Insulin complexes with PEGylated basic oligopeptides

Dimitris Tsiourvas a, Zili Sideratou a,⁎, Nikoletta Sterioti a, Athanasios Papadopoulos b, George Nounesis b, Constantinos M. Paleos a

a Institute of Physical Chemistry, NCSR “Demokritos”, 15310 Aghia Paraskevi, Attiki, Greece
b Biomolecular Physics Laboratory, IRRP, NCSR “Demokritos”, 15310 Aghia Paraskevi, Attiki, Greece

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ABSTRACT

Biodegradable oligolysine and oligoarginine-type homopeptides functionalized with PEG of two different molecular weights interact with insulin, at physiological pH, affording complexes studied by dynamic light scattering, (−)-potential, circular dichroism, FTIR spectroscopy, and isothermal titration calorimetry (ITC). High levels of insulin complexation efficiencies (>99.5%) were determined for all derivatives. FTIR spectra suggest that the positively charged homo-oligopeptide derivatives interact with B chain C-terminus of insulin leading to the formation of nanoparticles than can be traced even at low oligopeptide/insulin molar ratios. The ITC profiles are complex, displaying significant endothermic and exothermic contributions. Oligoarginine-type derivatives exhibit the strongest interactions, while PEGylation of either oligopeptide with the high molecular weight chains significantly affects the ITC profiles and leads to larger enthalpy changes. This may be attributed to PEG-induced aggregation of insulin due to the depletion attraction effect leading to the formation of stable nanocomplexes. Stabilization of complexed insulin against enzymatic degradation by trypsin and a-chymotrypsin is observed especially for the high molecular weight PEGylated arginine-based derivative. Insulin release rates in simulated intestinal fluid are controlled by the length of PEG chains and the presence of arginine end-groups. Released insulin retains its secondary structure as established by circular dichroism spectroscopy.

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1. Introduction

Diabetes mellitus is among the most serious metabolic diseases [1]. The standard treatment of insulin-dependent diabetic patients is the periodical subcutaneous injection of insulin. This regimen is associated with poor control of blood glucose level and patient compliance [1]. Therefore, the development of alternative modes for insulin administration is an area of intense research. Numerous attempts to administer insulin by routes avoiding injections have been reported in the literature [2–4].

Insulin oral administration is certainly the most attractive route, but the delivery of insulin to systemic circulation through oral administration is hindered by several obstacles such as the presence of proteolytic enzymes, the sharp pH gradients of the peptic system, and the low epithelial permeability. Extensive investigations have been conducted for addressing these problems as recently reviewed [5–7]. Several approaches have been employed in order to realize an effective insulin oral formulation. These include the application of absorption enhancers [8], protease inhibitors [9], and cell penetrating peptides [10] or the attachment of poly(ethylene glycol) chains to insulin [11] and the use of drug delivery systems such as liposomes [12], nanoparticles [13,14], hydrogels [15], or polymers that spontaneously form nanocomplexes with insulin in aqueous environment [16,17]. Protein delivery systems relying on complexation and assembly of proteins with linear or branched polymers present an alternative interesting approach. These polymers form nanoscale complexes with proteins through electrostatic and van der Waals interactions, protecting them against loss of biological activity with concomitant increase in transmucosal uptake. Several polymers such as chitosan [18,19], block-copolymers [20], or amphiphilic positive charged polymers [21] have been proposed for insulin delivery.

Previous studies have established that cell penetrating peptides (CPPs) including arginine-rich peptides, such as Tat-peptides and oligoarginines, are efficiently taken up by cells [22]. Additionally, conjugation of CPPs with poorly internalized macromolecules such as proteins and nanoparticles improves their permeability through cell membranes [23]. In this context, insulin-Tat hybrids were prepared and their intestinal absorption efficiency was found to be 6–8 times higher compared to normal insulin as tested on Caco-2 cell monolayers [24]. Insulin–Tat transportation was suggested to take place through an active and transcytosis-like mechanism. In another study, co-administration of insulin with

Fax: +30 210 6511766.
E-mail address: zili@chem.demokritos.gr (Z. Sideratou).

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CPPs and in particular octaarginine has been reported to improve insulin intestinal absorption in rats, without causing detectable damage on the intestinal mucosa [10]. Further studies [25, 26] indicated that the attachment of positively charged oligoarginine to negatively charged proteoglycans leads to insulin permeation through the ileal epithelial membrane. It was further reported that electrostatic interaction between insulin and octaarginine or \( \gamma \)-penetratin is associated with the enhancement of intestinal absorption. In this case, although the interaction results in the formation of aggregates, insulin absorption is improved due to disaggregation taking place in the presence of intestinal degradation enzymes. Based on these results, guanidinium end-functionalized derivatives of a poly(\( \gamma \)-lysine) dendrigrat having various numbers of arginine end-groups have been prepared [17]. These guanidine-lated derivatives spontaneously and efficiently interact electrostatically with insulin affording dendrigrat/insulin complexes. It was found that inulin release rates in simulated intestinal fluid can be controlled by the number of guanidinium end-groups and that released insulin retained its native conformation. Stabilization of complexed insulin against enzymatic degradation was observed, especially when using the derivatives with the highest number of guanidinium end-groups.

It is well-documented that the introduction of poly(ethylene glycol) (PEG) in proteins reduces immunogenicity and antigenicity, improves solubility, and increases plasma half-lives [27]. In addition, reduced enzymatic degradation of PEGylated proteins is reported [28], which is of special importance for oral protein delivery. This was attributed to steric hindrance of enzymes caused by the presence of PEG chains, resulting in improved resistance to proteolysis of peptide residues. However, PEG-conjugated insulin may loose its bioactivity and, therefore, a strategy in which insulin is complexed insulin against enzymatic degradation was observed, especially when using the derivatives with the highest number of guanidinium end-groups.

In the present work, the complexation of insulin with biodegradable PEGylated oligo(\( \gamma \)-lysines) or its guanidinylated oligoarginine-type counterparts at physiological temperature and pH values is reported. Specifically, oligo(\( \gamma \)-lysines) (OLys) was PEGylated affording two PEGylated derivatives bearing two different molecular weights, that is, OLys-PEG750 and OLys-PEG2000. These derivatives were subsequently guanidinylated affording two new oligoarginine-type derivatives bearing eight guanidinium end-groups, that is, OArg-PEG750 and OArg-PEG2000 (Scheme 1). The negatively charged insulin strongly interacts with the positively charged PEGylated oligopeptide derivatives affording complexes that were characterized by dynamic light scattering (DLS), \( \zeta \)-potential, isothermal titration calorimetry (ITC), and FTIR spectroscopy. Stabilization of complexed insulin against enzymatic degradation by trypsin and \( \gamma \)-chymotrypsin, as well as its release in enzyme-free simulated intestinal fluid will be presented.

### 2. Materials and methods

#### 2.1. Materials

**Oligo(\( \gamma \)-lysine) (M\(_{\text{w}}\) = 1,450, DPN = 8, polydispersity = 1.3)** bearing eight protected amino groups with TFA was kindly donated by Colcom SARL (Montpellier, France). 1H-pyrazole-1-carboxamide hydrochloride (99%), N,N-diisopropylethylamine (DIEPEA), trypsin and \( \gamma \)-chymotrypsin were purchased from Sigma–Aldrich Co. (St. Louis, MO, USA). N-hydroxybenzotriazole (HOBr) and 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyllumorium (HBHTU) were purchased from Anaspec (San Jose, USA). Methoxy poly(ethylene glycol)-amines of M\(_{\text{w}}\) = 750 and 2000 (PEG-amine) were obtained from Iris Biotech GmbH. Human insulin was kindly provided by Novo Nordisk (Denmark).

#### 2.2. Synthesis of PEGylated basic oligopeptide derivatives

The PEGylated derivatives of oligolysine (OLys) were prepared by reacting TFA protected OLys with methoxy poly(ethylene glycol)-amines of two different molecular weights, M\(_{\text{w}}\) = 750 or 2000, affording the TFA-protected OLys-PEG750 and OLys-PEG2000 derivatives that, after deprotection, afforded the final products (OLys-PEG750 and OLys-PEG2000, Scheme 1). Specifically, 2 mmol of protected OLys, dissolved in 10 mL of anhydrous DMF, was added to 2.5 mmol of PEG-amine, dissolved in 10 mL of dry DMF. Subsequently, HBTU (4 mmol) and HOBt (4 mmol) dissolved in 2 mL DMF were added to the mixture, followed by the addition of DIEPA (5 mmol). The mixture was allowed to react overnight, under argon atmosphere, at room temperature. The solvent was partially removed under vacuum, and the crude product was precipitated with diethyl ether. This solid was washed with water in order to remove unreacted PEG-amine. Then, the protected products were suspended in a mixture of H\(_2\)O (3 mL), NH\(_3\) (10 N, 2.5 mL) and MeOH (12 mL) and the mixture was stirred at 40 °C for 48 h. After completion of the reaction, the mixture was concentrated in vacuum to 1/10 of its initial volume to remove MeOH and NH\(_3\) and then freeze-dried to afford the final products (OLys-PEG750 and OLys-PEG2000) as white solids. The structure of OLys-PEG750 and OLys-PEG2000 was established by proton and carbon NMR spectroscopy.

\(^{1}\text{H} \text{NMR} \left( 500 \text{ MHz}, \text{D}_2\text{O} \right) \delta = 4.25 \ (m, \text{CHCONH}) , 3.65 \ (s, \text{CH}_2\text{CH}(\text{O})\text{H}), 3.50 \ (t, \text{CONHC}_3\text{H}_2\text{CH}(\text{O})\text{H}), 3.30 \ (s, \text{OCH}_3), 2.90 \ (t, \text{NHCH}(\text{CH}_3)), 1.50–1.85 \ (m, \beta \text{ and } \delta \text{CH}_2 \text{ of lysine}), 1.20–1.45 \ (m, \gamma \text{CH}_2 \text{ of lysine}). \text{ }^{13}\text{C} \text{NMR} \left( 125.1 \text{ MHz}, \text{D}_2\text{O} \right) \delta = 173.0 \ (\text{CO}), 69.2 \ (\text{CH}_2\text{CH}(\text{O})\text{H}), 54.5 \ (\text{OCH}_3), 53.0 \ (\beta \text{–CH}_2 \text{ of lysine}), 40.1 \ (\text{CONHC}_3\text{H}_2\text{CH}(\text{O})\text{H}), 39.0 \ (\text{NH}_2\text{CH}_2), 30.7 \ (\delta \text{–CH}_2 \text{ of lysine}), 26.3 \ (\beta \text{–CH}_2 \text{ of lysine}), 22.0 \ (\gamma \text{–CH}_2 \text{ of lysine}).

The previously prepared PEGylated oligo(\( \gamma \)-lysines) and the parent OLys were fully functionalized with eight guanidinium end-groups by a method analogous to one previously reported [17,29], affording two PEGylated oligoarginine-type derivatives, OArg-PEG750 and OArg-PEG2000. In brief, to 0.05 mmol of OLys or of PEGylated oligo(\( \gamma \)-lysines) derivatives dissolved in dry DMF (10 mL), a DMF solution (10 mL) containing 0.50 mmol 1H-pyrazole-1-carboxamide hydrochloride and 0.50 mmol N,N-diisopropylethylamine was added dropwise, and the solution was allowed to react for 24 h under argon atmosphere. Subsequently, the solution was concentrated by solvent distillation under reduced pressure and the product was precipitated with diethyl ether. The crude product was dissolved in water and was subjected to dialysis (mol. weight cut-off: 1200) to remove reaction by-products. Lyophilization afforded the final products OArg, OArg-PEG750, and OArg-PEG2000 (Scheme 1). The structure of these derivatives was established by \(^{1}\text{H} \text{NMR} \left( 500 \text{ MHz}, \text{D}_2\text{O} \right) \delta = 7.80–8.00 \ (\text{broad s, NH of guanidinium group}), 6.90–7.10 \ (\text{broad d, NH}_2 \text{ of lysine}), 4.25 \ (m, \text{CHCONH}), 3.65 \ (s, \text{CH}_2\text{CH}(\text{O})\text{H}), 3.50 \ (t, \text{CONHC}_3\text{H}_2\text{CH}(\text{O})\text{H}), 3.30 \ (s, \text{OCH}_3), 3.00–3.20 \ (t, \text{CH}_3\text{NH}(\text{NH}_2)_2), 1.50–1.85 \ (m, \beta \text{ and } \delta \text{CH}_2 \text{ of lysine}), 1.20–1.45 \ (m, \gamma \text{CH}_2 \text{ of lysine}). \text{ }^{13}\text{C} \text{NMR} \left( 125.1 \text{ MHz}, \text{D}_2\text{O} \right) \delta = 173.0 \ (\text{CO}), 157.0 \ (\text{NH}(\text{NH}_2)_2), 69.2 \ (\text{CH}_2\text{CH}(\text{O})\text{H}), 54.5 \ (\text{OCH}_3), 53.0 \ (\beta \text{–CH}_2 \text{ of lysine}), 41.0 \ (\text{CH}_3\text{NH}(\text{NH}_2)_2), 40.1 \ (\text{CONHC}_3\text{H}_2\text{CH}(\text{O})\text{H}), 27.4 \ (\text{CH}_3\text{CH}(\text{O})\text{H}), 26.3 \ (\beta \text{–CH}_2 \text{ of lysine}), 22.0 \ (\gamma \text{–CH}_2 \text{ of lysine}).

For performing control experiments, guanidinium terminated poly(ethylene glycol) derivatives were prepared by guanidinylation of PEG-amines having M\(_{\text{w}}\) = 750 and 2000, employing the same procedure as above.
2.3. Interaction of insulin with PEGylated oligopeptides and determination of insulin complexation efficiency

Stock solutions of insulin (0.36 mM, 2 mg mL\(^{-1}\)) and PEGylated oligopeptides (1 mM, 2–4 mg mL\(^{-1}\)) in 10 mM Tris buffer \(\text{pH} = 7.4\) were prepared. Insulin was allowed to interact with the oligopeptide derivatives by the addition of their stock solution to insulin solution at several oligopeptide derivative/insulin molar ratios under rigorous stirring. The interaction results in spontaneously formed nanoparticles that were separated from the aqueous phase by centrifugation at 11,000 RCF for 20 min. The obtained pellets were washed twice with water and dried under vacuum for 24 h. The supernatant containing the non-associated insulin was collected, and the amount of free insulin was quantified by reversed phase high performance liquid chromatography (HPLC)[30]. A Dionex HPLC system consisting of a helium out-gasser, a gradient pump GP 50, a LC30 oven equipped with auto injection port, 25 µL loop, a 218MR54 column (4.6 x 250 mm, 5 µm particle size, C18, Vydc, USA), and a PDA 100 photodiode array UV detector set at 218 nm was employed. The mobile phase consisted of 0.05% v/v TFA in deionized water (A) and 0.05% v/v TFA in HPLC grade acetonitrile (B). The gradient conditions were 27% B for 4 min and 27–36% B in the next 11 min and back to 27% B in 5 min, at a flow rate of 1 mL min\(^{-1}\) at 27 °C. Under these conditions, the retention time of insulin was 12.7 min. Insulin concentration in the aqueous solutions was determined from a calibration curve prepared from various standard solutions (2–100 µg mL\(^{-1}\), \(R = 0.9994\)). Insulin complexation efficiency was calculated for complexes obtained at various oligopeptide derivative/insulin molar ratios according to the equation [31]:

\[
\text{Percentage Complexation efficiency} = \frac{\text{Total amount of insulin} - \text{Free insulin}}{\text{Total amount of insulin}} \times 100
\]

Maximum complexation efficiency (>99%) was obtained at oligopeptide/insulin molar ratio of 0.20 for the OArg and 0.40 for the OLys derivatives. All samples were measured in triplicate.

2.4. Characterization techniques

Interaction between functionalized oligopeptide derivatives and insulin was investigated by dynamic light scattering (DLS), \(\zeta\)-potential measurements, isothermal titration calorimetry (ITC), Near UV (NUV-CD), and Far UV (FUV-CD) circular dichroism. Dynamic light scattering studies were performed employing an AXI-OS-150/EX (Triton Hellas) apparatus with a 30 mW laser source, and an Avalanche photodiode detector at an angle of 90°. For these experiments, 50 µL dispersion of the various oligopeptide derivative/insulin complexes obtained as described above was diluted with various quantities of Tris buffer (0.2–1.5 mL). Size differences were not observed upon dilution. At least ten light scattering measurements were collected for each dispersion, and the results were averaged. \(\zeta\)-potential measurements were conducted using ZetaPlus (Brookhaven Instruments Corp, USA). In a typical experiment, an oligopeptide solution (1 mM) was progressively added to insulin solution (0.36 mM, 2 mg mL\(^{-1}\), 10 mM Tris buffer, \(\text{pH} = 7.4\)). Following each addition, the mixtures were quickly agitated and introduced into the instrument cell. Ten \(\zeta\)-potential measurements were collected for each dispersion, and the results were averaged. Near UV circular dichroism spectra (NUV-CD) were recorded during titration of insulin solutions with oligopeptide derivatives, as described above for ITC experiments, employing a Jasco J-715 circular dichroism spectrophotometer coupled with a Peltier temperature control system (Jasco PTC-348Wi). The spectra were recorded at 25 °C using a 0.1-cm path length cell at 250–350 nm with a step size of 0.2 nm and a bandwidth of 1.0 nm. Experiments were run in triplicate, and 60 scans for each spectrum were signal averaged.

Scheme 1. Chemical structures of oligolysine (OLys) and oligoarginine-type (OArg) homopeptides and of their PEGylated derivatives.
For investigating the secondary structure of insulin, Far UV circular dichroism spectra (FUV-CD) were recorded at 25 °C using the same as above instrument parameters. To eliminate possible contributions from buffer and oligopeptide derivatives that were also present in solution, their CD spectra were recorded and subtracted from the spectra of the supernatants. Experiments were run in triplicate and 60 scans for each spectrum were signal averaged.

For FTIR studies, the nanoparticle formed at various oligopeptide/insulin molar ratios were separated from the aqueous phase by centrifugation at 11,000 RCF for 20 min, washed twice with water, and dried under vacuum for 24 h. Their spectra were recorded in a Nicolet 6700 Fourier Transformed Infrared spectrometer equipped with an Attenuated Total Reflectance accessory (ATR) with a diamond crystal (Smart Orbit™, Thermo Electron Corporation). Samples were firmly pressed against the diamond, and spectra were recorded at 4 cm⁻¹ resolution. A minimum of 64 scans were collected and signal averaged.

To establish the thermodynamic parameters for the binding of insulin to the oligopeptide derivatives, ITC experiments were carried out in a MCS-ITC calorimeter (Microcal, Northampton, USA) described previously [32], at 25 °C in Tris–HCl buffer (10 mM, pH = 7.4). In a typical experiment, an oligopeptide solution (1.50 mM) was loaded into the 250 μL injection syringe, while an insulin solution (0.50 mM or 1 mM) was placed in the 1.334 mL sample cell of the calorimeter. To avoid air bubble formation, all solutions were degassed under vacuum for 20 min immediately before the measurements. The oligopeptide solution was titrated into the sample cell at a sequence of 21 injections of 12 μL aliquots, respectively. Time intervals between successive injections were 300 s to assure that thermodynamic equilibrium is reached in the cell before the next addition. The contents of the sample cell were stirred throughout the experiment at 400 rpm to ensure thorough mixing. Raw data were obtained as a plot of power change (μcal s⁻¹) vs. time. The raw data were integrated to obtain a plot of enthalpy change per mole of injectant (ΔH, kcal mol⁻¹) vs. the oligopeptide derivative/insulin molar ratio. Enthalpies of dilution for all interacting molecules were determined in separate experiments and subtracted from the corresponding experimental curves prior to analysis. Raw data form these control experiments are displayed in Supplementary Data (Fig. 1). It can straightforwardly be observed that the dilution of PEGylated derivatives, especially the largest ones, exhibit significant exothermic traces due to the interaction of PEG with water molecules. The dilution enthalpies were subtracted from the apparent enthalpy obtained in each titration run, and all the net titration enthalpy curves were processed with the Origin® 5.0 software with embedded calorimetric fitting routines.

2.5. Insulin in vitro release studies

For determining in vitro release profiles of insulin in enzyme-free simulated intestinal fluid, complexes were incubated in 5 mL of simulated intestinal fluid without pancreatin (SIF, pH = 6.8, prepared according to USP31-NF26: monobasic potassium phosphate (6.8 g) and 0.2 N sodium hydroxide (77 mL) were mixed and diluted with water to 1000 mL) for 6 h at 37 °C in a Stuart Orbital incubator SI500 at 200 rpm. The complexes used in this series of experiments contained 2 mg insulin were prepared as described above, employing molar ratios that ensured maximum insulin complexation efficiency, that is, at oligopeptide derivative/insulin molar ratio of 0.20 for OArg and 0.40 for OLys derivatives. Aliquots were collected at predetermined time intervals and replaced by equal volumes of fresh incubation medium. For the determination of released insulin, samples were centrifuged at 11,000 RCF for 10 min, and 25 μL of the supernatant was analyzed by HPLC. In vitro release studies were carried out in triplicate.

2.6. Enzymatic degradation

A stock solution of trypsin (4.0 mg mL⁻¹) was prepared in 10 mM Tris–HCl buffer, pH = 8.0 (i.e., the optimal operating pH of trypsin). Oligopeptide derivative/insulin complexes were the same as those used in the in vitro release studies (Section 2.5). Subsequently, complexes, or free insulin (2.0 mg) as control, were incubated at 37 °C in 1.0 mL of 10 mM Tris buffer, pH 8.0, in the presence of trypsin at an enzyme/insulin molar ratio of 1:255 [33]. Aliquots (100 μL) were withdrawn at various time intervals and transferred into ice-cold vials containing 150 μL Tris buffer and 150 μL trifluoroacetic acid to stop enzymatic activity (pH = 2.5). Subsequently, 500 μL tert-butanol was also added to completely dissolve the complexes. Experiments were performed in triplicate, and non-degraded insulin was determined by reversed phase HPLC as described above.

An analogous study was performed using x-chymotrypsin. Specifically, a stock solution of x-chymotrypsin (0.1 mg mL⁻¹) was prepared in 1 mM HCl containing CaCl₂ (2 mM). Insulin complexes or free insulin (2.0 mg) were incubated at 37 °C in 1.0 mL of 10 mM Tris buffer, pH = 7.8, 10 mM CaCl₂, in the presence of x-chymotrypsin at an enzyme/insulin molar ratio of 1:215. Aliquots (100 μL) were withdrawn at various time intervals and treated as described above.

3. Results and discussion

3.1. Synthesis of PEGylated oligopeptides

Two PEGylated oligo(L-lysine) derivatives with different PEG molecular weights, OLy5-PEG750 and OLy5-PEG2000, were prepared by reacting the carboxylic end group of TFA-protected oligo(L-lysine) with the amino group of PEG using HBTU/HOBt/DIEA as coupling reagents and subsequent deprotection of lysine amines employing NH₃. The structure of these PEGylated derivatives was established by ¹H and ¹³C NMR. Specifically, the appearance of a singlet peak at 3.30 ppm corresponding to the terminal amine group, and the broad peaks centered at 3.65 ppm corresponding to the oxygenated methylenes of poly(ethylene glycol), confirmed the introduction of the PEG moiety. As expected, one PEG chain was attached at each oligolysine scaffold as confirmed by the integration of the peaks at 3.30 ppm attributed to the terminal oxygenated methyl group of PEG and the peaks at 2.90 ppm attributed to the α-methylene relative to primary amines. The introduction of one PEG chain to each oligo(L-lysine) molecule was also confirmed by the appearance in the ¹³C NMR spectrum of a strong peak at 69.2 ppm corresponding to the oxygenated terminal methyl group of the PEG chain. Additional signals appeared at 54.5 ppm corresponding to the oxygenated terminal methyl group of the PEG, and a peak at 40.1 ppm, corresponding to the α-carbon of methylene groups next to the newly formed amide group.

Subsequently, the primary amino groups of parent oligo(L-lysine) and PEGylated oligo(L-lysines) reacted with 1H-pyrazole-1-carboxamidine hydrochloride and N,N-diisopropylethylamine, affording the oligoarginine-type derivatives OArg, OArg-PEG750 and OArg-PEG2000, the structure of which was established by ¹H and ¹³C NMR spectroscopy. Specifically, the new triplet appearing at ~3.10 ppm is attributed to the α-CH₂ relative to the guanidinium group, and the broad peaks centered at ~7.00 and ~7.90 ppm are attributed to the guanidinium groups. The complete replacement of the primary amino groups by guanidinium groups was established by the integration of the peaks centered at ~3.10 ppm.
(α-CH₂ relative to guanidinium groups) and ~1.30 ppm (γ-CH₂ of lysine). This was also proved by the absence of the peaks at 2.90 ppm attributed to the protons of α-CH₂ relative to primary amino groups. Analogous results were obtained by ¹³C NMR spectroscopy. The peaks at α and β carbons relative to the primary amino groups at 39.0 and 30.7 ppm, respectively, were replaced by peaks at 41.0 and 27.4 ppm, attributed to the α and β carbons relative to guanidinium groups. Also, the carbon of the guanidinium moieties was observed at 157.0 ppm.

3.2. Interaction studies of oligopeptide derivatives with insulin

3.2.1. DLS and ζ-potential studies

The interaction of the negatively charged insulin with the positively charged oligopeptide derivatives was initially followed by DLS. Association of insulin with the PEGylated oligopeptides occurred spontaneously, leading to the formation of particles at the nanometer scale when low oligomeric derivative/insulin molar ratios were employed. As shown in Fig. 1, when OLys-PEG750 and OLys-PEG2000 were allowed to interact with insulin at molar ratios less than 0.02, nanoparticles of about 150–200 nm radii were detected by DLS. On the other hand, interaction of OArg-PEG750 and OArg-PEG2000 with insulin led to the formation of larger nanoparticles of ca. 200–300 nm radii, respectively, even at 0.005 molar ratios. As the oligopeptide derivative/insulin molar ratio increased, a plateau region was observed at molar ratios of 0.15–0.20 for the OLys derivatives and of 0.35–0.40 for the OArg derivatives (Fig. 2). Further increase in molar ratios resulted in a gradual increase in ζ-potential to positive values, reaching a final plateau at ~20 mV at molar ratios of 0.15–0.20 for the OArg derivatives and of 0.35–0.40 for OLys derivatives (Fig. 2). It is interesting to note that the overall behavior of the OLys derivatives follows the same general trend observed with the OArg ones; though, as a result of the absence of guanidinium groups and, consequently, of the weaker interaction between the primary amino groups with insulin, the observed ζ-potential increase is less abrupt and the plateau values are observed at higher oligolysine derivative/insulin molar ratios.

3.2.2. NUV circular dichroism spectroscopy (NUV-CD)

NUV-CD spectroscopy was used to study the proposed dissociation of insulin dimers upon interaction with the oligopeptide derivatives as a function of oligopeptide/insulin molar ratio. Insulin dimers and hexamers exhibit a characteristic tyrosyl CD signal at 220 nm [17]. Similar sizes were also reported upon insulin interaction with positively charged polymers [16,21,31,34]. The interaction of insulin with the PEGylated oligopeptides was also followed by monitoring the electrophoretic mobility of the resulting dispersions at various molar ratios. For all derivatives, the ζ-potential values of insulin complexes gradually increase on increasing molar ratios exhibiting two plateau regions (Fig. 2). The same behavior was observed upon interaction of insulin with positively charged polymers [16,21,31,34].

Specifically, at molar ratios of ~0.04–0.05, the ζ-potential values reach a plateau of ca. ~15 mV for OArg-PEG750 and OArg-PEG2000, while for OLys-PEG750 and Olys-PEG2000, a plateau of ~30 and ~25 mV was observed, respectively, at ~0.07–0.10 molar ratios. As first observed in DLS experiments and further discussed in the ITC and NUV-CD, at these molar ratios, insulin dimers dissociate and newly formed negatively charged insulin entities are available to interact with the gradually added positively charged oligolysine derivatives. As a result, ζ-potential values remain almost stable up to complete dissociation of dimers at molar ratios ranging from ~0.04–0.05 for the OArg derivatives to ~0.07–0.10 for the OLys derivatives (Fig. 2).

Further increase in the molar ratio results in a gradual increase in ζ-potential to positive values, reaching a final plateau at ~20 mV at molar ratios of 0.15–0.20 for the OArg derivatives and of 0.35–0.40 for OLys derivatives (Fig. 2). It is interesting to note that the overall behavior of the OLys derivatives follows the same general trend observed with the OArg ones; though, as a result of the absence of guanidinium groups and, consequently, of the weaker interaction between the primary amino groups with insulin, the observed ζ-potential increase is less abrupt and the plateau values are observed at higher oligolysine derivative/insulin molar ratios.

It should be noted that control experiments employing insulin and PEG, PEG-amines or guanidinium terminated PEG derivatives at the same concentrations and molar ratios did not show any nanoparticle formation by DLS or any modification of the surface charge of insulin by ζ-potential measurements underlining the necessity of a multifunctional oligo-amine or oligo-guanidine moiety for the complexes’ formation.

![Fig. 1. Mean hydrodynamic radii (nm) of oligopeptide derivative/insulin complexes formed following interaction of insulin with OLys-PEG750 (open circles), OLys-PEG2000 (open squares), OArg-PEG750 (solid circles), or OArg-PEG2000 (solid squares) at various molar ratios.](image)

![Fig. 2. ζ-Potential values of oligopeptide derivative/insulin complexes formed following interaction of insulin with OLys-PEG750 (open circles), OLys-PEG2000 (open squares), OArg-PEG750 (solid circles), and OArg-PEG2000 (solid squares) at various molar ratios.](image)
of the complexes (cf. curve b with curves a, c, d in Fig. 4). Thus, di-
which at this molar ratio are practically not traceable in the spectra
overlapping vibrational bands of the oligopeptide derivatives,
possible structural changes of insulin without the interference of
These molar ratios, additionally, offer the advantage of monitoring
changes in the corresponding NUV-CD spectra were registered.

3.2.3. Infrared studies

The FTIR spectra of insulin complexes with the oligopeptide
derivatives in the solid state were recorded at various oligopep-
tide/insulin molar ratios, typically 0.05–0.10, that is, near the first
plateau region observed by DLS and ζ-potential and before any
changes in the corresponding NUV-CD spectra were registered.
These molar ratios, additionally, offer the advantage of monitoring possible structural changes of insulin without the interference of overlapping vibrational bands of the oligopeptide derivatives, which at this molar ratio are practically not traceable in the spectra of the complexes (cf. curve b with curves a, c, d in Fig. 4). Thus, direct comparison of insulin before and after interaction with the various oligopeptide derivatives is possible. From the inspection of the spectra of all complexes, it is immediately evident that the profile of the Amide I band, centered at 1650 cm\(^{-1}\), does not change upon complex formation (Fig. 4), suggesting that no conformational changes in the secondary structure of insulin take place. Therefore, in this case and at least at these molar ratios, insulin is not destabilized by forming, for instance, highly beta-sheets rich amyloid-type structures as observed when positively charged dendrimers interact with insulin [37]. However, at higher molar ratios, possible conformational changes cannot be excluded. For instance, insulin interacting with polyethyleneimine–dextran sulfate nanoparticles can retain or alter its secondary structure depending on polymer ratios employed or pH values [38].

For the OArg-PEG750/insulin complexes at 0.05 and 0.10 molar ratios (Fig. 4, curve c), it is clear that the most prominent change of their spectra compared to the spectrum of pure insulin in the 1700–1000 cm\(^{-1}\) region is that the band at 1512 cm\(^{-1}\), which is assigned [39,40] to Tyr–OH ring vibration (ν(C=C), ν(CH)), lowers in intensity and shifts to 1516 cm\(^{-1}\). Additionally, the band at 1234 cm\(^{-1}\), which is also assigned to the Tyr–OH vibrations ν(C—O), ν(CC) vibration shifts to 1242 cm\(^{-1}\). In a similar manner, the spectra of the OArg-PEG2000/insulin complexes (Fig. 4, curve d) at 0.05 and 0.10 molar ratios exhibit the same as above changes with respect to the spectrum of insulin. However, the observed shifts of the Tyr–OH vibrations at 1515 cm\(^{-1}\) and 1239 cm\(^{-1}\), respectively, are less prominent in this case, apparently due to the presence of high molecular weight PEG. Regarding the oligolysine derivatives, the Olys-PEG750/insulin complex at 0.10 molar ratios shows basically the same differences in the FTIR spectrum compared to insulin’s spectrum as those of the OArg counterparts described above. The band assigned to the Tyr–OH ring vibration shifts to 1515 cm\(^{-1}\) and the band at 1234 cm\(^{-1}\) shifts to 1240 cm\(^{-1}\). As above however, when the oligolysine derivative with the high molecular weight

![Fig. 3](image)

Fig. 3. (A) NUV-CD spectra of free insulin (a) or of insulin following interaction with OArg-2000 at oligopeptide/insulin molar ratios of 0.01 (b), 0.03 (c), 0.05 (d), 0.07 (e), and 0.10 (f). (B) Variation of ellipticity at 275 nm as a function of oligopeptide/insulin molar ratio.
PEG is employed, the spectrum of the corresponding complex at 0.10 molar ratio shows less intense changes compared to the spectrum of insulin. Specifically, the intensity of the 1514 cm$^{-1}$ band is marginally lower than that of insulin, whilst the Tyr–OH band at 1234 cm$^{-1}$ shifts to 1238 cm$^{-1}$ suggesting reduced interaction. Overall, FTIR data indicate that interaction is taking place between the OH groups of tyrosine residue and the guanidinium or amino groups of OArg or Olys derivatives, respectively. This is confirmed by studying the interaction between p-creosol (representing the amino-acid residue of tyrosine) and octadecylguanidine or octadecylamine employing FTIR. The infrared spectra of p-creosol/octadecylguanidine or octadecylamine complexes clearly show that the band of creosol at 1511 cm$^{-1}$ decreases in intensity and shifts to 1515 cm$^{-1}$, while the band at 1233 cm$^{-1}$ shifts to 1258 cm$^{-1}$.

It is worth mentioning that insulin forms dimers in solution due to hydrogen-bonding between the C-terminus (B26–B30) of B chains [41–43]. Moreover, the B chain C-terminus is of great importance for receptor binding [44–47] and it is believed to possess inherent flexibility, which is critical for self-association of insulin molecules and the binding of insulin to its receptor [42,47,48]. In this C-terminus, a tyrosine amino acid residue (B26) is present, whose vibrational band is clearly affected upon complexation with the oligopeptide derivatives. It is therefore reasonable to suggest that the positively charged Olys or OArg derivatives interact with the available OH groups of the B chain C-terminus. It should be also noted that other interaction sites, for instance, interaction of the oligopeptides with the COOH group of the C-terminus cannot be excluded. However, such interaction is not possible to be observed unequivocally in the FTIR spectra since, according to Kunitake et al. [49,50], interaction of carboxyl group with guanidinium moiety leads to the appearance of a new band at about 1630–1650 cm$^{-1}$, that is, in the spectral region that is dominated by the amide I band of the protein.

Taken as a whole it can be suggested that oligopeptide derivatives interact with the C-termini B chain region of insulin molecules. Due to their multifunctional character, they can associate with more than one insulin molecule, which finally leads to insulin aggregation. However, significant interaction and nanoparticle formation is more prominent in PEGylated Olys or OArg, and this phenomenon is enhanced on increasing the molecular weight of the hydrophilic polymeric chain. It is generally accepted that protein–protein attraction can be induced by the presence of PEG due to the so-called depletion attraction mechanism [51–53]. Such interactions can result in protein aggregation, precipitation, or even protein crystallization. The effect of PEG, or other hydrophilic, flexible and non-absorbing macromolecules, is considered to be due to the dehydration of proteins, or a part of proteins’ surface, by the strongly hydrophilic macromolecules (depletion of water molecules), which leads to the attraction of the dehydrated surfaces and, finally, to phase separation or precipitation [54]. It has also been reported that attraction increases with PEG concentration and PEG molecular weight [55,56]. It is therefore reasonable to suggest that, in the case under investigation, although nominal PEG concentration is quite low to induce such phenomenon (as also confirmed by the absence of any interaction in the case of PEG, PEG–amine or guanidinium terminated PEG), the interaction between the oligopeptide part with insulin results in a local increase in PEG concentration on protein’s surface which in turn enhances the depletion attraction effect leading to the formation of nanoparticles and, at even higher concentrations, to the formation of large aggregates.

3.2.4. Isothermal titration calorimetry (ITC)

Isothermal titration calorimetry has previously been employed to study the thermodynamics of the binding of arginine end-functionalized poly(lysine) dendrigrafts to insulin [17]. The ITC profile of this interaction was shown to be complex involving at least two distinct processes. It was established that substituting one amino group by one guanidinium group in the dendrigraft architecture resulted in a favorable binding gain of 0.76 ± 0.08 kcal mol$^{-1}$. For the present system, the calorimetric data depict an overall complex behavior as well. The ITC result for the interaction of parent Olys with insulin is presented in Fig. 5 (upper panel). An endothermic interaction is recorded characterized by an enthalpy change $\Delta H = 1.7 ± 0.5$ kcal/mol, a relatively low binding constant $K = 2.2 ± 1.0 \times 10^4$ mol$^{-1}$ and a stoichiometry ratio (Olys/insulin) $N = 0.13 ± 0.02$, which is in agreement with the Olys eight possible binding sites ($1:8 = 0.125$). An endothermic ITC profile emerges for the interaction of insulin with Olys-PEG750 (Fig. 5, middle panel) as well. The endothermic signal shows a larger, overall, enthalpy change than in the case of Olys. Moreover, it displays a puzzling peak at molar ratio ∼0.2 that is likely significant of the fact that conformational changes or other binding process take place at higher molar ratios. Such processes may include the binding of Olys-750 to dimeric insulin that is present under the conditions used in the ITC experiments [57] as well as the dissociation of insulin dimers. $\Delta H$ is dramatically altered in the case of Olys-PEG2000. The ITC profile obtained (Fig. 5, lower panel) displays a low-molar-ratio endothermic component followed by a substantial exothermic contribution that becomes dominant at Olys/insulin molar ratios larger than 0.15. These ITC results provide evidence that PEGylation with the PEG-2000 chains of the Olys significantly affects the binding mechanism of insulin. The ITC results also suggest that the size of PEG directly interferes with the stabilization of the oligolysine/insulin complexes, even though ITC experiments (not shown here) studying the direct interaction of PEG, PEG–amine, or guanidinium-terminated PEG of either molecular weight with insulin did not reveal any calorimetric binding signal, demonstrating no direct insulin/PEG interaction. In this manner, the oligolysine derivatives’ capacity for binding insulin may be conveniently controlled by PEGylation. Therefore, the multivalent effect [58] exercised by the oligolysine moiety is essential for complex formation. The substantial exothermic contribution for the Olys-PEG2000 ITC experiments is likely associated with mechanisms such as the previously described depletion attraction effect, leading to PEG-mediated insulin aggregation and the formation of stable nanoparticles. Apparently, in the case of the low molecular weight PEG chain, higher concentrations of PEG at insulin surfaces are required for such mechanisms to take effect and, therefore, the exothermic contribution is not observed in the ITC data for Olys-PEG750 (middle panel of Fig. 5).

The ITC results obtained from the titration of OArg, OArg-PEG750, and OArg-PEG2000 with insulin are presented in Fig. 6. As in Fig. 5, the left column contains the raw, heat-flow calorimetric data and the right column the normalized results for the enthalpy per mol of OArg-derivative vs. the molar ratio (OArg-derivative/insulin). For the titration of OArg (top panel), the ITC profile is sharply different from the corresponding ones for Olys. The complex, V-shaped curve can only be analyzed by considering more than one thermodynamic process involved in the interaction mechanism, that is, an endothermic and an exothermic one. An accurate deconvolution of the ITC profile into these two components, using non-linear least-squares fits, is particularly difficult since there are too many parameters involved. Our attempts lead to the following results for $\Delta H$, $K$, and $N$: $\Delta H_{end} = 6.0 ± 0.5$ kcal/mol, $K_{end} = 1.2 ± 0.46 \times 10^6$ M$^{-1}$, $N_{end} = 0.18 ± 0.1$ for the endothermic process; and $\Delta H_{exo} = -9.0 ± 1.0$ kcal/mol, $K_{exo} = 2.8 ± 0.9 \times 10^6$ M$^{-1}$, $N_{exo} = 0.31 ± 0.5$ for the exothermic. In previous studies for the interaction of arginine end-functionalized poly(lysine) dendrigrafts with insulin [17], exothermic ITC signals have been associated with the interaction of insulin to the guanidinium group, while an endothermic component has been linked to the likely dissociation of insulin.

dimers. As previously stated, under the conditions used in the present ITC experiments, insulin exists predominately in a dimeric state [57]. The injection of OArg into the insulin solution can result in the dissociation of dimers [17] due to OArg interaction on the monomer–monomer interface. It should be noted that endothermic signals have also been observed [59,60] upon insulin addition into phosphate buffers and were attributed to the molecular dissociation of insulin oligomers upon dilution. In addition, the endothermic signals were enhanced upon insulin complexation with cyclodextrin derivatives present in solution [59,60]. It is also known that in insulin dimers, the B chain of one monomer packs against the B chain of the second monomer, and from FTIR data, it is suggested that OArg interacts with insulin in the B-chain region. Of course, the possibility of direct binding of the insulin dimers to OArg must also be considered. In any event, the complexity of the interaction limits our ability to accurately determine the (OArg/insulin) molar ratio for each process and thus estimate the values for $\Delta H$, $K$ and $N$.

The titration of OArg-PEG750 with insulin (Fig. 6, middle panel) gives an ITC profile that is quite analogous to what has been obtained for OArg. Once again the V-shaped titration curve is analyzed via a two-process mechanism leading to the following results for the endothermic and the exothermic components: $\Delta H_{\text{end}} = 12.0 \pm 1.0 \text{ kcal/mol}$, $K_{\text{end}} = 1.1 \pm 0.46 \times 10^6 \text{ M}^{-1}$, $N_{\text{end}} = 0.15 \pm 0.1$ for the endothermic; and $\Delta H_{\text{exo}} = -12.1 \pm 1.0 \text{ kcal/mol}$, $K_{\text{exo}} = 1.9 \pm 0.5 \times 10^7 \text{ M}^{-1}$, $N_{\text{exo}} = 0.27 \pm 0.3$ for the exothermic. It can be straightforwardly observed that with the exception of an increase in $\Delta H$ for both processes (even though the number of interacting sites has not been altered), the ITC profiles of OArg and OArg-PEG750 point towards equivalent interaction mechanisms.

Turning to the ITC results for the OArg-PEG2000 derivative (Fig. 6, bottom panel), it can be seen that PEGylation of the OArg with the higher molecular weight PEG chain results to a significant alteration of the ITC profile. Even though the ITC curve is once again V-shaped, it is characterized by a larger enthalpy change. Moreover, the ITC curve becomes sharper and shifts with respect to the molar ratio depending on the initial insulin concentration. This may be significant of the fact that the dissociation of the insulin dimers upon complexation, as described above, is important for the final ITC profile. The two-process analysis leads to following results: $\Delta H_{\text{end}} = 19.9 \pm 2.0 \text{ kcal/mol}$, $K_{\text{end}} = 1.5 \pm 0.61 \times 10^6 \text{ M}^{-1}$, $N_{\text{end}} = 0.11 \pm 0.1$ for the endothermic process; and $\Delta H_{\text{exo}} = -20.3 \pm 3.0 \text{ kcal/mol}$, $K_{\text{exo}} = 3.5 \pm 0.8 \times 10^7 \text{ M}^{-1}$, $N_{\text{exo}} = 0.27 \pm 0.3$ for the exothermic. These values indicate strong interaction between insulin and oligopeptide derivatives. For comparison, the binding constant of insulin with cationic protamine/insulin crystals at pH 7.5 is 15.5–37.7 $\times 10^5 \text{ M}^{-1}$ depending on buffer used [61], while the high affinity binding of the two first zinc ions to zinc-free insulin at pH 7.0 is $1.9 \times 10^6 \text{ M}^{-1}$ [62]. Moreover, the binding...
constants of insulin to amphiphilic polyesters having a positively charged polymer backbone were ranging from $4.68 \times 10^5$ M$^{-1}$ for low degree of amine substitution at the backbone, to $9.53 \times 10^6$ M$^{-1}$ for the higher degree of amine substitution at the backbone [21]. The additional PEG-dependent enthalpic contribution must be linked to the aggregation of insulin, via an interaction of their water-depleted surfaces. Therefore, according to the depletion attraction mechanism [51–54], it can be proposed that PEG affects OArg derivatives interaction by acting as a “glue”, triggering PEG/PEG and insulin/insulin interactions leading to the formation of the complexed nanoparticles that are detected by DLS.

### 3.2.5. Insulin complexation efficiency

The determination of insulin complexation efficiency as a function of oligopeptide derivative/insulin molar ratio employed was also studied since it is very important for the preparation of an effective insulin delivery system to exhibit high protein loading capacity. Insulin complexation efficiency expresses the amount of protein associated with the employed derivatives. High levels of insulin complexation efficiency was determined for all derivatives ranging from 99.5% to 99.8% at the relatively low molar ratio of 0.2 for OArg-PEG750 and OArg-PEG2000 and of 0.4 for OLys-PEG750 and OLys-PEG2000, corresponding to 0.1–0.2 mg of oligopeptide derivatives per 1 mg of insulin leading to insulin loading capacities from 83% for the OLys-PEG2000 to 93% for OArg-PEG750. Therefore, this molar ratio was used for enzymatic degradation and release studies. It should be noted that similar studies [13,18,21,31,63,64] concerning insulin interaction with positively charged polymers generally give complexation efficiencies of about 90% and up to 100% in the case of poly(L-lysine)-based hydrophilic star block co-polymer [65]. In addition, insulin loading capacity of about 55%, and up to 85% in the case of quaternized chitosan derivatives [21], was reported.

### 3.3. Insulin in vitro release profile

The release of insulin from the complexes was studied in enzyme-free simulated intestinal fluid (Fig. 7). When OLys-PEG750 was used for the complexation of insulin, a fast release rate was observed. Specifically, during the first 4 h, insulin was almost completely released from these complexes (~85%) at a steady rate, while in the case of OLys-PEG2000/insulin complex, ~60% of insulin was released within the first 3 h period. When OArg derivatives were employed, insulin was released at a significantly slower rate. Specifically, 40% and 30% of insulin was released at a steady rate from complexes with OArg-PEG750 and OArg-PEG2000, respectively, within the first 3 h, while a relatively low release of about 10% of insulin is observed during the next 3 h. It is evident that the insulin release in intestinal simulation fluid depends on the length of the PEG chains and on the presence of the strongly
interacting guanidinium groups on the oligopeptide backbone. More importantly, it is possible to control the release rate by modifying either the length of PEG and/or the type of positively charged amino acid residue of the oligopeptide scaffold.

Circular dichroism was employed to confirm that released insulin retains its conformation, based on the use of FUV-CD spectra as a fingerprint for the identification of various secondary structural elements [66]. CD spectra of insulin released from the complexes at the end of a 6 h period were recorded to monitor any conformational changes of insulin. FUV-CD spectra (Fig. 8) recorded in the range 200–250 nm, at 25 °C, display a minimum at 208 nm, attributed to the antiparallel β-structure of insulin, and a shoulder at about 223 nm which is characteristic of the α-helix. Any change in the secondary structure of insulin would be reflected as change in the intensity of the spectra in the vicinity of these peaks [20]. As clearly shown, the spectra of released insulin are identical to the original spectrum of insulin indicating that complexation with the oligopeptide derivatives does not significantly alter its conformation. It is interesting to note that the same observations were reported for insulin released from its complexes with cationic β-cyclodextrin polymers [13].

3.4. Enzymatic degradation studies

Protection of insulin against proteolytic enzymes was investigated in vitro by incubating the obtained oligopeptide derivative/insulin complexes in the presence of pancreatic enzymes such as trypsin and α-chymotrypsin. It is known that trypsin cleaves insulin at two specific sites, that is, at the carboxyl side of B29-Lys and B22-Arg residues, while α-chymotrypsin attacks the less accessible bonds of B15-Leu and A11-Cys residues located in the hydrophilic domain of insulin [33].

In the presence of trypsin, almost complete degradation of free insulin (control experiment) was observed after 60 min, while ~70% degradation of insulin was detected after a 2 h period when various PEGylated oligopeptide/insulin complexes were employed (Fig. 9A). This is attributed to the biodegradable nature of the oligopeptides used and to the fact that trypsin cleaves peptide chains at the carboxyl side of lysine or arginine. The degradation of complexed insulin is, however, observed at a considerably slower rate. Thus, OArg derivatives, especially the derivative with the higher PEG molecular weight, exhibit the slowest insulin degradation rates. Higher complexation efficiencies and, therefore, more stable complexes reduce the initial apparent degradation rates.

On the other hand, the oligopeptide derivatives are not hydrolyzed by α-chymotrypsin and, therefore, in this case enzymatic
4. Conclusions

Biodegradable PEGylated positively charged oligolysine and oligoarginine-type homopeptides interact spontaneously and efficiently with insulin at ambient temperature and physiological pH values, affording stable ionic complexes. The binding process and complex formation have been systematically studied for both types of oligopeptide derivatives. It was found that the PEGylated OArg yield the most stable complexes with insulin. Insulin association efficiency for all derivatives was found >99.5% at molar ratio of 0.2 for the OArg derivatives and of 0.4 for the OLys derivatives. FTIR spectra suggest interaction between the OH groups of tyrosine residue, most probably of the insulin’s B chain C-terminus, and the amino acid residues of OArg or OLys derivatives, respectively. In agreement with the spectroscopic findings, the ITC profiles show stronger interactions of the OArg derivatives with insulin compared to OLys. In all cases, the ITC results are complex revealing endothermic and exothermic contributions. The complex thermodynamic behavior may be attributed to the OArg-triggered dissociation of insulin dimers, evidence for which is also provided from CD spectroscopy results, and also to non-specific binding of the insulin dimers or possible conformational changes occurring during nanoparticle formation at high OArg-derivative/insulin molar ratios. The binding of OLys to insulin is weaker and is characterized by endothermic signals. The presence of PEG chains results in additional exothermic contribution, which is especially strong for PEG chains of high molecular weight. This is attributed to PEG-induced dehydration of insulin’s surface, generally known as the depletion attraction effect, resulting in water-depleted insulin surfaces that lead to protein aggregation and formation of stable nanoparticles.

Insulin release rates in intestinal simulation fluid were found to be strongly related to the length of PEG chains and to the presence of guanidinium end-groups. More importantly, it is possible to control the release rate by the presence of guanidinium groups on the oligopeptide scaffold and by modifying the molecular weight of PEG. Furthermore, the released insulin retains its native secondary structure as revealed by CD studies. The developed oligopeptide derivatives, being by themselves biodegradable as shown by the trypsin degradation results, offer stabilization of complexed insulin against enzymatic degradation by z-chymotrypsin. The presence of guanidinium end-groups and long PEG chains improves protection of insulin (up to 25% insulin degradation for a 3 h incubation time) due to strong complex formation and the resulting slower release rates.

Based on these results, stable complexes of PEGylated oligopeptides with insulin have significant potential to be further studied as a novel formulation for insulin delivery given the intestinal enzyme-degradable character of oligopeptides, the presence of arginine end-groups that are expected to enhance insulin intestinal absorption, slow insulin release, and improved protection of insulin against enzymatic degradation.

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Appendix A. Supplementary material

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