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Original Research Article

DEVELOPMENT OF GELATIN-SODIUM ALGINATE MICROPARTICLES FOR ORAL INSULIN DELIVERY

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ABSTRACT

Oral delivery of peptide drugs in the management of diabetes remained a major challenge among formulation scientists in the pharmaceutical industry. In this study, insulin-loaded microparticles for oral delivery were prepared with gelatin and sodium alginate and combined at different ratios using the double emulsion technique. The insulin-loaded microparticles (MPs) were evaluated for yield, and particle size, shape and thermal properties were determined. The *in vitro* release of insulin and blood glucose reduction after oral administration to diabetic rats were determined. The microparticles formed were spherical and pitted. The prepared insulin-loaded microparticles showed a lag phase before insulin release. The gelatin:sodium alginate (Na-Alg) ratio of 0:1 was the only formulation that released the most *in vitro*. The percentage blood glucose reduction for subcutaneously administered insulin was significantly greater than that for the formulation ($p < 0.05$). The reduction effect observed after orally administered insulin-loaded MPs in batch with Na-Alg-gelatin (1:1) was observed after 9 h. However, this effect was like the effect observed in

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subcutaneously insulin solution. There was gradual release with a sustain effect of lower blood glucose level for a period of 24 h. These results are indicative of its effectiveness as an alternative for the delivery of insulin.

INTRODUCTION

Insulin used in the management of diabetes is traditionally administered subcutaneously. This method, even though efficient, often leads to poor compliance and low quality of life for patients [1]. The most comfortable route of delivery for most patients is the oral route. However, passage through the gastrointestinal tract can lead to enzymatic and chemical degradation of the insulin moiety due to proteolysis. Several studies have attempted to improve the gastrointestinal passage and intestinal absorption of insulin. The explored strategies include permeation enhancers, protease inhibitors, mucoadhesives and polymeric drug carriers [2]. The last strategy has proven to be more successful but depends on polymers having key characteristics such as pH sensitivity and good gastroprotective properties. Therefore, it can be agreed that combining similar functional polymers for this purpose will likely have a gastroprotective effect [3].

Gelatin is a positively charged surface with high-density protein polymer, a product obtained from animal collagen. There are two types of extraction processes, namely, type A (acid hydrolyzed) and type B (alkaline hydrolyzed) [4]. The flexible nature of the gelatin and the numerous peptides allow it modification. In this context, it resists digestion or degradation in the GIT and is considered very compatible in biological systems [4]. It has good adhesive features, hence the ability to modify drug release and navigate any pH-related situation [3,4].

Na-Alg is an anionic polysaccharide of (1-4)-linked β-D-mannuronic acid (M) and a-L-glucuronic acid (G). These G residues have gelling properties that allow the formation of microparticle and nanoparticle matrices [5]. Moreover, in contrast to chitosan, alginate is soluble at high pH and insoluble at low pH. They are also mucoadhesive and offer better-controlled diffusion of the active ingredient. They therefore have better and more predictable release kinetics [6]. Considering all the above-mentioned findings, as well as the non-toxic and biodegradable nature of both gelatin and alginate, we hypothesized that their combination constitutes a versatile formulation for the encapsulation and controlled release of insulin. This system may display synergistic activities in preventing the early release of insulin in acidic regions after oral administration. Thus, this study aimed to formulate insulin-loaded microparticles for oral insulin delivery with gelatin-sodium alginate hybrids using the double emulsion method. The thermal properties of the polymer combinations were evaluated. The encapsulation efficiency, in vitro release of insulin and the ability to protect insulin offered by oral administration were also evaluated.

MATERIALS AND METHODS

Materials

Human recombinant insulin (Eli Lily, USA), paraffin oil (Moko Pharmacy Ltd., Lagos, Nigeria), sodium alginate (Mw = 380.000 g/mol, M:G ratio 25:75, Merck, Germany), gelatin (medium grade), Span 60 (WACO, Japan), acetone (Merck, Germany), potassium dihydrogen phosphate (98 %, May & Baker Ltd, Dagenham, England), sodium hydroxide (BDH, England), and GlucoPlus (GlucoPlus Inc, Canada) were used with no further purification. Water of Milli-Q grade (18.2 MΩ/cm) was used in all experiments.

Preparation of Microparticles

The ratios of gelatin and sodium alginate used for preparing the microparticles are shown in Table 1. The w/o/w double emulsion technique was used [2]. Specifically, 1 g of each gelatin and Na-Alg ratio (1:1) mixture was dispersed in 20 mL of distilled water to produce a 10% w/v homogenous dispersion. This mixture was sonicated at an amplitude of 100 rps for 60 s using an ultrasonic probe (Athena Technology, India). A 10 mL volume of insulin was added to this dispersion and gently sonicated using an ultrasonic probe (Athena Technology, India). The dispersion was gradually poured into a 100 mL beaker container with 50 mL of light liquid paraffin and stirred using (Silverson Machines Ltd, Waterside, England) at 300 rps for 60 s. A 400 mg weight of span-60 dissolved in 1 mL of ethanol was added to this stage of formulation and stirred using a magnetic stirrer at 300 rps for 60 s. The insulin-loaded gelatin-Na-Alg mixture in liquid paraffin was then poured into 200 mL of acetone maintained at -20 °C with continued homogenization using Ultrasonic Sonicator (Model: ATP 500, Mumbai, India) at 200 rpm for 10 min. The beaker was immersed in an ice jar to minimize the temperature increase that can lead to the degradation of insulin. The acetone was evaporated, and the resulting solution was brought up to 100 mL using distilled water. The microparticle formulation was centrifuged to remove the exogenous insulin. The supernatant was removed, and the formulation was remade to 100 mL using distilled water and stored at 4 °C for further study. The procedure was repeated using gelatin-Na-Alg ratio of 0:1 (Z1), 1:0 (Z2), 1:1 (Z3), 1:2 (Z4) and 2:1 (Z5).

Characterization of Insulin-loaded MPs

Recovery Value

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

$$\% \textbf{ Recovery} = \frac{\textbf{W1}}{\textbf{W2} + \textbf{W3}} \boldsymbol{x}\ \textbf{100} \quad \text{..Equation 1}$$

where W1 is the weight of the NPs (g), W2 is the amount of insulin (g) and W3 is the amount of carrier and additives (g).

Differential Scanning Calorimetry (DSC).

Insulin-loaded MPs so formulated were subjected to thermal study using a DSC (DSC-60, Shimadzu Co., Ltd., Japan). In the study, 5 mg of the various formulations were weighed, placed in a specimen pan and sealed under an inert atmosphere (N_2). The specimen and reference material were placed in the various sample holders. Evaluation was performed in the temperature range from 20–220 °C, with a heating rate of 10 °C/min. The measurements were performed under nitrogen flow at a rate of 20 mL/min. Empty pan were used for the baseline. Similar procedure was also used in the unloaded sample for comparison.

Morphology and Particle Size Evaluations

The morphology of the MPs was determined using scanning electron microscopy (SEM) (Hitachi Japan, Model 3400 N) to determine the surface morphology of the insulin MPs. Herein, 10 µL of the microparticle was placed on a glass slide. The samples were fixed to a stub and gold-sputtered to neutralize the charging effects before scanning via SEM at an acceleration voltage of 20 kV.

The particle sizes of the microparticles were determined by computerized image analysis using a polarized light microscope (Lieca, Germany) equipped with a Motic image analyzer (Moticam, China). Briefly, 10 µL of the gelatin/ Na-Alg MPs from each batch was placed on a slide (Mansfield, Germany). Then, the specimen was covered with a cover slip and observed under polarized light. The sizes of the particles were measured using automated software and the average values were reported.

Determination of Encapsulation Efficiency (EE) and Drug Loading Content (DLC)

The EE and DLC of insulin-loaded MPs were determined. Herein, 5.0 mL of the sample suspension was centrifuged at 5000 rpm for 30 min. The absorbance of these supernatants was determined in a UV spectrophotometer (Jenway, 605, Germany) at a wavelength of 270 nm, and the encapsulation efficiency (EE) and loading capacity (LC) were calculated using equations 2 and 3.

$$\textbf{EE } \% = \frac{\textbf{Actual drug content}}{\textbf{Theoretical drug content}} \times \textbf{100}$$

……..Equation 2

LC represents the ratio of the amount of entrapped drug to the total weight of the polymer. It is determined as follows:

$$\textbf{LC} = \frac{\textbf{Wa}}{\textbf{Wd}} \times \textbf{100} \quad \text{……Equation 3}$$

Where Wa is the weight of the polymer added to the formulation and Wd is the amount of insulin entrapped by the microparticle.

Physical Examination and Time-dependent pH Stability Study

The formulations were physically examined for color, homogeneity, and consistency. The pH values of the insulin-loaded MPs were measured using a digital pH meter (Horiba, Laqua, Japan). Measurements were made at predetermined intervals of weeks, samples were measured in triplicate, and the average values were reported.

Sedimentation Rate and Redispersibility Study

The formulations were allowed to stand undisturbed on a flat bench for 24 h, 7 and 30 days and the sedimentation rates (SR) were calculated using equation 4:

$$\text{SR} = \frac{(initial\ height - height\ of\ \ uppermost\ clear\ zone)}{Height\ of\ the\ formulation} \times 10 \quad \text{……..……Equation 4}$$

In vitro Release of Insulin from the Microparticles

The *In vitro* release profiles of the insulin-loaded microparticles were determined using a dialysis membrane technique. One millilitre of the formulation (containing 0.0157 mg of insulin) was added to a dialysis membrane (MWCO 8000 – 10000 Spectrum Labs, Germany) pretreated by soaking in phosphate buffer (pH 7.2) for 24 h before use. The membrane was clipped and lowered into 200 ml phosphate buffer at pH 7.2 and stirred using beads of a magnetic stirrer at 100 rpm and 37 °C ± 1 °C. At predetermined time intervals of 30 min, 1 h, 2 h, 4 h and 8 h, 0.5 ml of the sample was removed. The aliquots removed were also replaced with fresh phosphate buffer. The withdrawn samples were filtered through a 0.22 µm filter (Millipore®, USA). The concentration of insulin in the aliquot was determined by using a UV spectrophotometer at 271 nm. The cumulative amount of insulin release (CAR) at different time intervals was calculated using equation 5 and plotted against time to obtain the insulin release pattern. The above procedure was repeated using the remaining batches. All tests were carried out in triplicate.

$$\textbf{CAR} = \frac{C \times D}{Total\ amount\ of\ insulin} \times \textbf{100}$$

…… Equation 5

Where C and D refer to the concentration of insulin in the dissolution medium and the volume of the dissolution medium, respectively.

Determination of the Kinetic Release Mechanism

In vitro insulin release data were then fitted to zero-order, first-order, Higuchi and Korsmeyer-Peppas models (equations 6-9) to establish the insulin release mechanism of the microparticles.

Zero-order equation: $Q_t = Q_0 + K_0t$ Equation 6

First-order equation: $\text{Log } Q_t = \text{Log } Q_0 + Kt/2.303$ Equation 7

Higuchi equation: $Q = K_Ht^{1/2}$ Equation 8

Korsmeyer-Peppas model: $\frac{Mt}{M\infty} = Kt^n$ Equation 9

where Mt/M∞ denotes the fraction of drug released at the time (t), K is a constant showing the structural and geometric characteristics of the particles, and n is the release exponent indicating the diffusion mechanism. Release exponents (n) = 0.45 < n < 0.89 and 0.89 indicate Fickian (case I) diffusion, non-Fickian (anomalous) diffusion and zero-order (case II) transport, respectively.

In vivo Activity of Insulin-Loaded Microparticles in an Alloxan-Induced Diabetic Rat Model

All experimental procedures were carried out in accordance with the Federation of European Laboratory Animal Science Association (FELASA) Guide for the Care and Use of Laboratory Animals and the European Union (Council Directive 86/609/EEC) and the Animal Ethics Committee of the Faculty of Pharmaceutical Sciences, University of Nigeria, Nsukka, with the reference number DOR/UNN/17/00014 was assigned.

Induction of Diabetes

Wistar rats weighing 90-105 g were purchased from the Department of Pharmacology and Toxicology of the University and were housed at a temperature of 22 ± 1 °C and 45% RH. The rats were fed a standard diet (Feeds BC, Nsukka, Nigeria) and provided tap water ad libitum. Lighting was performed on a standard 12 h on/12 h off cycle.

Diabetes was induced in rats by a single intraperitoneal injection of 100 mg/kg of freshly prepared alloxan (50 mg/mL in pH 4.5 citrate). After 10 days of administration, rats with blood glucose levels above 250 mg/dL were considered diabetic and used for the experiments. The rats were fasted for 12 h before the experiments and remained fasted for 24 h during the experiment but had free access to water.

Protocol for the Administration of Insulin-Loaded MPs

Seven groups each containing 7 selected diabetic rats were used for the animal study. The different microparticle insulin-loaded formulations were dosed accordingly as follows:

Group 1 orally received insulin-loaded microparticles of gelatin/sodium alginate (1:0).
Group 2 orally received insulin-loaded microparticles of gelatin/sodium alginate (0:1).
Group 3 orally received insulin-loaded microparticles of gelatin/sodium alginate (2:1).
Group 4 orally received plain microparticles of gelatin/sodium alginate (1:1).
Group 5 received an oral insulin dispersion.
Group 6 received subcutaneous insulin.
Group 7 received distilled water only.

Assessment of the Hypoglycemic Effect of Oral Insulin on Diabetic Rats

Blood (0.1 mL) was collected from the tail vein, and the blood glucose concentration was determined using a glucometer (GlucoPlus Inc., Canada) at selected time intervals of 0.5, 1, 3, 6, 9, 12 and 24 h. The results were obtained in triplicate, and the average was calculated.

Statistical and Data Analysis

All experiments were repeated at least three times. The following statistical analyses were performed: one-way analysis of variance, Duncan's post hoc test and Dunnett's multicomparison test using the instant 2.00 Macintosh software (San Diego, California, US). Differences were considered significant when $p < 0.05$.

RESULTS

The formulation concept of insulin-loaded microparticles prepared with varying ratios of gelatin and Na-Alg was targeted at enhancing the smooth passage of insulin from the GIT, by providing a protective environment for insulin absorption and improved bioavailability of insulin for effective treatment of diabetes.

Particle Sizes and the Recovery Value

The particle sizes and the yield values of the formulations vary according to the polymer ratios and are depicted in Table 2.

Determination of Encapsulation Efficiency (EE) and Drug Loading Content (DLC)

The ability of the polymer and their combination to encapsulate the drug were evaluated and the corresponding capability shows high drug value and variation in drug loading. The data of the EE and DLC are presented in Table 3.

Differential Scanning Calorimetry (DSC).

The degree of crystallinity or melting transitions and changes in the heat capacity of the polymers and the formulations were determined using a calorimeter. It was observed that some of the polymer retain their peaks and the changes on the enthalpy were clearly shows which determine the transformation phase of the polymer as shown in Figure 1.

Morphology, Particle Size and Time-dependent pH Evaluations

The representative photomicrographs of the microparticles prepared with gelatin and Na-Alg for Z1 and Z5 of the insulin-loaded formulation are presented in Figure 2 and Figure 3. The shapes varied according to the polymer combination. In some particles, discrete free-flowing was observed. The pH also shows some variation in changes as per the time of the preparation

Sedimentation Rate and Redispersibility Study

The sedimentation rate was evaluated to establish the stability and the ease of redispersing the formulation over time. The result shows little variation among the formulations and is depicted in Figure 4

***In vitro* Drug Release Profile and Kinetic Study**

The release profile of insulin from the various insulin-loaded microparticles prepared from different gelatin–Na-Alg ratio mixtures and the release model are shown in Figure 5 and Table 4. The preparations exhibited a very little lag time which varying in the ratio of polymer used for the microparticles.

Assessment of the hypoglycemic effect of oral insulin on diabetic rats

The effectiveness of the oral insulin microparticles prepared with gelatin, Na-Alg, and their combination in varying ratios was assessed based on their effectiveness in blood glucose reduction in alloxan-made diabetic rats. The result of the decrease in blood varied according to the formulation and showed effectiveness in some batches as compared to the oral insulin solution.

DISCUSSION

In this study, we set out to establish the possibility of using some polymer for the effective delivery of insulin orally, as a means of diabetic treatment. To this end, we examined the yield value of the preparation, thermal properties (DSC), particle sizes, morphological characteristics, and *in vitro* release of the formulations. The blood glucose-lowering effects of the formulation were evaluated and compared to subcutaneous and oral insulin solution

The formulation's percentage yield or recovery rates were directly related to the methodology. As shown in Table 3, the maximum yield was observed for the loaded formulation. High values (> 70%) of the percentage of the MPs recovered from the formulations strongly indicate that the formulation technique adopted was reliable. It is worth mentioning that the role of any drug delivery system (DDS) is to deliver the incorporated drug to the target tissues intact with little or no toxic effect on other organs or systems [7].

The thermal analysis by DSC is an important thermometric tool for evaluating the compatibility of formulations as well as conformational changes of the drug when in the formulation. Figure 1 shows an overlayed DSC thermogram of the selected microparticles. In general, the thermal analysis of both gelatin and sodium alginate shows broad endothermic peaks corresponding to dehydration events centred at ca. 105 °C with an enthalpy of 2.5 and 2.1 mW/mg, respectively [8]. In our formulations, the mixture of gelatin and sodium alginate at an equal ratio of 1:1 (Z0) without insulin showed a melting temperature peak of 143.2 °C at 102.1 mW/mg enthalpy compared to that of insulin (no recrystallization was observed). The DSC results for insulin showed a melting peak at 139.5 °C (Tm) with a corresponding enthalpy of 97.7 mW/mg (no recrystallization was observed). This revealed the crystalline nature and purity of the drug compared with its previously reported characteristics. The DSC results of the drug-loaded microparticles showed different melting and thermal properties. As depicted in Figure 1, formulation Z0 (unloaded gelatin:sodium alginate 1:1), compared to its insulin-loaded batch with the same polymer ratio Z3 (loaded gelatin:sodium alginate 1:1), had an elevated melting peak temperature, and enthalpy of 143.2 °C and 102.2 mW/mg respectively, while Z3 had a melting peak temperature of 153.8 °C and an enthalpy of 51.4 mW/mg. This was, however, higher than that of the pure API, which indicates that there was little conformational change when the drug was encapsulated in the formulation. In summary, there was no change in crystallinity, which might affect the overall encapsulation efficiency, as there is a high chance that the drug can leak out of the microparticle. This might also affect the stability of the formulation and the ability of the polymers to confer protection on the insulin moiety. Batch Z1 (with a gelatin:sodium alginate polymer ratio of 0:1) had a melting temperature peak of 164.6 °C and an enthalpy of 174.6 mW/mg. When the alginate concentration increased, as observed for batch Z4 (with a gelatin:sodium alginate polymer ratio of 1:2), the melting temperature peak did not decrease compared with that of the API, as the values reached 147.8 °C, and the enthalpy increased to 51.4 mW/mg. This is still higher than that of the insulin drug, which shows the instability of the drug in the formulation. Compared with Z3,

Table 1. Representation of the polymer ratio used in the formulation[a]

Batch	Gelatin (g)	Sodium alginate (g)	Insulin (mL)	Liquid paraffin (mL)	Span-60	Water (mL)
Z0	1	1	0	50	0.4	100
Z1	0	1	10	50	0.4	100
Z2	1	0	10	50	0.4	100
Z3	1	1	10	50	0.4	100
Z4	1	2	10	50	0.4	100
Z5	2	1	10	50	0.4	100

Key: **Z0** (unloaded gelatin:Na-Alg polymer ratio of 1:1), **Z1** (insulin-loaded gelatin:Na-Alg polymer ratio of 0:1), **Z2** (insulin-loaded gelatin:Na-Alg polymer ratio of 1:0), **Z3** (insulin-loaded gelatin:Na-Alg polymer ratio of 1:1), **Z4** (insulin-loaded gelatin:Na-Alg polymer ratio of 1:2), **Z5** (insulin-loaded gelatin:Na-Alg polymer ratio of 2:1).

Table 2. Particle size of gelatin-sodium alginate microparticles[a]

Batch	Particle size (µm) after 24 h	Particle size (µm) after 1 week	Particle size (µm) after 4 weeks
Z0	11.67 ± 12.86	12.01 ± 5.67	13.59 ± 7.12
Z1	16.57 ± 11.56	16.97 ± 8.19	19.35 ± 5.13
Z2	13.47 ± 17.43	14.13 ± 9.24	18.24 ± 8.15
Z3	15.12 ± 16.92	16.27 ± 9.46	20.14 ± 9.14
Z4	27.57 ± 10. 72	28.14 ± 9.12	33.28 ± 8.16
Z5	21.59 ± 21.03	22.56 ± 4.12	28.14 ± 6.14

Key: **Z0** (unloaded gelatin:sodium alginate polymer ratio of 1:1), **Z1** (insulin-loaded gelatin:sodium alginate polymer ratio of 0:1), **Z2** (insulin-loaded gelatin:sodium alginate polymer ratio of 1:0), **Z3** (insulin-loaded gelatin:sodium alginate polymer ratio of 1:1), **Z4** (insulin-loaded gelatin:sodium alginate polymer ratio of 1:2), **Z5** (insulin-loaded gelatin:sodium alginate polymer ratio of 2:1).

Table 3. Encapsulation efficiency, percentage yield and loading capacity of gelatin:sodium alginate microparticles.[a]

Batch	EE (%)	DLC (%)	Yield (%)
Z1	83.98 ± 0.93	4.45 ± 0.45	87.46 1.57
Z2	79.84 ± 0.24	2.23 ± 0.96	76.43 2.93
Z3	89.34 ± 0.58	4.65 ± 0.35	82.35 1.35
Z4	89.34 ± 0.95	5.47 ± 1.19	93.47 ± 1.08
Z5	83.98 ± 0.93	4.45 ± 0.45	87.46 ± 1.57

*[a] Key: **Z1** (insulin-loaded gelatin:sodium alginate polymer ratio of 0:1), **Z2** (insulin-loaded gelatin:sodium alginate polymer ratio of 1:0), **Z3** (insulin-loaded gelatin:sodium alginate polymer ratio of 1:1), **Z4** (insulin-loaded gelatin:sodium alginate polymer ratio of 1:2), **Z5** (insulin-loaded gelatin:sodium alginate polymer ratio of 2:1).*

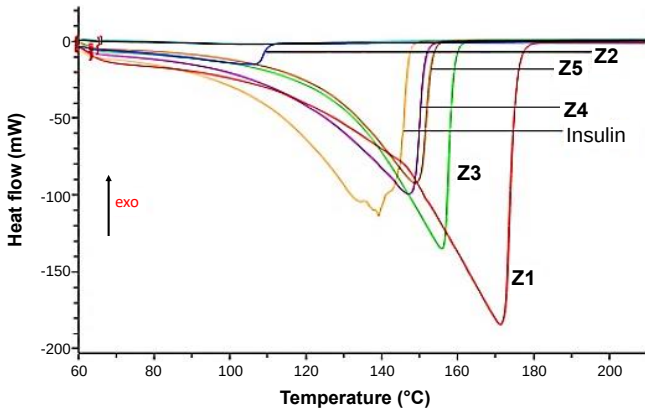

Figure 1. DSC thermograms of batches containing **Z1**, **Z2**, **Z3**, **Z4**, **Z5** sodium alginate, insulin, and gelatin in superposition.
*Note: **Z1** (0:1) is a microparticle-based ratio of 0:1 gelatin and sodium alginate, **Z2** (1:0) is a microparticle based ratio of 1:0 gelatin and sodium alginate, and **Z3** (1:1) is a microparticle-based on ratio of 1:1 of gelatin and sodium alginate with insulin. **Z4** and **Z5** are microparticle-based on ratios of 1:2 and 2:1, respectively, of gelatin and sodium alginate loaded with insulin.*

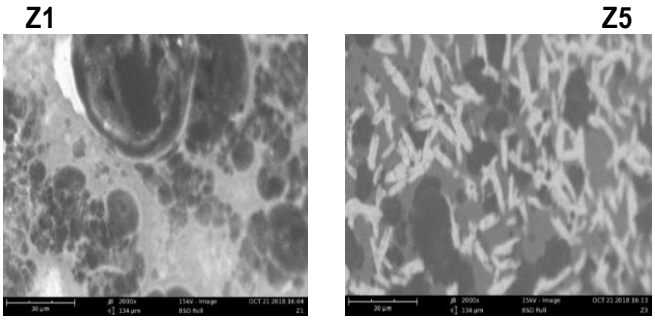

Figure 2. Morphology of **Z1** (2000x) (left) and **Z5** (20000x) (right) microparticles.
*Key **Z1** (insulin-loaded gelatin: sodium alginate polymer ratio of 0:1), **Z5** (insulin-loaded gelatin: sodium alginate polymer ratio of 2:1). Scale bars = 30 µm.*

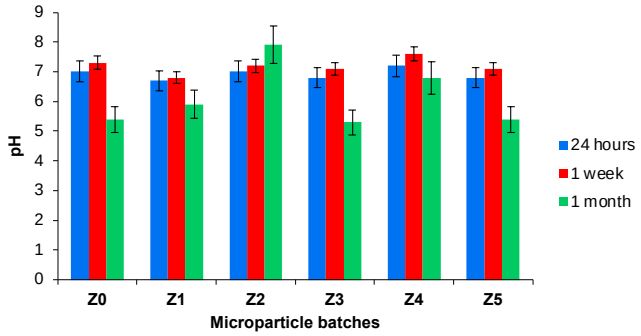

Figure 3. Time-dependent pH results of batch **Z** (gelatin:sodium alginate) containing 10 ml of insulin in gelatin and sodium alginate.
*Keywords: **Z0** (unloaded gelatin:sodium alginate polymer ratio of 1:1), **Z1** (insulin-loaded gelatin:sodium alginate polymer ratio of 0:1), **Z2** (insulin-loaded gelatin:sodium alginate polymer ratio of 1:0), **Z3** (insulin-loaded gelatin:sodium alginate polymer ratio of 1:1), **Z4** (insulin-loaded gelatin:sodium alginate polymer ratio of 1:2), **Z5** (insulin-loaded gelatin:sodium alginate polymer ratio of 2:1).*

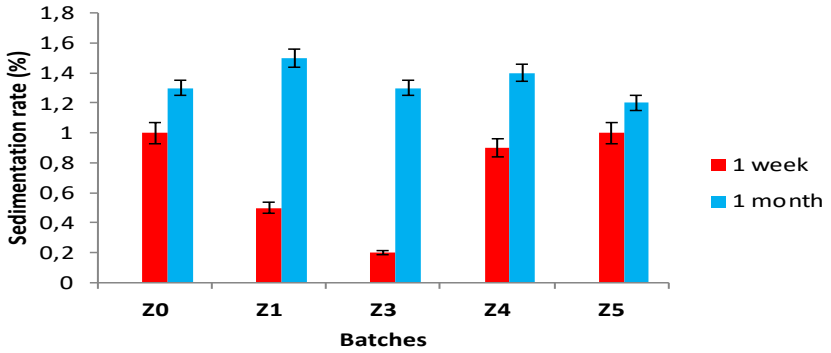

Figure 4. Sedimentation rate percentages of different formulation batches.
Keywords: ***Z0*** *(unloaded gelatin:sodium alginate polymer ratio of 1:1),* ***Z1*** *(insulin-loaded gelatin:sodium alginate polymer ratio of 0:1),* ***Z3*** *(insulin-loaded gelatin:sodium alginate polymer ratio of 1:1),* ***Z4*** *(insulin-loaded gelatin:sodium alginate polymer ratio of 1:2),* ***Z5*** *(insulin-loaded gelatin:sodium alginate polymer ratio of 2:1).*

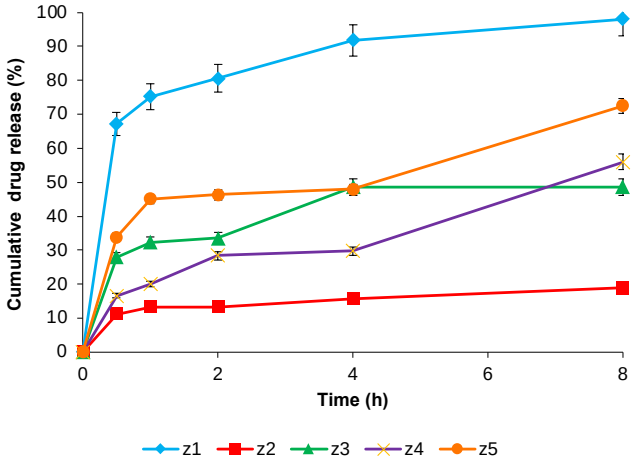

Figure 5. Comparison of *in vitro* release of insulin comparison from microparticles formulated with different ratios of gelatin:sodium alginate. The data are presented as the means ± SDs (n = 3).
Keywords: ***Z0*** *(unloaded gelatin:sodium alginate polymer ratio of 1:1),* ***Z1*** *(insulin-loaded gelatin:sodium alginate polymer ratio of 0:1),* ***Z2*** *(insulin-loaded gelatin:sodium alginate polymer ratio of 1:0),* ***Z3*** *(insulin-loaded gelatin:sodium alginate polymer ratio of 1:1),* ***Z4*** *(insulin-loaded gelatin:sodium alginate polymer ratio of 1:2),* ***Z5*** *(insulin-loaded gelatin:sodium alginate polymer ratio of 2:1).*

Table 4. R^2 values corresponding to the zero order, first order, Higuchi and KorsMeyer Peppas models for drug release determination of gelatin:sodium alginate microparticles.[a]

Batch[a]	Zero-order	First-order	Higuchi's	Korsmeyer's Peppers	
	R^2	R^2	R^2	R^2	N
Z1	0.8735	0.8735	0.9597	-	-
Z2	0.9527	09527	0.9670	0.9409	0.174
Z3	0.7761	0.7761	0.8594	0.8976	0.217
Z4	0.9574	0.9574	0.9155	0.9207	0.407
Z5	0.9066	0.9068	0.8724	0.7468	0.154

[a] Key: ***Z1*** *(insulin-loaded gelatin:sodium alginate polymer ratio of 0:1),* ***Z2*** *(insulin-loaded gelatin:sodium alginate polymer ratio of 1:0),* ***Z3*** *(insulin-loaded gelatin:sodium alginate polymer ratio of 1:1),* ***Z4*** *(insulin-loaded gelatin:sodium alginate polymer ratio of 1:2),* ***Z5*** *(insulin-loaded gelatin:sodium alginate polymer ratio of 2:1).*

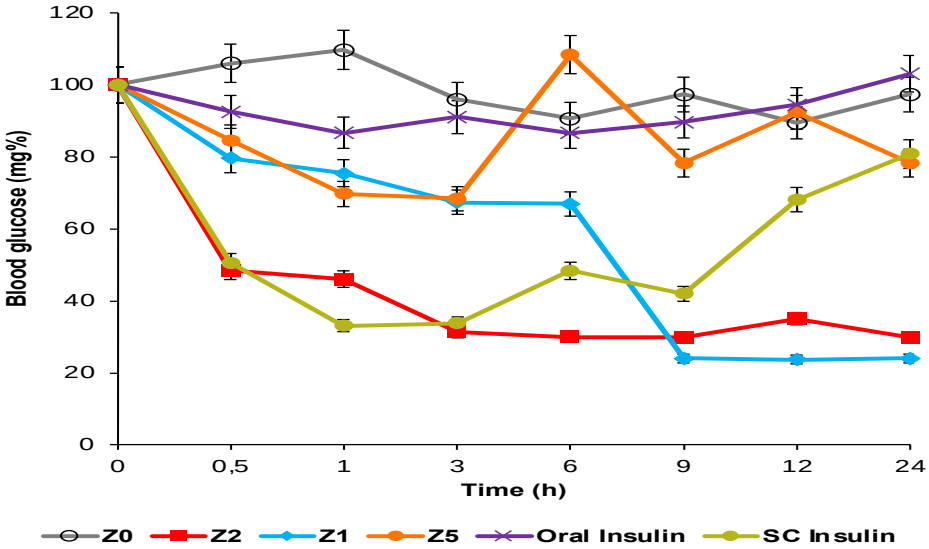

Figure 6. Blood glucose levels after a single oral administration of insulin solution (100 IU/kg), insulin-loaded microparticles at 100 IU/kg, unloaded microparticles and SC injection of insulin (2.5 IU/kg). The data are presented as the means ± SDs; n = 7 per group. *Keywords:* ***Z0*** *(unloaded gelatin:sodium alginate polymer ratio of 1:1),* ***Z1*** *(insulin-loaded gelatin:sodium alginate polymer ratio of 0:1),* ***Z2*** *(insulin-loaded gelatin:sodium alginate polymer ratio of 1:0),* ***Z5*** *(insulin-loaded gelatin:sodium alginate polymer ratio of 2:1).*

increasing the polymeric concentration of gelatin in Z5 (with a gelatin:sodium alginate polymer ratio of 2:1) led to a decrease in the temperature melting peak to 146.2 °C at an enthalpy of 89.1 mW/mg. However, when gelatin polymer was used alone, as seen in the formulation of batch Z2, the melting peak temperature was high at 145.4 °C at an enthalpy of 49.0 mW/mg. This suggests that the combination of the polymers did not confer conformational stability to the formulation [8].

The morphologies of the MPs were studied by scanning electron microscope (SEM). Several studies have demonstrated that the morphology and pore size of matrixes intended for drug delivery play an important role in the control of the release kinetics [9]. Figure 2 shows the morphological features of the insulin-loaded gelatin/sodium alginate microparticles based on optimized parameters of the in vitro dissolution profile as presented by the scanning electron micrograph. The physical stability and cellular uptake of microparticles are affected by particle size, which is influenced by several factors (see below). However, there was particle aggregation when the formulation was left on standing, indicating the possibility of physical instability. The surface of the Z1 microparticle was rough, and the microparticles were spherically shaped. Z5 microparticles had rough surfaces but were rod-like in shape. This can be attributed to the crystallization of the insulin drug in the formulation, which altered the shape of the formulation. Additionally, in the absence of steric hindrance or large molecular weight compounds, the net charges in cationic gelatin and anionic sodium alginate were not enough to prevent Ostwald ripening and particle aggregation [10].

The particle sizes of orally administered insulin-loaded microparticles, as previously mentioned, significantly affect their oral absorption and biodistribution, which ultimately determines their therapeutic efficacy. Generally, as the polymeric ratio increased, so did the particle size (Table 2). The unloaded batch, however, showed the least change in particle size, suggesting the presence of drug-induced crystallization and aggregation of the microparticles as time progressed [11]. Scientifically, particle size may be a function of one or more of the following: formulation excipients, degree of homogenization, homogenization pressure, rate of particle size growth, and crystal habit of the particle [10]. In terms of stability, the gelatin/alginate insulin-loaded microparticles were most stable in terms of pH, color, and odor. The presence of active drugs in the formulation and the need to achieve thermodynamic stability in the absence of electrostatic repulsion are usually motivating factors for particle size changes. Therefore, in the unloaded batch, Z0 was smaller than its loaded counterpart, and Z0 had little effect on the increase in particle size (Table 2). Ostwald ripening, according to the DLVO theory, explains the drastic increase in particle size growth after one month for batch Z4 (Table 2) [10]. These subbatches showed the least stability in the formulation and aggregated and increased in particle size for gelatin/sodium alginate microparticles: Z4 > Z5 > Z3 > Z1 > Z2 > Z0 (Table 2). The encapsulation efficiency (EE) and drug loading capacity (DLC) are important physicochemical properties used for determining the suitability of a drug carrier. The primary microparticles were observed to possess high structural integrity, which improved the encapsulation efficiency. The

formulated batches had encapsulation efficiencies greater than 79% (Table 3). This improved the therapeutic effect during the in vivo study [12] (Moeller and Jorgensen 2008). The overall drug loading capacity was low and showed significantly difference ($p < 0.05$) across the sub-batches. Additionally, there was no correlation between the encapsulation efficiency and the ratio of polymer used. This, therefore, suggested that insulin is a hydrophilic drug, and that double emulsion method used in the formulation could reduce drug loading [13].

All batches of the formulations were odorless, consistent and had uniform color. The pH value of the different batches of MPs was measured at 24 h, one week, and one month after preparation to ascertain the variation in pH with time, which could be a function of degradation of the API or excipients (Figure 3). Concerning the pH of the different formulations, a slight increase in the pH (at one week) followed by a reduction in the pH level (at one month) was observed (Figure 3). This can be explained by the increased quality of the glucuronic and mannuronic acid subunits, which reduces the acidity to a sufficient quantity, repressing microbial activity.

The decrease in the pH of the formulation loaded with the drug was significant ($p < 0.05$) compared to the decrease observed in the unloaded (drug-free) formulation. This indicates that a decrease in the pH may cause a change in the expected activity of the formulation. It is further suggested that such a change in the pH toward acidity could be overcome by the addition of a stabilizing agent, probably a buffer or a preservative, since the resulting instability might be of microbial origin during formulation. This could affect the pH and integrity of insulin, as this is above the accepted stable pH of 7.3 for insulin. Denaturation of the active compound can lead to loss of activity. It is be recommended that preservatives be added to this formulation, as there was a significant change in pH ($p > 0.05$) across the subbatches from 24 h to 1 month.

The sedimentation rate is usually affected by the particle size and viscosity of the suspension formulation, the presence of microparticles, and the wettability of the polymer. Sodium alginate/gelatin/insulin (batch Z) had no observable sedimentation rate after 24 h, hence only values obtained after 1 week and 1 month are shown in Figure 4. The formulation with the highest sedimentation rate was Z1, followed by Z4 and Z3. In all cases, a sedimentation rate below 1.6% was noted after one month. It was observed that upon gentle shaking, the formulation easily redispersed, indicating that the preparation was stable and did not undergo irreversible separation and phase inversion, which are considered very problematic in emulsion preparation [14]. The results clearly indicate that in the short-term stability study, the formulation parameters and ratios were properly selected and could withstand longer periods of storage with an even distribution when gently shaken.

Figure 5 shows the *in vitro* drug release profile of the insulin-containing gelatin-sodium alginate microparticles. The release of insulin from the microparticles was compared among the formulations for a period of 8 h. Insulin tends to ionize in a basic environment provided by phosphate buffer (pH 7.2). Sodium alginate is an anionic polymer that has high viscoelasticity and consistency but has the advantage of easy pore formation; hence, when used alone in pH 7.2 buffer, maximum release occurred as the polymer easily degraded at this pH. However, in the presence of gelatin, the pore size and outlet decreased, and as the concentration of the gelatin ratio increased, the percentage of drug released decreased. Concerning the above information, the decreasing order of release was as follows: Z1 > Z5 > Z4 > Z3 > Z2.

Interestingly, formulation Z1 was the only sub-batch for complete release, as it contained only sodium alginate, and at pH 7.2, the release was almost immediate. The addition of gelatin, however, reduced the rate of release by affecting the degradation and release of insulin. Thus, there was incomplete release of the sub-batches containing gelatin. It can be postulated that the addition of gelatin controlled the release of insulin by reducing the rate of erosion and blocking the channel pores of release through diffusion [15]. Additionally, the release of the entrapped protein is triggered by the degradation of the polymer by erosion, followed by the diffusion of the protein through the channels created in the process [10]. Thus, the erosion process is known to generate oligomers that can easily interact with the encapsulated protein, leading to its denaturation [10, 13].

The release data of insulin release from the formulation was fitted into mathematical models, such as zero-order, first-order, Higuchi's and Korsmeyer-Peppas models, as a way to investigate the kinetic mechanism as shown in (Table 4). According to the obtained R^2 values of the curves for various mathematical models, it was shown that there was a very good fit between the experimental data and the Korsmeyer–Peppas model ($R^2 > 0.9$). The Korsmeyer-Peppas model for the insulin-loaded microparticles had n values lower than 0.45, indicating that the release pattern occurred through Fickian diffusion through erosion of the surface of the microparticles. The release of insulin from the microparticles showed that it was pH dependent and factored in the erosion rate of the polymers during drug release. The percentage of Z1 (gelatin:sodium alginate 1:1) released exceeded 60% in the first 30 min, indicating that the rate of release was high. Additionally, Z4 and Z5 had release kinetics indicating a zero-order reaction ($R^2 > 0.9$), which indicated that the release of the formulation continued irrespective of the bioavailable concentration [16-18].

Figure 6 shows the hypoglycemic effect of insulin-loaded gelatinated polymer shell microparticles on the alloxan-treated diabetic mouse model. After subcutaneous insulin administration, the blood glucose level decreased significantly within 1 h and reached 45 mg% at the 2nd hour post-injection. The blood glucose level subsequently increased with time, returning to 80 mg% after 12 h. Oral insulin alone was unable to produce a hypoglycemic effect in diabetic rats. Unloaded

dose-treated diabetic rats did not show any significant changes in blood glucose levels during the long-term fasting. It also eliminated the probability of the polymers having hypoglycemic activity.

Gelatin/sodium alginate/insulin-loaded MP also significantly controlled the blood sugar level (BSL). Based on an *in vitro* release study, Z1 (gelatin:sodium alginate, 0:1) and Z5 (gelatin:sodium alginate, 3:1) were selected for the animal studies. Compared with Z5 (gelatin:sodium alginate, 3:1), alginate/gelatin/insulin microparticles Z1 (gelatin:sodium alginate, 0:1) improved the hypoglycemic response. Insulin-loaded Z1 microparticles efficiently and gradually decreased the blood glucose level except from one to 6 h, when the hypoglycemic effect was static. Thus, these phenomena are thought to be due to slow insulin release during swelling initiation of the core, i.e., alginate/gelatin/insulin-loaded microparticles reduced the level of blood glucose up to the 24th hour. Z5 insulin-loaded microparticles reduced the BSL to 60 mg% by the 3rd hour, but rapid degradation and excessive pore formation from the addition of the gelatin polymer reduced the protection offered to the active insulin component. There has been no study of the comparative advantage of an increasing concentration gradient of insulin. However, studies have shown that an increase in the concentration gradient can further improve intestinal absorption and hence bioavailability and pharmacological action [14, 16].

However, the formulation of Z2 containing insulin-loaded gelatin-sodium alginate ratios of 1:0, previous works have shown that gelatin alone can provide gastroprotection for the active drug but is not sufficient to control the release of the drug. However, the formulation continued to release after 24 h, suggesting that the release was controlled. This can be attributed to the fact that the gelatin used contained both high molecular weights and low molecular weights and thus created a polymer matrix sufficient to control the release. This observation was partly in agreement with the earlier work on oral insulin delivery using gelatin [14], and mucin obtained from snails [10]. Thus, owing to the ease of drug release, the blood glucose level decreased to 44 mg% at the 1 h mark and 23 mg% at the 6 h mark. The reduction in blood sugar concentration was gradually sustained throughout the evaluation period.

CONCLUSION

The results obtained in this study demonstrate that microparticles made from sodium alginate, gelatin and gelatin-sodium alginate can provide a versatile alternative to the formulation of insulin for oral use. The batches formulated in this work showed encapsulation efficiencies above 79%, with the drug homogenously dispersed in the gel matrix. Kinetics experiments indicated that the release pattern through erosion of the surface of the microparticles (Fickian mechanism) was pH dependent and tunable by the gelatin:sodium alginate ratio used. Moreover, the gelatin/alginate/insulin microparticles reduced the level of blood glucose up to the 24th hour. Overall, the foregoing results suggest that the formation of MPs of insulin with gelatin and sodium alginate is a potentially safe and promising combination system to protect insulin and enhance its oral delivery approach.

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AUTHORS' CONTRIBUTION

M.A.M. designed experiments and supervised the project. M.A.M., B.C.A., P.A.A., F.C.K., O.C.R. and J.A. performed experiments and characterization. M.A.M., B.C.A., F.C.K wrote the original draft. M.A.M., E.B., Y.M., C.S. and D.D.D. conceptualized the research, analyzed data and edited the final manuscript.

CONFLICT OF INTEREST

Authors declare no conflict of interest.

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