Journal of Controlled Release 143 (2010) 128–135

Contents lists available at ScienceDirect

Journal of Controlled Release

journal homepage: www.elsevier.com/locate/jconrel

Novel fibrillar insulin formulations for oral administration: Formulation and in vivo studies in diabetic mice

Y. Dekel, Y. Glucksam, R. Margalit *

Department of Biochemistry, the George W. Wise Faculty of Life Sciences, Tel Aviv University, Tel Aviv 69978, Israel

ARTICLE INFO

Article history:
Received 3 October 2009
Accepted 20 December 2009
Available online 5 January 2010

Keywords:
Diabetes
Drug delivery
Insulin fibrils
Oral delivery
ICR mice

ABSTRACT

The advantages of oral insulin are well-recognized, yet such formulations are still unavailable. Towards that goal we developed, and evaluated in diabetic ICR mice, two novel insulin microparticles for oral delivery. Although different in structure and shape, both microparticle formulations share: (i) hyaluronan on their surface (ii) fibrillar insulin, loaded at 50–100% efficiency over the insulin range of 1–10 mg/ml and (iii) high retention of insulin loads in simulated gastro-intestinal environments. BGL values in diabetic ICR mice were tested over a time span of 8 h, following a single oral dose of each formulation, using two protocols: the conventional (12 h pre-fasting and 8 h fasting); our revised protocol (no pre-fasting, meal at $t=4$ h). In both protocols, initial blood glucose levels (BGL) were 400–600 mg/dL and the novel formulations generated a continuous reduction of BGL. Results in the revised protocol, that mimics human eating habits, were more pronounced, providing stable (over several hours) glucose reductions approaching non-diabetic BGL values. These two fibrillar insulin formulations, and the fibrillar form for therapeutic proteins, merit further studies.

1. Introduction

Proper glycemic control is crucial for diabetic patients, to slow down disease progression and its long-term chronic complications. The need for life-long daily (or more) insulin injections — the standard treatment for type I and eventually for type II diabetics decreases patient compliance, can be painful and runs the risk of local infections [1,2]. Taking insulin by the oral route would be a significant improvement for diabetics, but is not currently-feasible. Like most peptides and proteins, insulin is prone to fast clearance and enzyme-catalyzed degradation by the harsh environments along the gastro-intestinal tract (GIT). Consequently, the share of the orally-administered insulin that does reach the blood is not sufficient to affect blood glucose levels [3]. Improving oral protein bioavailability is one of the most challenging goals in pharmaceutics and in drug delivery technology [4].

A major approach, among attempts underway to face this challenge, is to formulate insulin (native or chemically-modified) in a carrier that will endure the harsh GIT conditions. Such a carrier should possess mucoadhesive properties and act as a slow-release insulin-depot. Carriers tested for the task include hydrogels, microspheres, nanoparticles, microemulsions and liposomes [5]. Other approaches such as absorption enhancers and enzyme suppressors were also investigated [6,7]. Unfortunately, none of these yielded results that merited progression towards approved treatment modalities [5].

Here we describe, for the first time, two novel formulations of insulin based on its fibrillar state, and their use in the treatment of diabetic mice. Key properties of insulin fibrils — hydrophobic nature, intensely-packed conformation, and the ability to restore full biological activity when dissociated — make this insulin form a candidate for oral delivery [8,9]. To protect the insulin from the harsh GIT environments, and to target it to the intestinal mucosa, we developed two types of fibrillar insulin particles that, despite sharing some features, are distinctly different from each other [10,11]. In the first, denoted "coated insulin fibrils", the protein fibrils are coated by a mixture of phospholipids and lipid-hyaluronan conjugates. In the second, denoted "gagomeric insulin", the protein fibrils are encapsulated within a particle named gagomer [10,11]. In this particle, the exterior is made of hyaluronan and its interior contains lipid clusters and water regions. For targeting to the GIT epithelium both formulations utilize hyaluronan, known to be intestinal-mucoadhesive [12,13]. We also prepared and investigated plain (i.e., un-coated and un-encapsulated) insulin fibrils, as a control.

Our hypothesis, that targeted fibrillar insulin delivery systems can meet the basic requirements for oral delivery, was evaluated in simulated GIT environments and tested in diabetic ICR mice.

Abbreviations: BGL, Blood glucose levels; DPPE, 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine; HA-BAL, Hyaluronan bioadhesive liposomes; GIT, gastro-intestinal tract; SGF, simulated gastric fluid; SPC, soybean phosphatidyl choline; SIF, simulated intestinal fluid.

* Corresponding author. Tel.: +972 3 640 9822; fax: +972 3 640 6834.
E-mail address: rimona@post.tau.ac.il (R. Margalit).

0168-3659/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.jconrel.2009.12.018

2. Materials and methods

2.1. Preparation of fibrillar insulin

2.1.1. Plain insulin fibrils

Bovine pancreas insulin from (28.7 IU/mg) from Sigma Chemical Co. (St. Louis, USA) or human-recombinant insulin (27 IU/mg) from Boehringer-Manheim (Germany), at the concentrations of 1–10 mg/ml was dissolved in distilled water (DDW) acidified to pH = 2 (HCl) and agitated in an incubator at 37 °C for selected time spans. When desired, the initial insulin solution was doped with a trace of FITC-labeled insulin, 1:100 v/v (Invitrogen Corporation California, USA).

2.1.2. Coated insulin fibrils

Lyophilized powders of multilamellar hyaluronan bioadhesive liposomes (HA-BAL) prepared according to our previously-described procedure, was the start material [14,15]. Briefly, the selected lipids — SPC:DPPE at the mole ratio of 9:1 and at the concentration of 100 mg lipid/ml — were dissolved in ethanol. The lipid–ethanol solution was transferred to a round-bottomed flask and evaporated to dryness under low pressure in a rotary evaporator. Borate buffer (0.1 M, pH 9) was added to the dried lipid film and the system was incubated in a shaker bath for 2 h at 65 °C. A portion of these liposomes was set aside as a control and the remainder was taken to surface modification. HA was dissolved in acetate buffer (0.1 M, pH 4.5) at the concentration of 2 mg/ml and was pre-activated by the addition of the crosslinker EDC at a final concentration of 20 mg/ml. Incubation was for 2 h at 37 °C in a shaker bath (180RPM). The pre-activated HA was mixed with the liposome suspension, at the ratio of 1:1 (v/v). The mixture was incubated for 24 h in a shaker bath at 37 °C (180RPM). The HA-BAL were freed from excess materials and by-products by ultracentrifugation (4 °C, 40 min and 160,848 g) followed by several successive washes and re-centrifugations in 100 mM ammonium bi-carbonate at pH = 8. Aliquots of 1 ml of the resulting buffer-free salt-free HA-BAL were frozen for 2 h at −80 °C, followed by 24 h lyophilization. The resultant HA-BAL powders were stored at −18 °C until further use. When desired, the initial lipid solution was doped with a trace of DPPE-Rhodamine (1:1000 v/v).

The liposome powder was rehydrated with an aqueous solution of insulin (at pH = 2) and the process was continued as described in Section 2.1.1. When needed, the systems were spinned-down in a Sorval Discovery M120 SE (Thermo Fisher scientific MA, USA) ultracentrifuge at 4 °C, 40 min and 127,477 g. The supernatant was separated and saved, and the pellets — the coated insulin fibrils — re-suspended in DDW for use as is, or lyophilized until further use. The insulin concentration was evaluated by subtracting the insulin in the supernatant from the initial insulin concentration, using either absorbance at 280 nm or fluorescence (for the FITC-insulin doped systems) with excitation and emission at 480 nm and 530 nm, respectively (Synergy, Biotek, Winooski, Vermont, USA). When double labeling (insulin and lipid) was desired, the initial lipid solution was doped with a 1:1000 v/v trace of DPPE-Rhodamine from Sigma Chemical Co. (St. Louis, USA). The resulting lipid-coated fibrils were in the range of 10–100 μm and mostly elongated in shape. These systems were stable at 1% and at 5% DOC.

2.1.3. Insulin-gagomer particles

Insulin-gagomer particles were prepared in a two-step process. First, buffer-free salt-free gagomers were prepared and lyophilized according to previous work [10,11]. Briefly, HA pre-activation and lipid handling — dissolution, evaporation and final suspension in the borate buffer — were done as described under Section 2.1.2 above, but with the following critical differences: A single lipid species — DPPE — was used for the gagomers, and its concentration in the final aqueous suspension ranged from 0.6 to 12 mg/ml. The activated HA solution was added to the lipid suspension at a ratio of 1:1 (v/v), the pH was corrected back to 9 and the mixture was incubated in a shaker bath for 24h at 37 °C (150RPM). The resulting gagomer particles were freed from excess materials and by-products by ultracentrifugation (4 °C, 40 min and 160,848 g) followed by several successive washes and re-centrifugations in 0.1 M ammonium bi-carbonate at pH = 8. Aliquots of 1 ml of the gagomer suspensions (final gagomer concentrations of 0.6, 1.2 or 12 mg DPPE/ml) were frozen for 2 h at −80 °C, followed by 24 h lyophilization. The gagomer powders were stored at −18 °C until further use.

Next, the gagomer powder was rehydrated back to original pre-lyophilization volume, with an aqueous solution of insulin (pH = 2). Insulin loading efficiency was determined as described in Section 2.1.2 above. The resulting insulin-gagomer particles were in the range of 10–100 μm and tend to have vesicular shapes. These systems were stable at 1% and at 5% DOC.

2.2. Light, fluorescent and confocal microscopies

In all cases, to eliminate acidic quenching of FITC fluorescence, the pH of the test samples was raised to 8 prior to viewing. The same device, Leica DMRB upright microscope, (Leica Microsystems Gmbh, Wetzlar, Germany), was used for both light and fluorescent microscopies, and magnifications were 40 or 100 fold.

2.2.1. Light microscopy

Aliquots of 100 μl were pipetted into 96-well plates. The plates were taken “as is” or first left to dry at 37 °C, for viewing under the light microscope in the hydrated, or dry states, respectively.

2.2.2. Fluorescent microscopy

Aliquots of 100 μl were pipetted onto a microscope slide glass and covered with a cover glass. The slides were viewed under a fluorescence microscope. Excitation and emission were at 480 nm and 530 nm, respectively.

2.2.3. Confocal microscopy

The slides (similar to Section 2.2.2) were viewed under a confocal microscope (ZEISS LSM 510, Carl Zeiss MicroImaging Gmbh, Gottingen, Germany). Excitation and emission were as in Section 2.2.2.

2.3. Scanning electron microscopy (SEM) of insulin-gagomer particles

Samples (10 μL) were fixed with 2.5% glutaraldehyde in PBS, and subjected to the following processes: washing, dehydration in graded ethanol solutions, drying from CO_2 at critical point and coating with gold. The scanning electron microscope (SEM) was Jeol 480A (Tokyo, Japan).

2.4. Transmitting electron microscopy (TEM) of coated insulin fibrils

Samples (10 μL) were placed on 400-mesh copper grids (SPI supplies, West Chester, PA) covered by carbon-stabilized Formvar film. After 1 min, excess fluid was removed and the grids were negatively stained with 2% uranyl acetate in water for 2 min. Samples were viewed in a JEOL 1200EX electron microscope operating at 80 kV.

2.5. Thioflavin-S (Th-S) staining

Acidic suspensions of insulin fibrils were prepared as in Section 2.1 above. Samples (at the end of 48 h of incubation) were pipetted (100 μL aliquots) into 96-well plates and allowed to dry. Two hundred microliters of a 1% aqueous Thioflavin-S, Sigma Chemical Co. (St. Louis, USA) were added to each well. Three minutes later the Th-S was aspirated and each well was subjected to 3 successive washes with 200 μl water. The wells were then incubated for 20 min with 200 μl of