage Recovery Yield

The yield of each of the microparticle from the batches of the formulations was weighed after lyophilization, and the percentage yield was calculated using below:

$$\text{Percentage yield} = \frac{A1}{A2+A3} \times 100 \quad \ldots\ldots\ldots (1)$$

Where A1 = weight of microparticles recovered (g), A2 = weight of the drug, A3 = weight of polymer + another excipient

Differential Scanning Calorimetry (DSC) and Fourier Transform Infrared (FT-IR) Analysis

Thermal analyses of insulin-loaded MPs and control samples were carried using differential scanning calorimetry (DSC) (DSC-60, Shimadzu Co., Ltd., Japan). In brief, 3–5 mg of the corresponding samples was weighed and placed in an aluminum pan and hermetically sealed under inert atmosphere (N2). The specimen and reference sample were placed in the corresponding sample holder. Measurements were done in the temperature range from 20 to 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 MPs and control samples were recorded using a Shimadzu FT-IR 800 Spectrophotometer (Shimadzu, Tokyo, Japan). Herein, 150 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 =2cm−1).

Particle Size and Morphological Analysis

The particle size analysis was carried out on all the formulation batches 24 h after the formulation using a digital light microscope (Leica Germany) and an image captured with Moticam 1000 camera, China (magnification ×40). The morphology (shape and surface) of the particles was evaluated. All measurements were done in triplicate.

Time-dependent pH Stability Study

The pH of the formulations, drug-loaded and unloaded (drug free) MPs were determined in a time-dependent manner using a pH meter (Suntex TS-2, Taiwan) after 24 h, 4 weeks, and 12 weeks of storage condition in order to ascertain the stability of the formulations especially in the area of product degradation during storage.

Encapsulation Efficiency (EE%) and Drug Loading Capacity (DLC)

The EE % was evaluated using 50 mg of insulin-loaded MP added into a micro concentrator (Vivaspin 6, Vivascience Honover, Germany) and 5 mL of phosphate buffer was added to each. The dispersion was centrifuged at 3000 rpm for 60 min. The supernatant was filtered and analyzed using HPLC as described elsewhere (Mumuni et al., 2020). The amount of insulin was determined by high performance liquid chromatography (HPLC). The experiments were carried out in triplicate and the EE and DLC were calculated using the following formulas:

Drug loading capacity

$$(\%) = \frac{\text{Weight of drug in microparticles}}{\text{Theoretical weight of microparticles}} \times 100$$

Encapsulation efficiency

$$(\%) = \frac{\text{Weight of drug assayed in MP}}{\text{Weight of drug fed initially}} \times 100$$

***In vitro* Drug Release Study**

In vitro drug release study of the microparticles was evaluated in freshly prepared phosphate buffer solution (pH 7.2) using a dialysis membrane technique. The polycarbonate dialysis membrane (MWCO 8000–10,000 Spectrum Labs, Netherlands) used as a release barrier was soaked in a phosphate buffer solution for 24 h before its use in the study. Insulin loaded microparticles equivalent to 50 mg was placed in a dialysis membrane containing 5 mL of the dissolution media was secured at both edge with thread and was suspended in a 200 mL phosphate buffer in 500 mL-beaker at pH 1.2, agitation was provided by magnetic stirrer at speed of 100 rpm for a period of 3 h, thereafter the dissolution medium was changed to pH of 7.2, in all case, the experiment was maintained at 37.0 ± 0.5°C. At predetermine interval of time, 5.0 mL sample of dissolution medium was withdrawn, filtered through a 0.22 µm filter (Millipore®, USA) and assayed using a high-performance liquid chromatography (HPLC). At every withdrawer made, equal amount of the same medium was added to maintain a sinking condition. The above

procedure was repeated with the other batches of the formulation.

Antidiabetic Studies

Adult Wistar rats weighing 95-125 g were purchased from the Department of Pharmacology and Toxicology, University of Nigeria, Nsukka, and were maintained at standard housing conditions (room temperature, 25^{o}C) with 12 h light. The animals were allowed to acclimatize for a week, during which they were fed with a commercial diet (Feeds BC, Nsukka, Nigeria) and water. All animal experimental procedure followed the Animal Ethics Committee of the Faculty of Pharmaceutical Sciences, University of Nigeria, Nsukka, and in line with the European Community guidelines (EEC Directive of 1986; 86/609/EEC).

Alloxan monohydrate solution (Sigma, USA) was freshly prepared before injection. A stock solution of alloxan monohydrate was prepared by dissolving 10 g of alloxan to 100 mL of normal saline (0.9% w/v NaCl) to obtain a concentration of 100 mg/mL. The rats were rendered diabetic before the study by intraperitoneal injection of 1 mL of the stock solution of alloxan. Thereafter, the blood glucose levels were regularly monitored at various intervals; four times daily for three days using glucometer (ACCU-CHECK, Roche, USA). Food intake was measured in (g), water (mL), and urine volume (mL) daily. The rats were confirmed diabetic 5-7 days after the administration of solution of alloxan when the glucose levels were in the range of 220 – 250 mg/dL.

The diabetic rats were enlisted into the study and were randomly divided into five groups of five rats per group. All animals were fasted for 12 h before the experiment but had free access to water throughout the study. The rats in group one (positive control) received 2.5 IU/kg insulin subcutaneously, group two (negative control) were given 25 IU/kg of plain insulin through the oral cavity, group three and four (Test samples) were given 25 IU/kg of insulin loaded microparticles (FS1 and FS3), respectively using oral nasogastric tube, and group five received 25 IU/kg of unloaded formulations (FS-0). The blood glucose level was determined using Accu check glucose meter at predetermined time intervals and was expressed as a percentage of the baseline plasma glucose level.

Statistical and Data Analysis

Data were analyzed using SPSS Version 16.0 (SPSS Inc. Chicago, IL). All values were expressed as mean ± SD. Differences between means were assessed using one way ANOVA and student's t-test. $p < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Recovery Yields

The insulin-loaded MPs were prepared via the self-gelation method using snail cyst and chitosan at varying ratios. The snail cyst was obtained from African giant snail, via physical removal from the snail and used in the formulation by hybridization with chitosan. All the preparation yields high, indicates the formulation parameters are very reliable.

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 thermogram of pure insulin solution showed a melting peak at 136.2 ^{0}C with corresponding enthalpy of -32.5686 mw/mg. The DSC thermograms of insulin-loaded

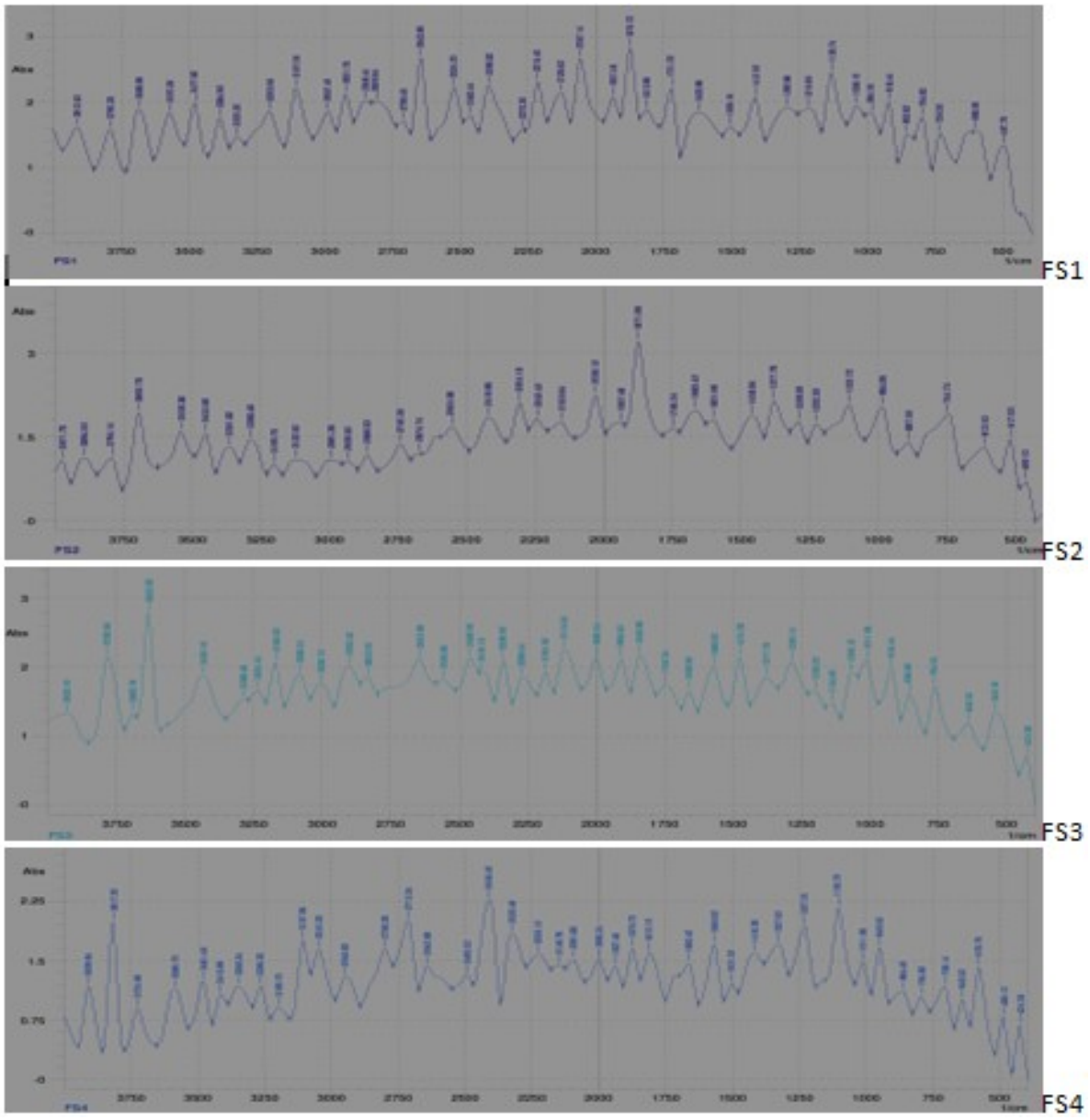
MPs showed different melting peaks and thermal properties (Thermographs not shown). FS1(SE:CS 1:1) showed melting peak of 130.6 ^{0}C with corresponding enthalpy of - 46.2967 mw/mg, FS2 (SC:CHS ratio of 1:2) showed melting peak of 74.9 ^{0}C with corresponding enthalpy of -0.07572173 mw/mg, FS3 (SE:CS 1:3) showed melting peak of 74.9^{0}C with corresponding enthalpy of -1.52044 mw/mg, while FS4 (SE:CS 1:4) showed melting peak of 128.9 ^{0}C with corresponding enthalpy of -4.81407 mw/mg. Comparing the thermal properties of insulin (136.2 ^{0}C) and the unloaded MPS or drug free sample 'FS0' (137.752^0) with those of the loaded formulation showed that the latter possessed better crystal properties. This is because insulin-loaded sample gave lower melting peaks than pure insulin and unloaded batch. Interestingly, the melting peak of the insulin-loaded batches shows a low melting peak as compare to pure insulin solution and unloaded batch FS0. Study has shown that decrease in melting point value indicates less ordered crystal structure resulting in high payload of drug and a possible disorientation of the crystalline arrangement of the drug, suggesting a change in the lattice structure (Kenechukwu *et al*., 2018). Additionally, it is also a pointer to show that insulin existed in amorphous form in the formulation and could be considered as stable and properly solubilised in the polymer mixture. In summary, the results indicate that all the MPs prepared had low enthalpy changes which suggest system of low crystalinity. The DSC results also show that there was no strong chemical interaction between the drug and the excipients.

For the FTIR spectrum (Supp 1) of insulin-loaded MPs carriers (containing chitosan and snail epiphragm); FS1 showed principal characteristic absorption peaks at 3570.26 cm^{-1} and 3477.62 cm^{-1} (O-H stretch of alcohol), 3477.62 cm^{-1} (N-H stretch of amine), 2921.78 cm^{-1} (C-H stretch of alky), 2643.86 cm^{-1} (O-H stretch of carboxylic acid), 2057.14 cm^{-1} (CΞC stretch of alkyne), 1620.96 cm^{-1} and 1412.52 cm^{-1} (C=O stretch of carboxylic acid), and 1620.96 cm^{-1} (C=C stretch of alkene). FS2 showed principal characteristic absorption band at 3539.39 cm^{-1} (O-H stretch of alcohol), 3288.48 cm^{-1} (N-H stretch of amine), 2555.08 cm^{-1} (O-H stretch of carboxylic acid), 2030.12 cm^{-1} (CΞC stretch of alkyne), 1871.86 cm^{-1} (C=O stretch of carboxylic acid), 1663.42 cm^{-1} (C=C stretch of alkene). FS3 showed principal characteristic absorption band at 3632.02 cm^{-1} (O-H stretch of alcohol), 3436.16 cm^{-1} (N-H stretch of amine), 2902.48 cm^{-1} (C-H stretch of alky), 2643.86 cm^{-1} (O-H stretch of carboxylic acid), 2115.04 cm^{-1} (CΞC stretch of alkyne), 1566.92 cm^{-1} and 1474.28 cm^{-1} (C=C stretch of benzene ring). FS4 showed principal characteristic absorption band at 3585.70 cm^{-1} and 3481.48 cm^{-1} (O-H stretch of alcohol), 3481.48 cm^{-1} (N-H stretch of amine), 3045.30 cm^{-1} (C-H stretch of alkene), 2713.34 cm^{-1} (O-H stretch of carboxylic acid), 2223.12 cm^{-1} (C=C stretch of alkyne), 1663.42 cm^{-1} and 1566.92 cm^{-1} (C=O stretch of carboxylic acid), and 1563.06 cm^{-1} (C=C stretch of benzene ring).

Particle Size and Morphology

The particle size of the insulin-loaded snail epiphragm grafted chitosan microparticles is shown in Table 1. The results show that particle size ranged from 121.0 ± 0.04 to 142.6 ± 0.20 µm in all the formulation (FS1 to FS4). The photomicrographs shown in Figure 1, revealed that the microparticles have a spherical shape with a smooth surface and all the particle sizes were within the acceptable range that would facilitate oral absorption of insulin. Results also revealed that an increase in chitosan concentration insignificantly decreased the

particle size ($p > 0.05$) of formulations. The result showed that an increase in the concentration of polymers resulted in a decrease in particle size. However, the particle characteristics of a formulation are necessary to ensure the production of stable products of suitable quality since physical stability and cellular uptake of particles are affected by particle size (Garg *et al.,* 2005; Zhang *et al.,* 2013).



Suppl 1: Fig. 3. FT-IR spectra of SE-CS-loaded insulin microparticle. Abbreviations: FS1 = SE:CS (1:1), FS2 = SE:CS (1:2), FS3 = SC:CHS (1:3), FS4 = SE:CS (1:4) containing snail epiphragm: chitosan

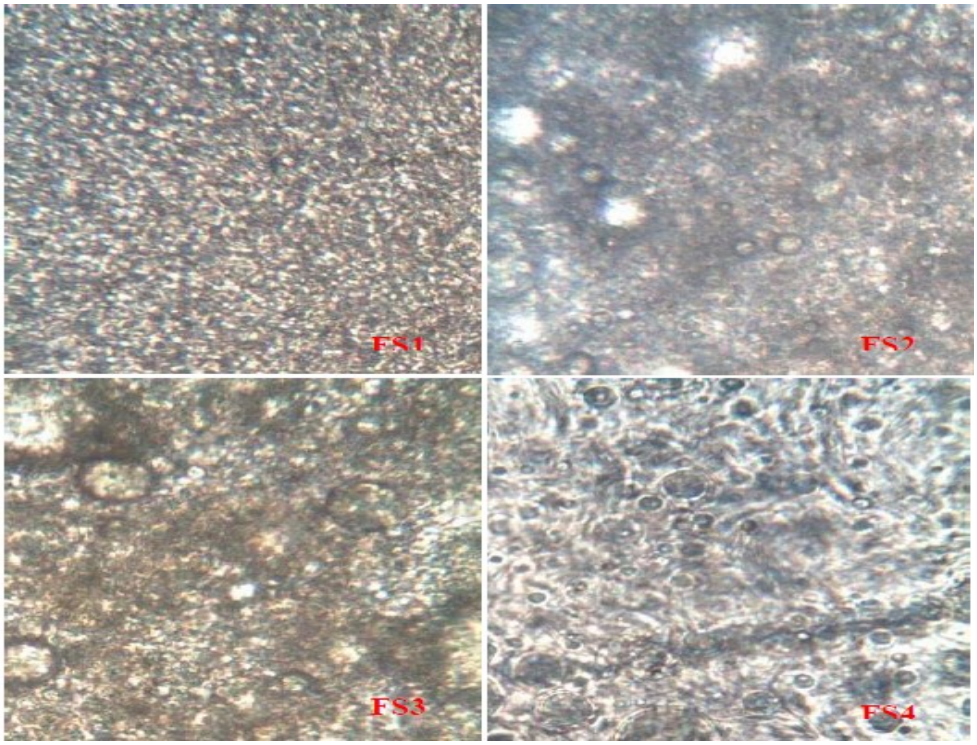


Fig. 1; Representative photomicrographs of insulin-loaded microparticles. Abbreviations: FS1 = SE:CS (1:1), FS2 = SE:CS (1:2), FS3 = SE: CHS (1:3), FS4 = SE:CS (1:4) containing snail epiphragm: chitosan Data are presented as the mean ± standard deviation (n=5).

Encapsulation Efficiency and the Loading Capacity

The encapsulation efficiency (EE%) and loading capacity (DLC) of the insulin-loaded MPs were very good, implying that the formulation procedure and selection of the polymers 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 *et al.* (2011), reported that ionic interaction of chitosan nanoparticles with hydroxypropyl methylcellulose phthalate 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 (Franklin *et al.,* 2018).

Table 1: Recovery Values (RV), Particle Size (PS), Encapsulation Efficiency (EE) and Drug-loading Capacity (DLC) of Insulin-loaded MPs (n=5)

Batch	RV (%)	PS (µm)	EE(%)	DLC(%)
S0	71.16	120.0 ± 0.02	---	---
S1	74.20	121.0 ± 0.12	93.2 ± 0.11	21.1 ± 0.17
S2	76.11	133.0 ± 0.01	90.5 ± 0.03	27.4 ± 0.52
S3	87.28	149.7 ± 0.53	92.6 ± 0.12	30.5 ± 1.12
S4	74.16	156.0 ± 1.06	97.7 ± 0.22	31.3 ± 2.22

Abbreviations: FS1 = SC: CHS (1:1), FS2 = SC: CHS (1:2), FS3 = SC: CHS (1:3), FS4 = SC: CHS (1:4) containing snail cyst: chitosan Data are presented as the mean ± standard deviation (n=5).

Time-dependent pH Stability Studies on the Formulations

The pH of the different batches of MPs were measured 24 h, 4 week, and 8 weeks after preparation to ascertain the variation of pH with time, which could be a function of degradation of the incorporated drug or excipients (Table 2). There was a slight decrease in the pH of the formulations within the storage period. This was also applicable to unloaded formulation (drug free) samples whose pH continued to decrease throughout the period of observation. The shifting of the pH toward alkalinity is evidence of probably no much degradation of the polymer components of the formulation toward acidity. However, the slight increase in the pH of the formulation loaded with drug is not significant ($p > 0.05$) as compared to the increase observed in the unloaded (drug free) formulation. This indicates that the increase in the pH may not be enough to cause any changes in the expected activity of the formulation (Ahmed TA, Aljaeid BM, 2016).

Table 2: Periodic pH Values as a Function of Stability Study (n=5)

Batch	12h	2 weeks	4 weeks	12 weeks
S0	4.8 ± 0.11	4.6 ± 0.01	4.1 ± 0.11	5.9 ± 0.12
S1	5.6 ± 0.10	5.7 ± 0.14	5.9 ± 0.11	6.1 ± 0.14
S2	4.9 ± 0.22	4.9 ± 0.01	5.1 ± 0.03	5.3 ± 0.22
S3	4.5 ± 0.01	4.5 ± 0.21	4.8 ± 0.12	4.8 ± 1.12
S4	4.6 ± 0.11	4.6 ± 0.11	4.8 ± 0.22	4.7 ± 2.22

Abbreviations: FS1 = SE:CS (1:1), FS2 = SE:CS (1:2), FS3 = SE:CS (1:3), FS4 = SE:CS (1:4) containing snail epiphragm: chitosan Data are presented as the mean ± standard deviation (n=5).

***In vitro* release Study**

The release profile of insulin-loaded microparticles formulated using S-extract of snail mucin and chitosan (FS batches) are shown in Figure 2. The *in vitro* release studies of insulin from the insulin-loaded microparticles numbering FS1-FS4 were performed in phosphate buffer (pH 7.2) at temperature of 37^0C. The result obtained showed a rapid release of insulin from the micro-MPs within the first 30 min, this was followed by a gradual release of insulin for a period of 8 hrs. The rapid release may be possibly due to the leaching out of the unencapsulated drug adhering to the surface of the microparticles at the initiation of the release study. However, at 1 h, all the batches showed a gradual and sustained the release of insulin in the dissolution medium, this was possibly due to the interconnectivity between negatively charged snail scale and the chitosan, resulting in firm insulin entrapment into the microparticle and thereby limiting insulin migration into the dissolution medium.

Additionally, the gradual insulin released could be also attributed to the role of mucin network in retardation of drug expulsion due to its high viscoelasticity and consistency as explained by an earlier researcher Adikwu, (2005); Builders *et al.,* 2008; Alessandra *et al.,* 2012. In summary, the results showed that the maximum percentage of insulin released from the loaded microparticle within the evaluation period ranges from 50-89 %, with batch FS1and FS2 showing minimum and maximum of 50 and 89 % released respectively. This clearly indicates that none of the batches of the formulation

had 100 % released of the loaded drug. All the batches of the formulation showed a steady and prolonged release in the buffer solution of pH 7.2. Interestingly, our preliminary evaluation in buffer of pH 1.2, shows no significant released (data not shown), which indicate that, though, chitosan was known to undergo protonation when used alone, however hybridization with snail epiphragm has prevented the early release of insulin in acidic pH.

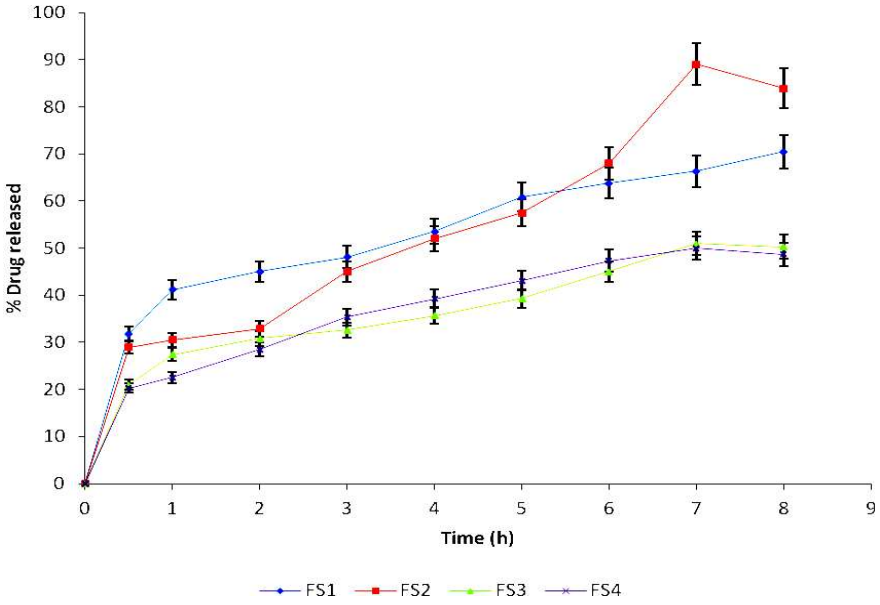


Figure 2: *In vitro* release profile of insulin from microparticles prepared with different ratios of snail cyst and chitosan in phosphate buffer at pH 7.2. Abbreviations: FS1 = SE:CS (1:1), FS2 = SE:CS (1:2), FS3 = SE:CS (1:3), FS4 = SE:CS (1:4) containing snail epiphragm: chitosan Data are presented as the mean ± standard deviation (n=5).

In vivo Bioactivity of Insulin-loaded MPs

Figure 3 showed changes in plasma glucose levels versus time after oral administration of unloaded micro particles, insulin loaded FS-1, FS-2, and FS-3, pure insulin solution and subcutaneous administration of insulin to overnight fasted diabetic rats. The mean plasma glucose baseline value was taken as 100 % level. After subcutaneous injection of insulin solution (2.5 IU/kg), the blood glucose level decreased to 50.6 % after 1 h, to a maximum decrease of 33.15 % after 1h and then increased gradually. Administration of pure solution of insulin via oral route at a dose of 25 IU/kg only resulted in maximum decrease of 86.71±4.41 % and 86.59±7.13 % at 1h and 6 h respectively. This is expected since a small fraction of insulin could be absorbed through the intestinal wall before degradation by the proteolytic enzymes (Alessandra *et al.,* 2012; Fei *et al.,* 2015; Depeng *et al.,* 2017).

Oral administration of 25 IU/kg of insulin loaded FS1 batch resulted in rapid decrease in plasma glucose level, from 83.61±1.25 % within the 0.5 h to 67.85±3.75 % in 1 h. However, the plasma glucose reduction appeared more gradual after 1 h, attaining a maximum decrease of 38.40 ± 2.89 % in 12 h, consistent with insulin release profile *in vitro* (Fig. 2). The rapid reduction was probably due to the presence of un-entraped insulin at the surface of the particles. After oral administration of insulin loaded FS2

batch, the glycaemia reduction was approximately similar with that of batch FS1, causing a rapid decrease in plasma glucose of 85.06 ± 3.46 % within 0.5 h to 69.91±5.45 % in 1 h, followed by a gradual decrease to a maximum of 34.67±3.65 % in 12 h. However, when insulin loaded FS3 was administered; a gradual reduction in plasma glucose level of 90.61±1.76 % in 0.5 h was observed. This gradual decline in glycaemia level was observed throughout the duration of the experiment with maximum blood glucose level of 44.05±7.06 % after 24 h. The administration of unloaded microparticles (unloaded FS0) to the diabetic rats shows no significantly decreased in blood glucose lowering effect as compared to subcutaneous injection and pure insulin respectively, this indicate no significant antihyperglycemic effect of pure chitosan (Bin *et al.,* 2012; Navarro *et al*., 2017), in conjunction with mild hypoglycemic effects of snail cyst (Mumuni *et al.,* 2020).

Insulin loaded microparticles for oral administration had a significant blood glucose reduction compared to the pure oral insulin between 1 to 24 h ($P < 0.05$), indicating that the microparticles protected insulin against enzyme degradation in the gastrointestinal tract in agreement with previous reports (Abdallah *et al*., 2011; Alessandra *et al.,* 2012). The insulin loaded MPS also varied significantly in blood glucose reduction at 0.5, 1, 12 and 24 h compared to the subcutaneous injection of insulin ($p < 0.05$). Insulin subcutaneous injection (positive control) showed a faster percentage decrease in initial glycaemia level than the insulin loaded microparticles up to 6 h, indicating a better hypoglycaemic property compared to the oral formulated microparticles, which is in line with earlier reports (Mumuni *et al.,* 2020). However, the microparticles had a gradual and prolonged blood glucose reduction as compared to the subcutaneous injection (positive control). These prolonged and gradual plasma glucose reduction effect observed with the formulated microparticles (FS1, FS2 and FS3), with FS2 showing maximum at 12 h as shown in Fig 3. This clearly suggests that snail cyst modified with chitosan was able to stabilize insulin and prevent it from degradation in the harsh conditions of the stomach, thereby allowing a significant fraction of intact insulin to reach the site of absorption for biological activity (Carino and Mathiowitz, 1999).

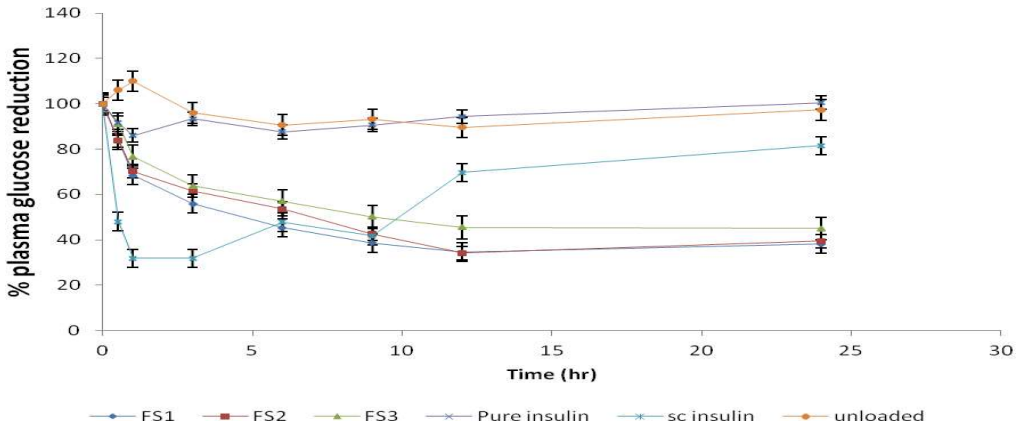


Fig. 3. Percentage blood glucose levels in diabetic rats after orally administered test agents. Abbreviations: FSI (1:1), FS2 (1:2) and FS3(1:3) loaded MPs contain snail cyst and chitosan. pure insulin = insulin-solution; SC-insulin = insulin administered subcutaneously; unloaded MPs (no insulin added). Data are presented as the mean ± standard deviation (n=5).

CONCLUSION

In the present study, we successfully prepared a novel functional biomaterial microparticles composed of SE:CS, and it potential for delivery of insulin orally in diabetes treatment was investigated *in vitro* and *in vivo*. In this study, snail epiphragm hybridized with chitosan based microparticles were prepared by w/o/w double emulsion. The recovery values were high and the microparticle with fairly spherical shapes, and good encapsulation efficiency were obtained. *In vitro* evaluation showed a sustained release pattern. The blood glucose level of alloxan-induced diabetic rats after oral administration of the formulation was lowered, though not comparable to the subcutaneous injectable insulin. The ability of the test sample to reduce the glucose level is an indication that the insulin was preserved in the microparticles and could be considered as a stable form of preparation. Snail epiphragm modified with chitosan based microparticles appears promising as a suitable carrier system for the administration of insulin through the oral route.

Conflict of interest

The authors declare no conflict of interests.

Acknowledgements

This study was financially supported by Tertiary Education Trust Fund (TETFUND)-Institution Based Research (IBR), University of Nigeria with grant number TETF/ DR&D/CE/UNI/ NSUKKA/BR/ 2020/VOL.1.

References

Abdallah, M., Yuichi, T., Hirofumi, T. (2011). Design and evaluation of novel pHsensitive chitosan nanoparticles for oral insulin delivery. *Eur J Pharm Sci, 42, 445–451.*

Abdel-Rahman, R.M., Abdel-Mohsen, A.M., Hrdina, R., Burgert, L., Fohlerova, Z., Pavlinak, D., et al., (2016). Wound dressing based on chitosan/ hyaluronan / nonwoven fabrics: preparation, characterization and medical applications. *Int J Biol Macromol, 89, 725.*

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. *Nig J Pharm Res, 4, 60–69.*

Ahmed, T. A., Aljaeid, B. M. (2016). Preparation, characterization, and potential application of chitosan, chitosan derivatives, and chitosan metal nanoparticles in pharmaceutical drug delivery. *Drug Des Dev Ther, 10, 483–507.*

Alessandra, M., Lucia, Z., Maria, D., Anastasia, F., Andrea, G. (2012). Oral colon delivery of insulin with the aid of functional adjuvants. *Adv Drug Deliv Rev, 64, 540–556.*

Andreani, T., Miziara, L., Lorenzón, E. N., De Souza, A. L., Kiill, C. P. (2015). Effect of mucoadhesive polymers on the in vitro performance of insulin loaded silica nanoparticles: Interactions with mucin and biomembrane models. *Eur J Pharm and Bioph, 93,118–126.*

Bin, X., Guohua, J., Weijiang, Y., Depeng, L., Yongkun, L., Xiangdong, K. (2017). Preparation of poly(lactic- co-glycolic acid) and chitosan composite nanocarriers via electrostatic self assembly for oral delivery of insulin. *Mat Sci and Eng C, 78, 420–428.*

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

Carino, G. P., Mathiowitz, E. (1999). Oral insulin delivery. *Adv Drug Del Rev, 35, 249–257.*

Depeng, L., Guohua, J., Weijiang, Y., Lei, L., Zaizai, T., Xiangdong, K., et al. (2017). Oral delivery of insulin using CaCO3-based composite nanocarriers with hyaluronic acid coatings. *Mat Let, 188, 263–266.*

Fei, Y., Yang, L., Chang, S.L., Qin, C., Gui, H., Wang, W., 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.*

Franklin, C. K., Anthony, A. A., Emmanuel, C. I., Petra, O. N., Chukwuebuka, E. U., Emmanuel, M. U., et al. (2018). Surface-modified mucoadhesive microgels as a controlled release system for miconazole nitrate to improve localized treatment of vulvovaginal candidiasis. *Eur J Pharm Sci, 111, 358–375.*

Garg, S. K., Ellis, S. L., Ulrich, H. (2005). Insulin glulisine: A new rapid-acting insulin analogue for the treatment of diabetes. *Exp Opin Pharmaco, 6, 643–651*

Jimtaisong, A., Saewan, N. (2013). Utilization of carboxymethyl chitosan in cosmetics. *Int J Cosm Sci, 36, 12–21.*

Khan, I., Ullah, S., Oh, D. H. (2016). Chitosan grafted monomethyl fumaric acid as apotential food preservative, *Carbohydr. Polym. 152, 87*

Mumuni, M. A., Kenechukwu, F. C., Ofokansi, K. C., Attama, A. A., and Díaz, D. D. (2020). Insulin-loaded Mucoadhesive Nanoparticles Based on Mucin-Chitosan Complexes for Oral Delivery and Diabetes Treatment. *Carbohydr Polym 229, 115506.*

Navarro, L. A., French, D. L., Zauscher, S. (2018). Advances in Mucin Mimic Synthesis and Applications in Surface Science. *Curr Opin Coll Interf. Sci. 38, 122–134.*

Zambanini, A., Newson, R. B., Maisey, M., Feher, M. D. (1999). Injection related anxiety in insulin-treated diabetes. *Diab Res Clin Prac, 46, 239–246.*

Zhang, L., Zhang, Z., Li, N., Wang, N., Wang, N., Tang, S., et al. (2013). Synthesis and evaluation of a novel B-cyclodextrin derivative for oral insulin delivery and absorption. *Int J Biol Macro, 61, 494–500.*