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Research paper

Reversible pegylation of insulin facilitates its prolonged action *in vivo*

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Abstract

We attempted to engineer a novel long-acting insulin based on the following properties: (i) action as a prodrug to preclude supraphysiological concentrations shortly after injection; (ii) maintenance of low-circulating level of biologically active insulin for prolonged period; and (iii) high solubility in aqueous solution. A spontaneously hydrolyzable prodrug was thus designed and prepared by conjugating insulin through its amino side chains to a 40 kDa polyethylene glycol containing sulfhydryl moiety (PEG_{40}-SH), employing recently developed hetero-bifunctional spacer 9-hydroxymethyl-7(amino-3-maleimidopropionate)-fluorene-*N*-hydroxysucinimide (MAL-Fmoc-0Su). A conjugate trapped in the circulatory system and capable of releasing insulin by spontaneous chemical hydrolysis has been created. PEG_{40}-Fmoc-insulin is a water-soluble, reactivatable prodrug with low biological activity. Upon incubation at physiological conditions, the covalently linked insulin undergoes spontaneous hydrolysis at a slow rate and in a linear fashion, releasing the nonmodified immunologically and biologically active insulin with a $t_{1/2}$ value of 30 h. A single subcutaneous administration of PEG_{40}-Fmoc-insulin to healthy and diabetic rodents facilitates prolonged glucose-lowering effects 4- to 7-fold greater than similar doses of the native hormone. The beneficial pharmacological features endowed by PEGylation are thus preserved. In contrast, nonreversible, "conventional" pegylation of insulin led to inactivation of the hormone.

Keywords: Diabetes; Glucose-lowering profiles; Long-acting insulin; Normoglycemia; Pro(drug)-insulin

1. Introduction

Most polypeptide drugs, in particular nonglycosylated proteins of molecular mass less than Mr = 50,000, are short-lived species *in vivo*, having a circulatory half-life of 5–20 min [1]. The short lifetime of proteins *in vivo* is attributed to several mechanisms, including glomerular filtration, particularly of small proteins, in the kidney and proteolysis at several levels [2]. Insulin is degraded primarily in the liver, through a mechanism known as receptor-mediated endocytosis [3,4]. This mechanism is an efficient route for terminating the action of the hormone after the levels of glucose and other nutrients have been normalized. Chemically modified insulins with negligible receptor binding affinities are therefore longer-lived species within the circulation but are biologically ineffective [5].

Abbreviations: MAL-Fmoc-OSu, 9-hydroxymethyl-7(amino-3-maleimidopropionate)-fluorene-*N*-hydroxysucinimide; IDDM, insulin-dependent diabetes mellitus; HSA, human serum albumin; HPLC, high-performance liquid chromatography; STZ, streptozocin; TCA, trichloroacetic acid; MIB·NHS, *m*-maleimido benzoic acid·*N*-hydroxysuccinimide ester; DTNB, 5,5′-dithiobis(2-ntirobenzoic acid); PEG, polyethyleneglycol; PEG_{40}-SH, a Mr = 40,000, branched polyethyleneglycol containing a sulfhydryl moiety; PEG_{40}-OSu, a Mr = 40,000, branched polyethyleneglycol *N*-hydroxy succinimide; Fmoc·OSu, fluorenylmethoxycarbonyl-*N*-hydroxy-succinimide.

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IDDM patients receive multiple daily subcutaneous administrations of 'rapid' and long-acting insulins [6,2,7,8]. Prolonged-acting preparations of insulin are needed to maintain the low basal levels of circulating insulin between meals and during nighttime, a physiological requirement for lowering triglyceride breakdown and suppressing hepatic glucose output under 'resting' conditions [9–11].

Most prolonged-acting insulins currently in use are suspensions of crystals, produced either by Zn^{2+} ions or by the addition of basic protamine. Such injected suspensions have decreased rates of absorption from the subcutaneous compartment to the circulatory system. Slow dissolution at the site of injection brings about the protracted effect [12,13,2].

In recent years major efforts have been devoted to developing prolonged-acting insulins that are soluble in aqueous buffered media. In one such novel approach, two arginine moieties were covalently linked to insulin to shift the isoelectric point of the hormone upward. The derivative thus obtained is formulated in a soluble form in a slightly acidic media, and is crystallized and precipitated immediately after injection, at the physiological, neutral pH of the subcutaneous space [14–16]. In a second approach, soluble, long-chain fatty acid-acylated insulins were prepared. These derivatives associate with albumin ($K_a \sim 10^5\ M^{-1}$) and therefore exhibit lower rates of clearance and moderate degree of protracted action *in vivo* following subcutaneous administrations [17–21].

Therapeutically, insulin differs from quite many other polypeptide drugs as overdosing may lead to severe hypoglycemia [22–24]. To avoid that, only a limited dosage of insulin can be applied each time. Such a dosage is often insufficient to maintain the basal insulin level (i.e. at nighttime) needed to tackle with hyperglycemia at dawn [6–8]. Thus, developing a new preparation of insulin that is soluble in aqueous media, facilitates prolonged glucose-lowering effects *in vivo*, as well as being in an inactive state at early time points of the administration is of high therapeutic value.

The covalent linkage of high molecular weight PEG-chains to peptide or protein drugs may prolong conjugate lifetime *in vivo* from minutes to hours [25]. This advantageous effect stems, from a significant decrease in renal clearance and reduced proteolytic degradation. The pegylation approach, however, becomes irrelevant if the protein drug is inactivated upon pegylation [26]. Conventional pegylation inactivates insulin (i.e. this study). In previous recent studies from our laboratories we have searched for an approach that would overcome this drawback. Toward this aim we have designed and studied a strategy by which attached PEG-chains can be released by a spontaneous hydrolysis under physiological conditions with a consequent generation of the parent intact protein/peptide. Using this novel approach, i.e. reversible pegylation, the *in vivo* action of interferon α2 and of exendin-4 were most significantly prolonged [27,28]. As the degradation of insulin occurs primarily following receptor-mediated endocytosis, and not via proteolysis by serum enzymes, we wanted to examine whether its survival and function in circulation can be elongated by reversible pegylation. Here we present in detail the engineering of PEG_{40}-insulin prodrug, capable of undergoing hydrolysis and reactivation upon incubation, with a desirable pharmacokinetic pattern at physiological conditions.

2. Experimental

2.1. Research design and methods

2.1.1. Materials

Human (Zn^{2+}-free) insulin was donated by Novo Nordisk (Bagsvaerd, Denmark) or by Biotechnology General (Rehovot, Israel) and was used without further purification. D-[U–^{14}C]Glucose (4–7 mCi/mol) was obtained from Du Pont-NEN (Boston, MA). Type I collagenase (134 U/mg) was purchased from Worthington (Freehold, NJ). 9-Fluorenylmethoxycarbonyl-*N*-hydroxysuccinimide (Fmoc-Osu) and di-*tert*-butyldicarbonate (t-Boc)$_2$O were obtained from Novabiochem (Laüfelfingen, Switzerland). Dansyl-glycine, N_ε-dansyl-L-lysine and dansyl-L-phenylalanine were purchased from Sigma Chemical Co. (Ness Ziona, Israel). Chemicals used in this study were of analytical grade.

2.1.2. Biological methods and procedures

Iodination of insulin by [^{125}I] iodine was performed using the chloramine-T method [29]. Rat adipocytes were prepared from fat pads of male Wistar rats (100–200 g) by collagenase digestion [30]. Lipogenesis (incorporatin of [U–^{14}C] glucose into lipids) was performed by the procedure of Moody et al. [31]. Diabetes was induced by a single intravenous injection of freshly prepared solution of streptozocin (STZ, 55 mg/kg body wt) according to the method of Meyerovitch et al. [32]. Only STZ-rats having blood glucose level >390 mg/dl in the fed state and >115 mg/dl in the fasting state, 2 days after STZ injection, were used. STZ-rats meeting these criteria have 1.5 ± 0.3 μg insulin/g pancreatic tissue, namely ~2% of that found in control, nondiabetic rats (unpublished observation). Rats were maintained at 24 °C under conditions of controlled lighting and were fed *ad libitum*. Blood samples for the analysis of blood glucose were taken from the tail vein and measured with a glucose analyzer (Beckman Instruments, Fullerton, CA) by the glucose oxidase method. Groups consisted of five rats each. Data are presented as means ± SE.

2.1.3. Quantitation of immunoreactive insulin

Immunoreactivity insulin was analyzed by a standard radioimmunoassay (RIA) using guinea pig anti-porcine insulin antibodies (Sigma Chemical Co., St. Louis, MO); ^{125}I-labeled human insulin with human standard was used.

The minimal detectable level for human insulin was 0.32 ng/ml. In this RIA, human, porcine and bovine insulins are identical with respect to immunoreactivity.

2.1.4. Synthesis of 9-hydroxymethyl-7-(amino-3-maleimidopropionate)-fluorene-N-hydroxysuccinimide (MAL-Fmoc-NHS)

2.1.4.1. 9-Hydroxymethyl-2-amino fluorine I. 9-Hydroxymethyl-2-amino fluorine I was synthesized in 84% yield as described in detail by Albericio et al. (Fig. 2) [33] (TLC, chloroform/methanol, 9:1 [v,v] $R_f = 0.38$; ESMS, calc. 211.26 Da, found, [M+1], 212.1 Da).

2.1.4.2. 9-Hydroxymethyl-2-(amino-3-maleimidopropionate)fluorene 3-maleimidopropionic anhydride. 3-Maleimidopropionic acid (1 g, 5.9 mmol) and DCC (0.69 g, 2.95 mmol) were dissolved in 10 ml drug DMF and incubated for 4 h. Dicyclohexylurea (DCU) was filtered out and the anhydride thus formed was kept at 4 °C.

9-Hydroxymethyl-2-amino fluorene (0.4 g, 1.9 mmol) and sodium hydrogen carbonate (0.74 g, 8.85 mmol) were dissolved in water and 3-maleimidopropionic anhydride in DMF (12 ml, 2.95 mmol) was added. The reaction mixture was stirred for 40 min and product formation was monitored by analytical HPLC on a Chromolith column, $R_t = 4.46$ min, 10–100% B in 10 min, 3 ml/min). The crude product was purified using preparative HPLC (C_{18} column, 10–100% acetonitrile: water [75:25], 60 min, 12 ml/min). Yield, 57%, 1.08 mmol, 0.39 g. ESMS, calc. Mr = 362.38, found Mr = 362.42).

2.1.4.3. 9-Hydroxymethyl-2-(amino-3-maleimidopropionate)fluorene-N-hydroxysuccinimide III: (MAL-Fmoc-OSu). Pyridine (0.167 ml, 2 mmol) was added dropwise to a stirred solution of 9-hydroxymethyl-2-(amino-3-maleimidopropionate)fluorene (0.37 g, 1.02 mmol) and triphosgene (0.425 g, 1.43 mmol, 4.2 equiv) in dry THF (10 ml). After 20 min the precipitated pyridine hydrochloride salt was filtered out and the THF was removed in vacuum. The oil obtained was dissolved in 10 ml dry THF with *N*-hydroxysuccinimide (0.61 g, 5.3 mmol). Pyridine (0.26 ml, 3.2 mmol) was then added and the solution was stirred for 20 min. The precipitated pyridine hydrochloride salt was filtered out and the THF was removed by vacuum. The oil obtained was dissolved in chloroform (100 ml) and washed with an aqueous sodium hydrogen carbonate solution (0.5 N, 3× 50 ml), HCl (0.1 N, 3× 50 ml), water (2× 50 ml) and saturated NaCl solution. The chloroform was removed by vacuum and the product desiccated. Yield, 89%, 0.9 mmol, 0.45 g. HPLC (Chromolith column) $R_t = 5.7$ min (10–100% B in 10 min, 3 ml/min). Calculated ESMS Mr = 503, found ESMS for $M+Na^+$ = Mr = 526.38, found for $M+K^+$: Mr = 542.30.

2.1.4.4. PEG_{40}-SH. PEG_{40}-Osu was dissolved at a concentration of 40 mg/ml in an aqueous solution of cystamine–di-HCl (1 M) and brought to pH 8.5 with solid $NaHCO_3$. Reaction was carried out for 2 h at 25 °C. The product thus obtained was dialyzed overnight against 0.1 M $NaHCO_3$, treated with 30 mM dithiothreitol (25 °C, 1 h), redialyzed against 0.01 M HCl containing 10 mM ascorbic acid and lyophilized. PEG_{40}-SH was obtained with a 93% yield. It contained one mol sulfhydryl moiety per mol of PEG_{40} as determined with 5,5′-dithiobis(2-nitrobenzoic acid). MALDI-TOF-MS analysis yielded a MW of 43818 D.

2.1.4.5. Preparation of PEG_{40}-Fmoc-insulin. To a stirred solution of Zn^{2+} free insulin (5.9 mg in 1.0 ml, 0.1 M phosphate buffer, pH 7.2) was added 505 μg (one equivalent) of MAL-Fmoc-OSu (50.5 μl from a fresh solution of MAL-Fmoc-OSu in DMF, 10 mg/ml). The reaction was carried out for 20 min at 25 °C. PEG_{40}-SH was then added to a final concentration of 0.417 mM (0.6 mol/mol insulin). The reaction was carried out for 2 h, and then dialyzed overnight against H_2O at 7 °C. PEG_{40}·Fmoc·insulin thus obtained was then purified from unreacted insulin and/or from insulin-Fmoc-mal that did not react with PEG_{40}-SH by a preparative HPLC procedure, using a spectra-physics SP8800 liquid chromatography system equipped with an Applied Biosystem 757 variable wavelength absorbance detector. A wavelength of 220 nm was used. A pre-packed VYDAC RP-4 column (250 × 22 mm Hesperia, CA) was used. The column was eluted with a binary gradient of 20–100% solution B (acetonitrile·H_2O 75:25 in 0.1% TFA) over a period of 60 min at a rate of 10 ml/min (Solution A was 0.1% TFA in H_2O). The fractions corresponding to PEG_{40}-Fmoc-insulin were collected and lyophilized.

2.1.4.6. Determining the sites of attachment of PEG_{40}-Fmoc to the insulin molecule. PEG_{40}-Fmoc-insulin (5 mg solid material) was dissolved in 2.0 ml of 1 M $NaHCO_3$ (pH 8.5) and cooled to 0 °C. Aliquots (10 μl each) of acetic-anhydride were then added to the stirred solution over a period of 1 h to obtain 500 molar excess of acetic-anhydride over the conjugate. The reaction mixture was then dialyzed overnight against H_2O. Acetylated PEG_{40}-Fmoc-insulin was dissolved in 0.1 M Na_2CO_3 (pH 10.3) and reacted with dansyl-chloride (final concentration 2 mM) over a period of 7 h at 70 °C. Under these experimental conditions, PEG_{40}-Fmoc is detached from the insulin molecule. The reaction mixture was then dialyzed overnight against H_2O, acid-hydrolyzed in 6 M HCl at 110 °C for 22 h, evaporated to dryness and analyzed for the presence and contents of dansyl amino-acids by HPLC analysis.

2.1.4.7. Preparation of PEG_{40}-BENZ-insulin. This (irreversible) PEG_{40}-insulin conjugate was prepared under identical conditions to those used for PEG_{40}-Fmoc-insulin except for the hetero-bifunctional agent used, *m*-maleimido-benzoate-*N*-hydroxysuccinimide ester (MIB-NHS). The reagent (32 μl from a fresh solution of MIB-NHS 10 mg/

ml) reacted with insulin for 20 min at 25 °C prior to the addition of PEG_{40}-SH (see above). HPLC-purified PEG_{40}-Benz-insulin was characterized by MALDI-TOF-MS. A mass of Mr = 49,649 was found (calculated mass for 1:1 conjugate is Mr = 49,858).

2.1.4.8. PEG_{40}-insulin. This irreversible PEG_{40}-insulin conjugate was prepared by reacting insulin (5.81 mg/ml in 0.1 M phosphate buffer, pH 7.2) with one equivalent of solid PEG_{40}-OSu (44 mg). Reaction was performed with stirring over a period of 20 h at 7 °C. PEG_{40}-insulin was then separated from unreacted insulin by preparative HPLC procedure and the tubes containing the conjugate were lyophilized. The conjugate was obtained in 40% overall yield. It has $\varepsilon_{280} = 5800$, similar to that of the native hormone. PEG_{40}-insulin contains one PEG_{40} residue per insulin, as determined by MALDI-TOF-MS analysis (PEG_{40}-Osu; Mr = 43,626; Insulin, Mr = 5807; calculated Mr = 49,333; found Mr = 49,166).

3. Results

3.1. Engineering a PEG_{40}-insulin conjugate that releases insulin at physiological conditions

The covalent linking of insulin via its amino functions to PEG_{40}-OSu, or of PEG_{40}-SH to insulin via *m*-maleimidobenzoate, yielded a PEG_{40}-insulin conjugate that has less than 1% the biological potency of insulin (subsequent paragraphs). We therefore designed and synthesized a hetero-bifunctional agent consisting of Fmoc-OSu in which a maleimide group was attached to the fluorenyl backbone. The steps involved in the synthesis and the structure of MAL-Fmoc-OSu are illustrated in Fig. 1. MAL-Fmoc-OSu enables linkage to proteins via their amino side chains to cysteinyl moieties (i.e. to PEG_{40}-SH). Fmoc moieties, linked to the amino side chains of peptides and proteins, hydrolyze slowly at physiological conditions, generating the nonmodified parent peptides and proteins [34,35].

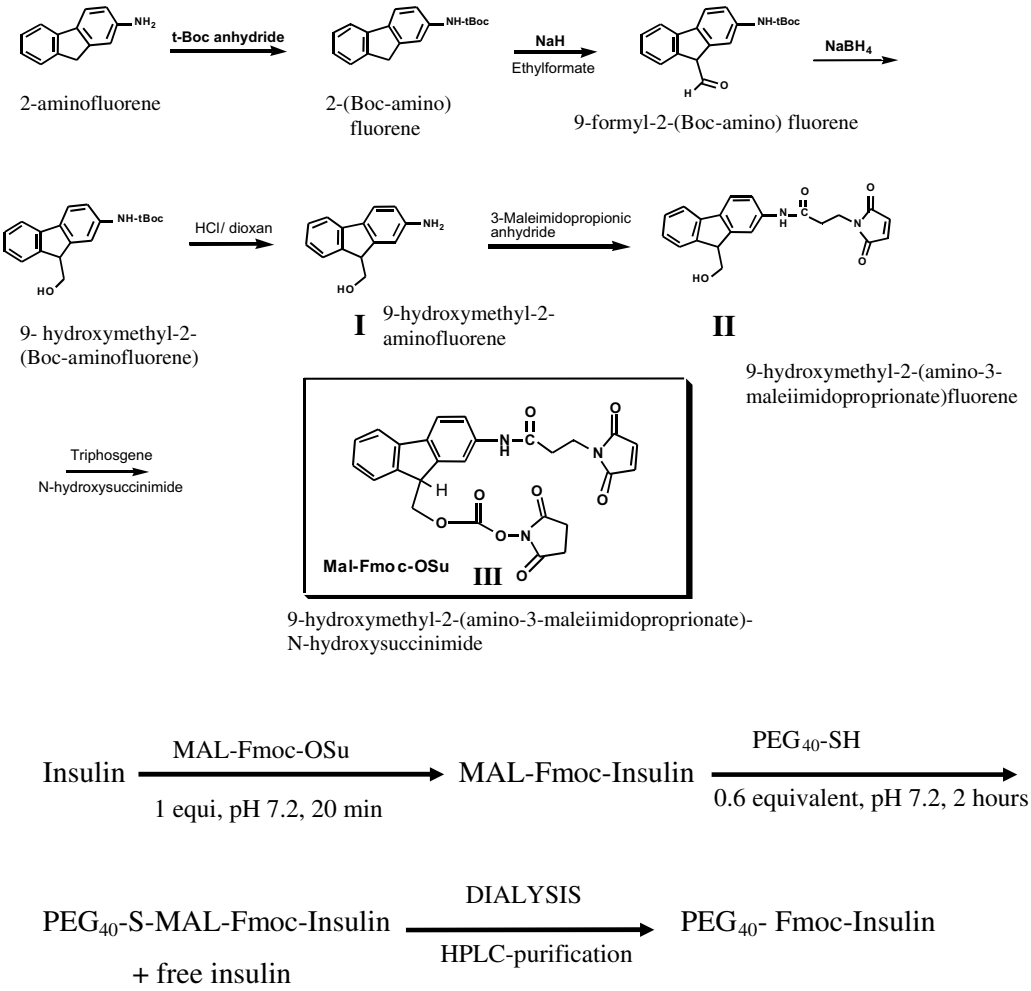

Fig. 1. Schematic illustration of the synthesis and structure of 9-hydroxymethyl-2-(amino-3-maleimido-propionate)-fluorene-*N*-hydroxysuccinimide (Mal-Fmoc-Osu, (A)) and the experimental procedure for preparing PEG_{40}-Fmoc-insulin (B).

The procedure described in detail in Section 2 and summarized in Fig. 1 was found optimal for coupling equimolar amount of insulin to PEG_{40}-SH. In brief, MAL-Fmoc-OSu reacts first stoichiometrically with insulin for 20 min at pH 7.2, namely at a pH value where the alkylating capacity of MAL remains stable for several hours [36]. PEG_{40}-SH is then added in a stoichiometry of 0.6 mol/mol derivatized insulin to ensure quantitative coupling of insulin-Fmoc-MAL to PEG_{40}-SH. Unreacted insulin and/or insulin-Fmoc-MAL that has not been linked to PEG_{40}-SH are removed by a semi-preparative HPLC procedure (Section 2).

3.2. General features of PEG_{40}-Fmoc-insulin

Table 1 and Fig. 2 summarize several characteristic features of HPLC-purified PEG_{40}-Fmoc-insulin prepared by us. It is a highly water-soluble derivative (>1 mM) in buffered solutions near neutrality (pH 6–7). MALDI-TOF-MS analysis revealed the presence of a main peak having a molecular mass of Mr = 49.977 (calculated mass for the 1:1 PEG/insulin conjugate is Mr = 50,028). PEG_{40}-Fmoc-insulin contains 53.4 ± 4 μg covalently linked insulin/mg conjugate as determined by acid hydrolysis and amino acid analysis. The calculated value of insulin for the 1:1 conjugate is 116 ± 7 μg/mg conjugate, indicating that our preparation contains also PEG_{40}-Mal-Fmoc that has not been linked to insulin. This point is also verified by the molar extinction coefficient found ($\varepsilon_{280} = 28{,}350$, calculated $\varepsilon_{280} = 18{,}200$; Table 1). PEG_{40}-Fmoc-insulin has $7 \pm 2\%$ the immuno-reactivity of the native hormone, and $11 \pm 3\%$ of the biological potency of insulin (ED_{50} value = 0.0454 nM) as shown in a lipogenic assay in rat adipocytes (Fig. 2). Incubation of PEG_{40}-Fmoc-insulin in PBS buffer, pH 7.4, containing 0.2% HSA for a period of 70 h nearly fully activated PEG_{40}-Fmoc-insulin ($ED_{50} = 0.05$ nM; Fig. 2, summarized in Table 1).

Table 1
Biological and chemophysical features of PEG_{40} Fmoc insulin

Amino acid composition		Identical to insulin
Solubility in aqueous buffer (pH 7.0)		>1 mM
MALDI-TOF-MS analysis[a]	Calculated	Mr = 50,028
	Found	Mr = 49,977
Molar extinction coefficient	Calculated[b]	$\varepsilon_{280} = 18{,}200$
	Found	$\varepsilon_{280} = 28{,}000$
Covalently bound insulin (μg/mg conjugate)	Calculated	116
	Found	53.4 μg
Relative immunoreactivity (%)		7 ± 1
Relative biological potency[c] (%)		11 ± 2
Biological potency following incubation (PBS-buffer, pH 7.4–0.2% HSA, 37 °C, 70 h; %)		96 ± 3

[a] MALDI-TOF-MS analyses were carried out with Bruker-reflex-reflection model.
[b] Molar extinction coefficient for PEG_{40}-Fmoc-insulin was calculated by combining the ε_{280} values of insulin ($\varepsilon_{280} = 5800$) and that of MAL-Fmoc-Osu ($\varepsilon_{280} = 12{,}400$). PEG_{40}-SH has no absorption at 280 nm.
[c] Determined in a lipogenic assay in rat adipocytes.

The attachment of a single PEG_{40} chain to insulin, either by reacting the hormone with PEG_{40}-OSu (PEG_{40}-insulin) or by linking PEG_{40}-SH to insulin through the nonhydrolizable MIB-NHS linker (PEG_{40}-Benz-insulin), yielded PEG_{40}-insulin conjugates having negligible amount of biological potencies (ED_{50} values = 6.55–10 nM, 0.5–0.8% the biological potency of native insulin, Fig. 2). Obviously, these conjugates were not reactivated upon incubation (not shown).

3.3. Identifying the sites of attachment of PEG_{40}-Fmoc to the insulin molecule

Fig. 3 shows the HPLC analysis of the three dansyl amino acids, corresponding to the amino side chains of insulin; and Table 2 summarizes and quantitates the dansyl amino acids obtained from PEG_{40}-Fmoc-insulin, following acetylation, detachment of the PEG_{40}-Fmoc chain from the insulin molecule, dansylation and acid hydrolysis (Section 2). PEG_{40}-Fmoc was found to be covalently linked primarily to the α-amino side chain of Phe^{B1} (0.66 mol/mol), and to a lesser extent to the α-amino side chain of Gly^{A1} and to the ε-amino group of $lysine^{B29}$ (Table 2). Quantitation of the three dansyl amino acids yielded a ratio of 0.66, 0.167 and 0.174 for dansyl-L-phenylalanine, dansylglycine and N_{ε}-dansyl-lysine, respectively (Table 2).

3.4. Rate of hydrolysis of PEG_{40}-Fmoc-insulin

In the experiment summarized in Fig. 4, PEG_{40}-Fmoc-insulin (1 mg/ml containing 53.4 μg covalently linked insulin/mg) was incubated at 37 °C in 0.1 M phosphate buffer, pH 7.43, containing 20 mg/ml HSA. Under these incubating conditions the hydrolysis rate is similar to that obtained in normal human serum at 37 °C (not shown). At the indicated time points, aliquots (100 μl) were analyzed for the amount of insulin released, using HPLC on an RP-4 column. Insulin was released from the conjugate in a nearly linear fashion (Fig. 4). About 40% of the covalently linked insulin was released after 15 h and nearly all (~95%) was released following 70 h of incubation (Fig. 4). Fig. 5 shows the exponential fit of this data, using Kaleidograph version 3.6. A rate constant (k) of 0.023 ± 0.07 has been calculated. Thus, using the equation $t_{1/2} = \ln 2/k$ at pH 7.43 (37°) in a medium containing 20 mg/ml HSA, insulin is released from the conjugate with a $t_{1/2}$ value of 30 h.

3.5. Reactivation of PEG_{40}-Fmoc-insulin upon incubation

Fig. 6 shows the time-course of reactivation of PEG_{40}-Fmoc-insulin. Aliquots from incubated PEG_{40}-Fmoc-insulin (0.1 M phosphate buffer, pH 7.43–0.2% HSA, 37 °C) were withdrawn at different time points and analyzed for their biological potencies in a lipogenic assay in rat adipocytes. As shown, the conjugate undergoes reactivation

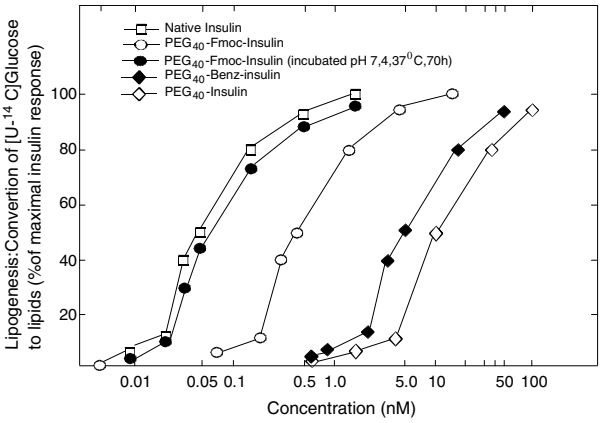

Fig. 2. Dosage-dependent stimulation of lipogenesis in rat adipocytes. Lipogenesis was carried out for 2 h at 37 °C in plastic vials containing 0.5 ml of fat cell suspensions (1.5×10^5 cells) and 0.2 mM [U–^{14}C] glucose in the presence or absence of the indicated concentrations of native insulin, PEG_{40}-insulin, PEG_{40}-BENZ-insulin and PEG_{40}-Fmoc-insulin prior to and following incubation at pH 7.4, 37 °C, over a period of 70 h. Results are expressed as the percentage of maximal stimulation. Insulin (17 nM) stimulated lipogenesis 4- to 5-fold above basal level.

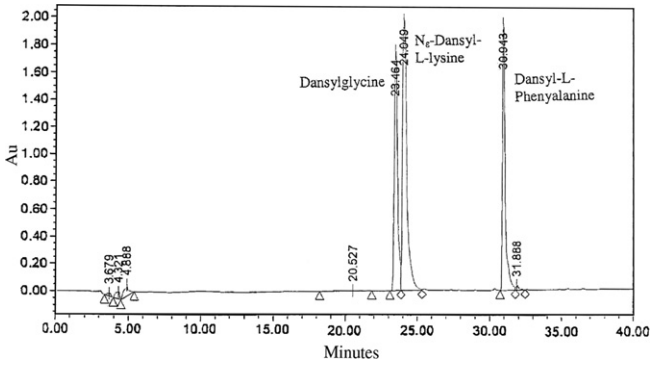

Fig. 3. Separation of dansylated phenylalanine, glycine and ε-dansyl lysine by HPLC-Chromatography. The three dansylated amino-acids corresponding to the insulin molecule were resolved by HPLC-Chromatography. HPLC-analysis was conducted using C_{18} column (5 μm, 250 × 4 mm) developed by a linear gradient of 5% to 90% solution B (0.1% TFA in acetonitrile–H_2O 75:25 v/v) over a period of 40 min, at a flow rate of 0.8 ml/min. The effluent was monitored at 220 nm.

Table 2
Identification and quantitation of the dansyl amino acids corresponding to the sites of attachment of PEG_{40}-Fmoc to the insulin molecule[a]

Derivative[b] obtained	R_t-value[c] (min)	ng/sample[d]	% of total
Dansylglycine	25.6	54.9	16.7
N_ε-Dansyl-L-lysine	26.4	57.2	17.4
Dansyl-L-phenylalanine	29.5	215.4	66

[a] PEG_{40}-Fmoc-insulin was subjected to a sequential procedure of acetylation, detachment of PEG_{40}-Fmoc from the insulin molecule, dansylation and acid hydrolysis (Section 2).

[b] Dansyl amino acid derivatives were resolved by HPLC analysis, as described in the legend to Fig. 3.

[c] Verification of the R_t-values was obtained by coadministration with the corresponding authentic dansyl amino acid derivatives.

[d] Quantitation of dansyl amino acids was carried out using surface areas of 6750 ± 300 mav/ng for dansylglycine, 11.150 ± 500 mav/ng for N_ε-dansyl-L-lysine, and 6500 ± 250 mav/ng for dansyl-L-phenylalanine.

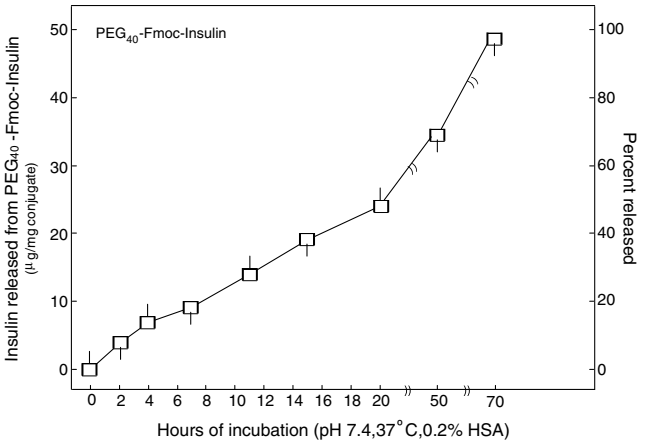

Fig. 4. The rate of insulin release from PEG_{40}-Fmoc-insulin upon incubation at pH 7.43–0.2% HSA, 37 °C. A solution of PEG_{40}-Fmoc-insulin (1 mg/ml) was incubated in 0.1 M phosphate buffer at pH 7.43–0.2% HSA, 37 °C. At the indicated time points, aliquots (100 μl) were applied to analytical HPLC. The peak area of the released insulin as a function of time was calculated. Results are expressed as the amount of insulin released/mg PEG_{40}-Fmoc-insulin. Under our running conditions, peak areas were found to be 187,000 ± 9000 mav/μg insulin.

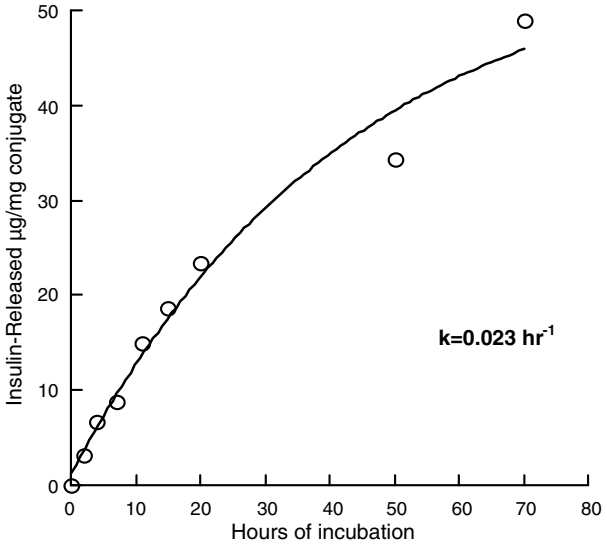

Fig. 5. Exponential fit of discharged insulin from PEG_{40}-Fmoc-insulin at pH 7.4–0.2% HSA at 37 °C.

upon incubation in a nearly linear fashion. Thus, starting from 11 ± 2% at time 0, lipogenic potency is elevated to 22 ± 2%, 67 ± 4% and 80 ± 5% following 10, 20 and 40 h of incubation, respectively. Upon 70 h of incubation, PEG_{40}-Fmoc-insulin regained nearly all of its biological potency (Fig. 6).

A single subcutaneous administration of PEG_{40}-Fmoc-insulin facilitates prolonged glucose-lowering pattern in mice. In Fig. 7, we compare the glucose-lowering pattern of PEG_{40}-Fmoc-insulin to that of the native hormone, following a single subcutaneous administration. A dose of 0.3 mg/mouse, namely 16.2 μg covalently linked insulin, which is equivalent to 1.8 μg of biologically active insulin

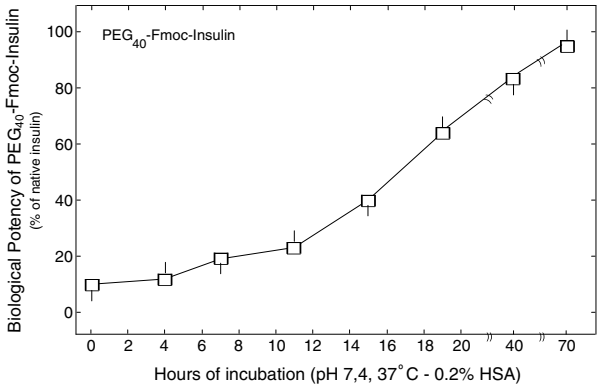

Fig. 6. Time-course of reactivation of PEG_{40}-Fmoc-insulin. PEG_{40}-Fmoc-insulin (1 mg/ml in 0.1 M phosphate buffer, pH 7.43–0.2% HSA) was incubated at 37 °C. Aliquots were withdrawn at the indicated time points and each analyzed at several dilutions for its biological activity in a lipogenic assay with rat adipocytes. Under our assay conditions, insulin activated lipogenesis 4- to 5-fold above basal level with an ED_{50} value = 0.05 ± 0.03 nM. An aliquot of PEG_{40}-Fmoc-insulin exhibiting ED_{50} of 0.5 nM was considered as having 10% the native biological potency.

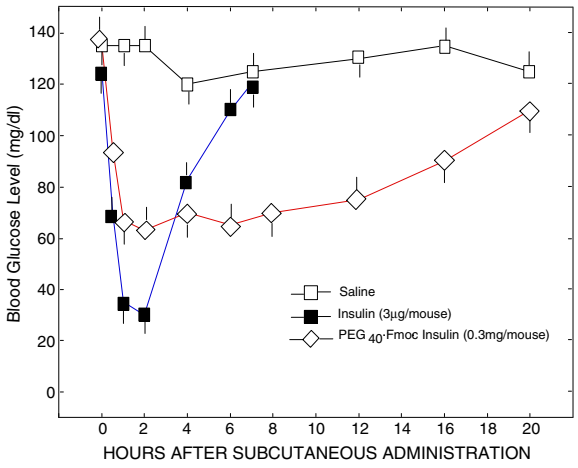

Fig. 7. Circulating glucose levels in mice following a single subcutaneous administration of PEG_{40}-Fmoc-insulin. Mice ($n = 5$ per group) received, subcutaneously, Zn^{2+}-free insulin (3 μg/mouse in 0.2 ml saline) or PEG_{40}-Fmoc-insulin (0.3 mg/mouse). Blood glucose levels were determined at the indicated time points. Each point represents arithmetic mean of five mice ± SEM.

at the time of administration, was compared to a dose of Zn^{2+}-free insulin (3 μg/mouse). As shown in the Figure, PEG_{40}-Fmoc-insulin facilitates a prolonged and stable glucose-lowering pattern with $t_{1/2}$ values of 4 ± 0.4 and 17 ± 0.1 h for Zn^{2+}-free and PEG_{40}-Fmoc-insulin, respectively, exceeding 4.25 times the duration obtained by the native hormone.

3.6. Glucose-lowering pattern of PEG_{40}-Fmoc-insulin in STZ-rats

Fig. 8 shows the glucose-lowering pattern of the conjugate after a single subcutaneous administration in streptozocin-treated, hyperglycemic rats. Here we compared a conjugate dose that is equipotent to a dose of Zn^{2+}-free insulin at the time of administration. A conjugate dose of 2 mg/STZ-rat was calculated to be equipotent to 12 μg insulin/STZ-rat, based on 53 ± 3 μg covalently linked insulin/mg PEG_{40}-Fmoc-insulin having 11% the biological potency of the free hormone (Table 1 and Fig. 2). As shown in Fig. 8, PEG_{40}-Fmoc-insulin facilitates about 4 times more prolonged glucose-lowering effect than that of the native hormone. Circulating glucose levels reached a maximal decrease at 1–2 h after administration (120 ± 20 mg/dl). A low blood glucose level was then preserved for many hours before hyperglycemia reoccurred with a $t_{1/2}$ value of 15 ± 1 h. The area under the curve of the saline or PEG_{40}-Fmoc-ala treated groups, following PEG_{40}-Fmoc-insulin administration, exceeds ~4 times that obtained with the native hormone (integrated from Fig. 8). Thus, PEG_{40}-Fmoc-insulin is considerably more effective than insulin in lowering circulating glucose levels in insulin-deficient diabetic rats.

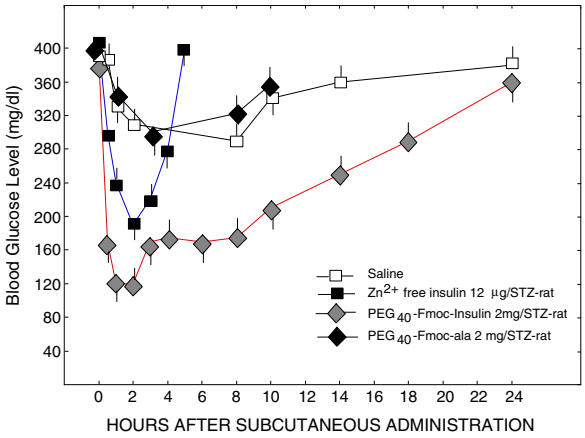

Fig. 8. Effect of a single subcutaneous administration of PEG_{40}-Fmoc-insulin on blood glucose level in STZ-rats. STZ-rats received subcutaneously saline solution (control rats), Zn^{2+}-free insulin (12 μg/STZ-rat), PEG_{40}-Fmoc-insulin (2 mg/STZ-rat) or PEG_{40}-Fmoc-alanine (2 mg/STZ-rat). Blood glucose levels were determined at the time points indicated. Each point represents the arithmetic mean of the blood glucose levels of five STZ-rats ± SEM. Each STZ-rat received 0.2 ml, subcutaneously.

3.7. PEG_{40}-Fmoc-insulin is also long-acting after intravenous administration to STZ-rats

The prolonged action of PEG_{40}-Fmoc-insulin following subcutaneous administration can be attributed, in part, to a lower rate of absorption of this high-molecular-weight derivative from the subcutis to the circulatory system. In Fig. 9, we bypassed the subcutaneous compartment by administrating intravenously the same doses of insulin and PEG_{40}-Fmoc-insulin applied in Fig. 8 subcutaneously. As shown in Fig. 9, PEG_{40}-Fmoc-insulin is a long-acting derivative as well following intravenous administration to STZ-rats. Its glucose-lowering action exceeded that obtained by insulin nearly 8 times, owing to the shorter-

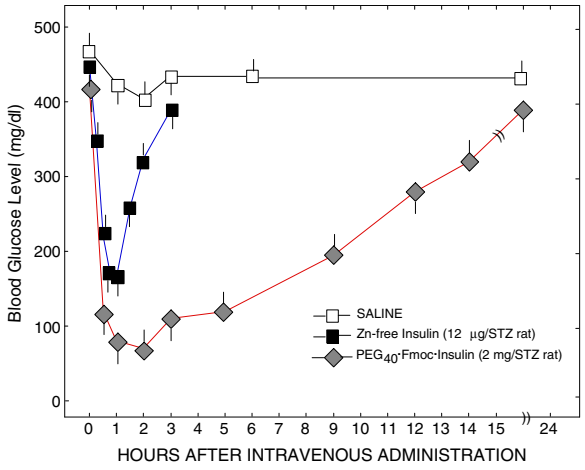

Fig. 9. Effect of a single intravenous administration of PEG_{40}-Fmoc-insulin on blood glucose levels in STZ-rats. STZ-rats received intravenously either saline, Zn^{2+}-free insulin (12 μg/STZ-rat), or PEG_{40}-Fmoc-insulin (2 mg/STZ-rat). Blood glucose levels were determined at the time points indicated in the figure. Each point is the arithmetic mean of the blood glucose level of five STZ-rats ± SEM. Each STZ-rat received 0.2 ml, intravenously.

lived character of intravenously administered insulin ($t½ = 1.5 \pm 0.3$ h) and the prolonged acting nature of intravenously administered PEG_{40}-Fmoc-insulin ($t½ = 11 \pm 1$ h, Fig. 9). Thus, the prolonged action of PEG_{40}-Fmoc-insulin is the outcome of two processes acting in harmony: reduced clearance rate of the macromolecule by glomerular filtration, and decreased receptor-binding and therefore endocytosis and degradation of the hormone, prior to its fall-off from the conjugate by spontaneous hydrolysis.

4. Discussion and conclusions

The covalent linkage of PEG chains to peptide and protein drugs was shown to exhibit clinical properties superior to those of their corresponding unmodified parent proteins, most notably lifetime prolongation *in vivo*, in some instances from minutes to hours. The steric protection conferred by pegylation protects conjugates from proteolysis, shields them from the immune system, and decreases their rate of clearance from the circulatory system via intracellular uptake and kidney filtration [reviewed in [37–41,25,42–44,26]. The linkage of a single PEG chain of 40 kDa to a peptide or protein prolongs lifetime in rodents 7- to 10-times as we [27,28] and others [39,40,26] reported. In spite of those advantages, only a small number of pegylated polypeptide drugs are used clinically, as pegylation often drastically decreases the biological or pharmacological potency of the protein [41,42,44]. This is particularly valid to peptides and small proteins as a proportionally larger section of their surface must be engaged to enable binding to their receptors. Linking a PEG chain at a noninteracting surface may inactivate a peptide agonist as well by inducing steric hindrance [39,42].

Insulin, by virtue of having an extended surface area engaged in receptor binding [45,46], also falls into this category. When PEG-chains of 40 kDa are covalently linked to insulin, the resultant conjugates have less than 1% the biological potency of the hormone (Fig. 2). Thus, the option of applying this powerful pegylation technology to insulin for obtaining a derivative supplying a basal circulatory level of the hormone over many hours after administration is lost.

Previously, we have covalently linked Fmoc moieties to the amino side chains of peptides and proteins and found that in aqueous media these moieties were hydrolyzed at slow rates generating the native nonmodified parent peptides and proteins [34,35]. Rates of Fmoc hydrolysis were dictated exclusively by the pH, temperature, and the composition of the serum – three parameters that are maintained in mammals in strict homeostasis [47]. Indeed, the linked proteins were released (i.e. in normal human serum at 37 °C) in a nearly linear fashion (reviewed in [16]).

Based on this Fmoc technology, we have prepared a bifunctional agent (MAL-Fmoc-Osu, Fig. 1) which binds to peptides and proteins through a slowly hydrolyzable bond and enables the attachment of PEG_{40}-SH to its MAL moiety. By using this approach, we have prepared the monopegylated insulin derivative PEG_{40}-Fmoc-insulin. PEG_{40}-Fmoc-insulin is a water-soluble, low-activity conjugate that is stable in aqueous solutions (i.e. in pH 6.0) for prolonged periods. At physiological conditions (i.e. in phosphate buffer, pH 7.4–0.2% HSA, at 37 °C) the conjugate is hydrolyzed, releasing the native hormone at a low rate and in a homogeneous fashion (Fig. 4), with a K_d value of $0.023 \pm 0.07\ h^{-1}$ (Fig. 5). PEG_{40}-Fmoc hydrolysis is accompanied by linear generation of insulin possessing full biological potency (Fig. 6). A single subcutaneous or intravenous administration of PEG_{40}-Fmoc-insulin to mice or to STZ-rats facilitates prolonged, nonhypoglycemic glucose-lowering effects exceeding ~4- to 7-times those obtained by similar doses of native insulin (Figs. 7–9).

Thus, the principal drawback of conventional pegylation, which precluded the option of preparing active PEG-insulin conjugates, is solved here by this development. Moreover, the 10-times lower biological potency of PEG_{40}-Fmoc-insulin at the time of administration (Table 1, Fig. 2) allows the administration of high dosages with less concern for hypoglycemia, although more ideal prodrug is desirable particularly with regard to insulin (subsequent commentary). The prolonged glucose-lowering action is then maintained by the slow generation of biologically active insulin.

Calculation based on the hydrolysis rate of PEG_{40}-Fmoc-insulin at 37 °C, in 0.1 M phosphate buffer, pH 7.43–0.2% HSA, revealed that about 1.67% of the native hormone is released every hour. We are synthesizing at present several hetero-bifunctional agents, introducing electron-donating or electron-withdrawing groups to positions 2 or 9 of the Fmoc compounds (in preparation). Linking PEG_{40}-SH to insulin through generation of these

new hetero-bifunctional agents may yield PEG_{40}-insulin conjugates that have even more protracted actions *in vivo*.

5. Commentary and future plans

From optimal pharmacology standpoint, we wished to obtain a prolonged-acting insulin derivative, which is a classical prodrug as well (namely, fully inactive at time = 0). This appears highly desirable with regard to insulin as slight overdosing of this hormone may lead to severe hypoglycemic episodes. This last goal has not been fully fulfilled in this study. As we have learned here, the covalent introduction of a large macromolecule, such as PEG_{40}-Fmoc, that is being linked primarily to the α-amino side chain of phe^{B1} (Table 2) ended-up with an insulin derivative that preserves significant amount of its biological potency (11 ± 3%, Fig. 2). This specific shortcoming is to be overcome by linking PEG_{40}-Fmoc to the α-amino side chain of $glycine^{A1}$. We are currently attempting to reach this goal. The latter amino-side chain is located well in the contact area of the hormone with its receptor site, and its derivatization is expected to be accompanied with full loss of binding and the biological potency of insulin [45,46].

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