Preparation of snail cyst and PEG-4000 composite carriers via PEGylation for oral delivery of insulin: An in vitro and in vivo evaluation

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Abstract

Purpose: To develop PEGylated mucin as a carrier system for oral insulin delivery.

Methods: Varied ratios of snail cyst were molecularly modified with polyethylene glycol 4000 (PEG 4000). Briefly, in each case, 20 g quantities of snail cyst and PEG 4000 were separately dispersed in distilled water, stirred and allowed to stand for 24 h to produce a homogeneous dispersion and clear solution, respectively. The solution of PEG was added to the snail cyst dispersion, stirred and allowed 12 h for molecular interaction. The mixture was added to a 250-mL beaker containing 100 mL of light liquid paraffin. The microparticles were obtained after stirring and removing the paraffin using chilled acetone. The obtained PEGylated mucin matrices, which were subsequently loaded with insulin using a diffusion method, characterized for particles size, drug loading, encapsulation efficiency, in vitro drug release and evaluated for oral application in diabetic rats.

Results: The polymer hybrids improved insulin encapsulation efficiency (max 82.3 %), gave polydispersity indices that ranged from 0.11 ± 0.1 to 0.24 ± 0.2, zeta potential values between 28 ± 0.3 and 38 ± 1.1 mV. Insulin release was highest (68 % in 6 h) for batch C and was sustained for 10 h in simulated intestinal fluid. The optimized batch (C-5) showed higher hypoglycaemic activity (56.5 %) than control (0.5 %) in diabetic rats.

Conclusion: The results suggest that PEGylated mucin can potentially be developed as a platform for oral insulin delivery.

Keywords: Snail-cyst, PEGylation, Diabetes, Microparticles, Archachatina marginata

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INTRODUCTION

Excipients are generally considered as one of the cardinal factors in effective drug delivery [1]. 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 utilisation 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 [2–4]. In this case, the modified excipients or their combinations are expected to have an improved drug delivery properties.

Polymeric nano/microparticles have been developed to facilitate clinically applicable oral delivery of sensitive drugs like insulin. Polyethylene glycol (PEG) is a non-toxic polymer that has been used in various pharmaceutical drug delivery systems because of its good biocompatibility and biodegradability [5].

Snail cyst (SC) 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 [6].

In recent times, SC has gained attention in the area of drug delivery along with the mucin obtain from the mucous content of snail slime as a result of their mucoadhesive properties [6]. 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. In this study insulin-loaded snail cyst and PEG 4000 microparticles were prepared via molecular PEGylation method and characterized for in vitro release and in vivo hypoglycaemic activity.

**EXPERIMENTAL**

**Materials**

Polyethylene glycol 4000 (Germany), insulin (Eli-Lily and Company Indianapolis, USA), sorbic acid, sorbitol (BDH, England), and polysorbate-80 (Uniqema, Belgium). All other reagents were used as procured. Double distilled water (Lion Water, Nigeria) was used throughout the study.

**Preparation of snail cyst (SC)**

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. The snail cysts were dried and pulverised 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 SC.

**Preparation of PEGlyated snail cyst**

A 20 g quantity of snail cyst and PEG-4000 at ratios shown in Table 1 was dispersed in 100 mL beaker containing distilled water to produce a 20 % w/v homogeneous dispersion. The SC dispersion was added into 250 mL beaker containing 20 mL of soft liquid paraffin and thoroughly mixed using Silverson mixer (Adelphi 4R, England) at 2000 rpm 10 min. A 20 mL of the PEG-4000 solution was gradually added to the snail cyst dispersion with continue stirring under magnetic stirrer at a stirring speed of 350 rpm for 20 min. The dispersion was further homogenised using an Ultrasound sonicator (AT-500 Sonicator, India) at 60 W for 5 min in an ice bath. The microparticles formed was precipitated using chilled acetone, filtered using a filter paper (Whatman, No 1, USA), and washed off of liquid paraffin several times with a mixture of acetone and n-hexane (3:1). The final microparticles were recovered, air dried and lyophilised.

**Insulin loading**

A diffusion loading method was used to prepare insulin-loaded microparticles. Briefly, 5.0 g quantity of prepared microparticles was placed in a 10 mL clean glass plate and a 5.0 mL volume of the insulin solution pH 7.3, 100 IU/mL was gradually added to 5 g microparticles in a plate, which was gently shaken. Thereafter, the mixture was allowed to stand for 10 min to enable drug diffusion into the microparticles. The excess insulin solution was gently removed and the insulin-loaded microparticles were lyophilized. The recovered insulin-loaded microparticles was collected and used for further studies. The composition of insulin-loaded MPs is depicted in Table 1.

<table>
<thead>
<tr>
<th>Batch</th>
<th>SC (g)</th>
<th>PEG-4000 (g)</th>
<th>Insulin (mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C1</td>
<td>1.0</td>
<td>1.0</td>
<td>5.0</td>
</tr>
<tr>
<td>C2</td>
<td>1.0</td>
<td>2.0</td>
<td>5.0</td>
</tr>
<tr>
<td>C3</td>
<td>1.0</td>
<td>3.0</td>
<td>5.0</td>
</tr>
<tr>
<td>C4</td>
<td>1.0</td>
<td>4.0</td>
<td>5.0</td>
</tr>
<tr>
<td>C5</td>
<td>1.0</td>
<td>5.0</td>
<td>5.0</td>
</tr>
</tbody>
</table>

SC = snail cyst, PEG = polyethylene glycol, Insu-MPs = insulin microparticles
Differential scanning calorimetry (DSC)

The thermal property of insulin-loaded MPs was determined by differential scanning calorimetry (DSC) (Shimadzu DSC 204 F1, Japan). A 5.0 mg quantity each of the insulin-loaded MPs was weighed and placed on an aluminum pan, hermetically sealed, and the thermal behaviour determined over a temperature range of 20 – 220 °C under a 10 ml/min nitrogen flux at a heating rate of 10 °C/min. The baselines were corrected using an empty pan, and all the thermograms were baseline corrected.

Evaluation of surface morphology of insulin-loaded MPs

The surface morphological characteristics of insulin-loaded MPs were evaluated using a scanning electron microscope (SEM-1000, Japan) following the standard procedure as stated in an earlier work [7]. The results were presented using a three-dimensional model.

Determination of micromeritic properties

A 10 gram quantity each of the batches of the preparations was weighed and used to evaluate the tapped and bulk density of the microparticles. The angle of repose (AR) and Carr’s index (CI) were also evaluated using standard methods [7]. Briefly, a rubber funnel was properly clamped on the retort stand. In each case, microparticle samples with known weight were gradually placed into the funnel with the orifice of the funnel closed. Upon opening the orifice, the entire microparticles were allowed to flow freely through the orifice on plain sheet of paper. Thereafter, the height and radius of the microparticle was measured using a meter rule. The angle of repose (θ) and Carr’s compressibility index (CI) were calculated using Eqs 1 and 2, respectively.

\[ \theta = \tan^{-1}\left(\frac{\text{HP}}{\text{RHP}}\right) \]  

\[ \text{CI (CI)} = \frac{\text{HP} - \text{RHP}}{\text{HP}} \times 100 \]  

where HP and RHP are height of powder and radius of heap of powder respectively.

Assessment of encapsulation efficiency (EE) and drug loading (DL)

The EE (%) and DL of insulin-loaded MPs were studied following an earlier method. Briefly, 50 mg of the insulin-loaded MPs was placed inside a pre-treated polycarbonate dialysis membrane bag of 20 kDa, molecular weight cut off of 8000 (MWCO 8000) containing 20 mL of deionized water and centrifuged (Kubota model RL-1700, Japan), at 11,000 rpm for 30 min at 10 °C. The amount of free insulin in the supernatant was measured using a UV spectrophotometer at 271 nm (Jenway 65042, Germany). The concentration of free insulin in the supernatant was calculated from a standard curve of insulin. EE (%) and DL were calculated using Eqs 3 and 4, respectively.

\[ \text{EE} = \frac{\text{MIA} - \text{MIS}}{\text{MIA}} \times 100 \]  

\[ \text{DL} = \frac{\text{MIA} - \text{MIS}}{\text{TMM}} \times 100 \]  

where MIA = mass of insulin added, MIS = mass of insulin in suspension

In vitro release studies

In vitro release of insulin was evaluated in two different buffer solutions according to an earlier work with slight modification [8]. In brief, phosphate buffered saline (PBS, pH 7.2) was prepared to simulate the gastrointestinal tract (GIT) environment. Approximately 30 mg of the prepared insulin-loaded microparticles was placed in a polycarbonate dialysis membrane and a 2.0 mL volume of the dissolution medium was added. The membrane was properly secured at both ends by tying it with thread to prevent leakage. The prepared dialysis membrane was suspended in 200 mL of PBS and at different time intervals, 2 mL aliquot was withdrawn and replaced with same solution. The withdrawn samples were filtered, appropriately diluted and analysed using high pressure liquid chromatography (HPLC) as previously described [9]. The concentration of insulin released in the medium was calculated and plotted against time. All the experiments were performed in triplicate.

In vivo diabetic study

Wistar albino rats weighing 180 were obtained and were allowed to acclimatize to the new experimental environmental condition. The entire animals were weighed and they had access to food and water before the study. Prior to the induction of diabetes, they were fasted overnight and the baseline glucose level was determined with a glucometer (Accu-check Roche, USA). The rats were made diabetic using alloxan monohydrate solution as previously reported [10]. Rats with blood glucose level above 220 ml/dl were considered diabetic and were enrolled in this investigation.

Oral administration of insulin-MPs

Four groups of seven rats per group were used in this investigation. Rats in group I received saline, those in groups II and III received insulin solution orally and subcutaneously (s.c), respectively. Rats in group IV received Insu-MPs (C-5) equivalent to 40 I.U/kg with the aid of an intragastric tube. Blood samples were collected from the tail of the rats and the blood glucose concentration was obtained as a percentage of the baseline using the glucometer (Accu-check, Roche, USA). All the animal experiments in this study was approved by the institutional (University of Nigeria) Review Board for Use of Animal Subjects under the Directorate of Research (no. DOR/UNN/17/00014) and were in compliance with the Federation of European Laboratory Animal Science Association and the European Community Council Directive (Publication No (86/609/EEC) of November 24, 1986 (11).

Statistical analysis

All experimental data were expressed as (mean ± standard error (SE, n = 3). Statistically significant differences were evaluated using one-way analysis of variance (ANOVA) or a two-tailed t-test using SPSS Version 11 (SPSS Inc, Chicago, IL, USA). Differences were considered significant at p < 0.05.

RESULTS

Micromeritics and recovery rate of the microparticles

Table 2 depicts the results of micromeritics properties and the recovery rate of the microparticles. The recovery rate was very high, the maximum and minimum recovery rate were 88.0 and 76.2 % for batch C3 and C1 respectively. However, there was no significant difference (p < 0.05) among the batches of the preparation. Hence, none of the formulations showed up to 100 % recovery rate, due to losses that occurred during the processes of scrapping, pulverising and harvesting the microparticles.

Particle size, zeta potential and polydispersity index of the microparticles

Table 3 shows the particle properties of the formulations. The particles sizes and the zeta potential of the preparation range from 261.3 - 321.5 µm and 28 - 38 mV, respectively. The polydispersity indices range from 0.11 ± 0.1 to 0.24 ± 0.2.

Encapsulation efficiency and loading capacity

The encapsulation efficiency (EE) and loading capacity (DC) as shown in Table 3, ranged from 61.0 - 82.3 % and 23.2 - 42.6 %, respectively. EE and DC increased as the concentration of the PEG-4000 increased. However, there was no significant difference among the batches of the formulation.

Table 2: Micromeritic properties and recovery rate of insulin-loaded microparticles

<table>
<thead>
<tr>
<th>Batch</th>
<th>BD (g/cm³)</th>
<th>TD (g/cm³)</th>
<th>CI (%)</th>
<th>AR (%)</th>
<th>RR (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C1</td>
<td>0.351</td>
<td>0.481</td>
<td>12.12</td>
<td>28.40</td>
<td>76.21</td>
</tr>
<tr>
<td>C2</td>
<td>0.341</td>
<td>0.491</td>
<td>19.17</td>
<td>29.21</td>
<td>81.17</td>
</tr>
<tr>
<td>C3</td>
<td>0.361</td>
<td>0.487</td>
<td>26.11</td>
<td>26.41</td>
<td>88.03</td>
</tr>
<tr>
<td>C4</td>
<td>0.411</td>
<td>0.512</td>
<td>18.91</td>
<td>30.11</td>
<td>81.27</td>
</tr>
<tr>
<td>C5</td>
<td>0.431</td>
<td>0.514</td>
<td>24.11</td>
<td>29.16</td>
<td>89.25</td>
</tr>
</tbody>
</table>

Table 3: Mean size, zeta-potential (ZP), polydispersity index (PDI), drug-loading (DL) and encapsulation efficiency (EE %) of insulin-loaded microparticles (n = 5)

<table>
<thead>
<tr>
<th>Batch</th>
<th>PS (µm)</th>
<th>ZP (mV)</th>
<th>PDI (%)</th>
<th>DL (%)</th>
<th>EE (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C1</td>
<td>261.3 ± 1.3</td>
<td>32 ± 0.2</td>
<td>0.12 ± 1.1</td>
<td>23.2</td>
<td>61.0</td>
</tr>
<tr>
<td>C2</td>
<td>268.4 ± 4.0</td>
<td>28 ± 0.3</td>
<td>0.16 ± 0.7</td>
<td>30.0</td>
<td>70.8</td>
</tr>
<tr>
<td>C3</td>
<td>277.2 ± 2.3</td>
<td>29 ± 1.0</td>
<td>0.22 ± 1.4</td>
<td>34.1</td>
<td>70.9</td>
</tr>
<tr>
<td>C4</td>
<td>311.3 ± 0.1</td>
<td>33 ± 0.9</td>
<td>0.11 ± 0.1</td>
<td>34.4</td>
<td>74.2</td>
</tr>
<tr>
<td>C5</td>
<td>321.0 ± 0.3</td>
<td>38 ± 1.1</td>
<td>0.24 ± 0.2</td>
<td>42.6</td>
<td>82.3</td>
</tr>
</tbody>
</table>

Thermal properties

The thermal properties of the prepared microparticles are shown in Figure 1. The thermograms of the insulin-loaded MPs were intense endothermic peaks that ranged from 95.0
95.5 °C in all the formulation. This clearly shows no much difference among the insulin microparticles prepared.

Figure 2, which shows irregularly-shaped and free-flowing particles, with increasing size in the order of C1 < C2 < C3 < C4 < C5.

In vitro drug release from microparticles

In vitro release of insulin in phosphate buffer of pH 7.2 from the various batches of the microparticles prepared with snail cyst is graphically represented in Figure 3. All the batches of the formulation showed sustained release characteristics over a period of 10 h. Interestingly, there was an initial burst release of the insulin from the microparticles, which was more pronounced in the batch with high concentration of PEG to snail cyst (5:1). This was followed by more sustained drug release. Microparticles prepared with high content of PEG to mucin ratio (batch C-5) had the tendency to fully sustain the release of insulin for prolonged period of time.

In vivo anti-diabetic activity

In vivo hypoglycaemic properties of the optimized batch (C-5) and the reference samples were investigated using alloxan-induced diabetic rat model and the results are presented in Figure 4. Results showed that there was no significant hypoglycaemic effect after oral administration of saline solution. On the contrary, rats dosed with subcutaneous injection of insulin showed a sharp and remarkable blood glucose level reduction. The onset of the pharmacological effect was very fast and attained a maximum effect at 4 h with nadir effect or decrease of 61 % from the initial 100 %. After the period of 5 h, the glucose level began to rise again and at 10 h, it reached 88 %; nearly getting to the starting point of 100 %. In contrast, insulin solution administered orally, showed no significant blood glucose reduction when compared to the insulin-sc. Interestingly, oral administration (40 IU/kg) of the insulin-

Figure 1: DSC thermograms for insulin-MPs batches (C1, C2, C3, C4 and C5)

Figure 2: A representative photomicrographs of the morphology of insulin-MPs (A1 = C1, B = C2, C= C3 and D= C5. C4 is very similar to that of C5. The arrows indicate the particle shape of the various MPs

Figure 3: Insulin release profiles from microparticles in phosphate buffer pH 7.2 from PEGylated-snail cyst

Morphological features of the microparticles
loaded MPs exhibited delayed onset of activity, but had prolonged hypoglycemic activity when compared to the insulin-sc administration (Figure 4). For insulin-loaded MPs, the hypoglycemic effect was maximum at 10 h with nadir effect of 54%, and this activity was sustained for a longer period of time (> 10 h).

The thermal properties of the formulations indicate that the polymers are well blended resulting in a new polymer entity with a narrow PDI.

Morphological results indicate that there was no homogeneity in the morphology of the particles. It is worthy of note that increase in the amount of PEG-4000 created a significant increase in the size of the particles. The batches with lower concentration of PEG exhibited smaller particle size than batches with higher PEG-4000 concentration.

Results of in vitro drug release showed that insulin release from the microparticles prepared using the remote loading process as used in this work presented with the challenge of initial burst effect. This is because there is every tendency for part of the drug to adhere to the surface of the microparticles. In such cases, the interaction of microparticles with the dissolution medium will cause an early release of the peripheral drug on the outer surfaces of the microparticles resulting in an initial burst effect. Importantly, burst release resulting in biphasic release pattern may be utilized in therapeutic design of dosage forms especially in the management of disease condition like diabetes.

Results of anti-diabetic study showed that there was no significant hypoglycaemic effect

![Figure 4: Effect on blood glucose levels in diabetics animal model after orally administered of insulin-loaded microparticles (batch C-5), insulin solution (ins-sol), insulin subcutaneous (Ins-sc) and saline](image)

DISCUSSION

The results of micromeritics properties and the recovery rate showed that the formulation method adopted in this study was good. The size of the particles is very important in the drug carrier, as it forms one of the key determinants of the amount of drug loaded in the formulations which directly affects the release and the pharmacokinetic activities of the drug. More so, the zeta potential is an important parameter of the microparticles, since the surface charge gives an indication of the electrostatic stability of the preparation and the degree in which the particles will interact with the biological media [10]. From the study, the polydispersity indices indicate a unimodal size distribution of the formulations. Additionally, the majority of the particle sizes are within the lower micrometer range and their zeta potentials indicate that the formulations are stable.

The EE and DC of the formulations clearly showed that PEG-4000 had direct influence on the amount of drug incorporated into the polymer. In all cases, the EE and DC of the preparations were very high, which further proved that the formulation method was reliable and could be exploited further for possible future commercial preparations.

Clinically, the objective of diabetes management is always to lower the blood sugar level and maintain it at a particular level that is not harmful. This is possible if a loading dose of the antidiabetics is administered, but this must not be confused with dose dumping. The subsequent slow release as seen in this formulation (Figure 4) would serve as the maintenance dose, which would further sustain the availability of the insulin in the plasma for a long period of time. It is worthy of note that none of the formulations emptied the entire drug in the form of dose dumping just after about 1 h.

Results of anti-diabetic study showed that there was no significant hypoglycaemic effect
observed after oral administration of saline solution because it did not contain any anti-diabetic agent; rather it served as the negative control. However, subcutaneously administered insulin which served as positive control exhibited significant hypoglycemic effect. The rebound effect of rise in blood glucose observed in the study could be attributed to the relatively short circulating plasma half-life of the drug and the possibility of rapid clearance of the administered insulin from the system by the kidneys [13,14]. In contrast, insulin solution administered orally showed no significant blood glucose reduction when compared to the insulin-sc. This clearly indicates that oral insulin solution could not exert any meaningful hypoglycemic effect and the reasons are attributed to; (i) intestinal enzymatic degradation of the peptides due to molecular cleavage sites when crossing the intestinal mucosa via active transport or passive diffusion, (ii) the high molecular weight of insulin hindering absorption into biological system and (iii) acidic environment of the gastrointestinal tract (GIT) affecting the stability of the insulin causing it to lose its biological activity.

Although the oral dose administered was higher than the dose for subcutaneous injection of insulin in diabetes treatment, this is due to lower absorption of oral route of drug administration. Pharmacologically, the onset of action for orally administered insulin was slow compare to the subcutaneously administered insulin, and this can be explained as follows: the preparation underwent a gradual swelling by the polymers and subsequently released the insulin from the microparticles via diffusion mechanism. Moreover, the lag or delay onset time could also be attributed to the time for the microparticles to reach the site where the released insulin could be absorbed [13]. The prolonged effect was attributed to the snail cyst (as the carrier) and its combination with PEG in the form of PEGylation or complexation. The mucoadhesive property of the snail cyst allowed a closer intimacy of contact between the drug and mucosal wall of the GIT at the absorption area, and increased the residence time of the drug, and thus enhanced the permeability of the encapsulated drug.

Previous studies have shown that the polyethylene glycol chains have the ability to increase the apparent molecular size of peptide drugs and alleviate their high hydrophilicity thereby increasing their bioavailability and enhancing their cell membrane permeation [13,14]. Additionally, it has been established that polyethylene glycol has the capability to limit the globular filtration of the absorbed insulin in the system thereby prolonging the plasma circulation time [15,16]. This assertion was in agreement with previously reported PEG inclusion strategies to prevent the early clearance of the peptide from the system [17,18].

The results of the hypoglycaemic activity in this study has further lent credence to the fact that steric hindrance of PEG network would prevent the early clearance of insulin and that its interaction with the mucus of the GIT played a synergistic role in protecting the environmental degradation of insulin thereby increasing its biological activity.

CONCLUSION

Insulin-loaded MPs for oral delivery have been successfully prepared with snail cyst and PEG-4000 and evaluated for better diabetes management. The results showed the effectiveness of PEGylated mucin microparticles as a carrier system for oral insulin delivery as it was comparable to subcutaneously administered insulin after 6 h. The findings also indicate that insulin-loaded PEGylated microparticles protect insulin from degradation in the GIT, facilitate its delivery in biologically active form, prolong its action and enhance its oral absorption in a diabetic animal model. Further studies will seek to evaluate the efficacy of the formulation in higher animals, with a view to surmounting the challenge of oral delivery of insulin.

DECLARATIONS

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Conflict of interest

The authors have no conflict of interest regarding this research paper. The research was financed by TETFund-NRF, Government of Nigeria.

Contribution of Authors

The work was original designed by Mumuni A Momoh, Franklin C. Kenchukwu, Kenneth A. Ofokansi and Anthony A. Attama, and the studies were carried out by same people. Ossai C. Emmanuel, Azubuike C. Onyeto and Youngson Darlington; were involved in the in vivo / in vitro evaluations. The writing of the manuscript was...
done by the first four authors. All authors read the manuscript before it was submitted for publication.

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