Physical PEGylation to prevent insulin fibrillation

Francesca Mastrotto, Federica Bellato, Valentina Andretto, Alessio Malfanti, Mariangela Garofalo, Stefano Salmaso, Paolo Caliceti.*

Department of Pharmaceutical and Pharmacological Sciences, University of Padova, via F. Marzolo 5, 35131 Padova – Italy.

***Corresponding author:**
Stefano Salmaso
Department of Pharmaceutical and Pharmacological Sciences,
University of Padova,
via F. Marzolo 5,
35131 Padova – Italy.
e-mail stefano.salmaso@unipd.it

Abstract

Insulin is one of the most marketed therapeutic proteins worldwide. However, its formulation suffers from fibrillation, which affects the long-term storage limiting the development of novel devices for sustained delivery including portable infusion devices. We have investigated the effect of physical PEGylation on structural and colloidal stability of insulin by using two PEGylating agents terminating with polycyclic hydrophobic moieties, Cholane and Cholesterol: $mPEG_{5kDa}$-Cholane and $mPEG_{5kDa}$-Cholesterol, respectively. Microcalorimetric analyses showed that $mPEG_{5kDa}$-Cholane and $mPEG_{5kDa}$-Cholesterol efficiently bind insulin with binding constants (Ka) of $3.98\ 10^4$ and $1.14\ 10^5$ M^{-1}, respectively. At room temperature, the two PEGylating agents yielded comparable structural stabilization of α-helix conformation and decreased dimerization of insulin. However, melting studies showed that $mPEG_{5kDa}$-Cholesterol has superior stabilizing effect of the protein conformation than $mPEG_{5kDa}$-Cholane. Furthermore, the fibrillation study showed that at a 1:1 and 1:5 insulin/polymer molar ratio $mPEG_{5kDa}$-Cholesterol delays insulin fibrillation 40 and 26% more efficiently, respectively, as compared to $mPEG_{5kDa}$-Cholane which was confirmed by TEM imaging. Insulin was released from the $mPEG_{5kDa}$-Cholane and $mPEG_{5kDa}$-Cholesterol assemblies with comparable kinetic profiles. The physical PEGylation has a beneficial effect on the stabilization and shielding of the insulin structure into the monomeric form, which is not prone to fibrillation and aggregation.

Keywords: physical PEGylation, insulin fibrillation, insulin stability.

Introduction

The in vivo structural rearrangement of physiologic proteins and peptides into ordered 3-dimensional fibrils is usually associated to the development of neurodegenerative diseases including Alzheimer's and Parkinson's diseases.[1,2] In vitro, therapeutic protein fibrillation occurs during manufacturing and storage which hampers the development and translation into biopharmaceutics.[3-5] Consequently, the identification of excipients and technological strategies that inhibit protein fibrillation is crucial to generate medicines that possess adequate stability and biopharmaceutical profile.

Insulin can easily undergo chemical degradation and physical denaturation during storage and delivery above room temperature, namely in intraperitoneal pumps.[6] For such a reason, insulin formulations are shipped and stored at 4–8 °C, and, when in use, must be stored below 30 °C and discarded in 30 days (15 days in the case of diluted solutions of Humalog® or Novolog®).

The physical denaturation of insulin that mostly occurs through fibrillation phenomena, is a complicate process difficult to be controlled.[6,7] The artificial modification of the amino acid sequence has been found to enhance the biopharmaceutical properties of this protein. Amino acid substitutions at the level of the flexible regions of insulin, which are sensitive to chemical degradation, have been found to enhance the protein stability.[7] Furthermore, the mutational destabilization of subunit interfaces within the insulin hexamer obtained by amino acid substitution has been found to enhance the protein absorption,[8-12] which results in enhanced bioavailability.[13,14] Nevertheless, insulin analogues used in portable devices such as Humalog® or Novolog® have been found to undergo fibrillation due to accelerated disassembly of the insulin physiological oligomers and misfolding, which yields pump occlusion.[13,15-17] Therefore, despite the modification of amino acid sequence generates insulin analogues with enhanced physicochemical stability and bioavailability, additional strategies are required to enhance the physical stability of these analogues. Post-translational chemical modifications such as glycosylation and covalent conjugation of hydrophilic polymers have been found to enhance the chemical and physical stability of insulin as well as its bioavailability.[18-20] However, chemical alterations in the protein structure, either by sequence modification or by conjugation, may reduce the insulin activity.[21]

Enhanced shelf-life of a liquid formulation of peptide/protein with poor physico-chemical stability can be achieved by using stabilizing excipients such as amino acids, sugars, buffer/pH and ionic strength.[22] Additionally, amphiphilic macromolecules, namely surfactants, are known to physically associate with several therapeutic proteins via electrostatic and/or hydrophobic interactions that prevent surface adsorption-induced denaturation and aggregation.[23-25] Accordingly, PEGs bearing terminal hydrophobic moieties have been used to formulate proteins and peptides including rhGH, GCSF and calcitonin.[26-28] A variety of reports from the literature showed that physical PEGylation can provide advantages over chemical PEGylation where covalent conjugation of the therapeutic peptide attenuates the binding affinity with the target through steric inhibition and/or associated structural changes.[29] Furthermore, physical PEGylation can circumvent the need for additional, usually complicate, chemistry, or the introduction of amino acids conducive to conjugation (e.g. lysine).[30]

Recently, we physically PEGylated a vasoactive intestinal peptide (VIP) mutant that was chemically derivatized with a palmitoyl moiety (VIP-palm). The functionalization with the palmitoyl moiety was aimed at promoting the binding to the hydrophobic pockets of human serum albumin (HSA) and consequently increase the *in vivo* half-life.[31] However, the palmitoylation was found to induce the VIP-palm gelation and fibrillation under acidic conditions.[32,33] The terminal hydrophobic moiety of

$mPEG_{5kDa}$-Cholane was found to associate with the lipid moiety of VIP-palm[33] and remarkably slowing the peptide aggregation/fibrillation.

The present work aims at elucidating the ability of two amphiphilic PEG based polymers, namely $mPEG_{5kDa}$-Cholane and $mPEG_{5kDa}$-Cholesterol, to physically associate to insulin and inhibit the protein aggregation and fibrillation. For the first time, we proved that these PEGylation technologies can conformationally stabilize a non-mutated and non-post-translationally modified polypeptide, i.e. insulin, and prevent its spontaneous fibrillation.

Materials

Linear 5 kDa monoamino-monomethoxy-polyethylene glycol ($mPEG_{5kDa}$-NH_2) was obtained from Iris Biotech GmbH (Marktredwitz, Germany). Bovin insulin (MW 5733.49 Da), 5β-cholanic, cholesterol, Triethylamine (TEA), trinitrobenzensufonic acid (TNBS), thionyl chloride (SO_2Cl) and all reagents and solvents of analytical grade were purchased from Aldrich (St. Louis, MO, USA).

Methods

Solubility studies.

Insulin solubility at different pHs. The solubility of insulin at pH 2.0, 5.3 and pH 7.4 was preliminary assessed. Fifty mg of insulin was dispersed in 900 μL of 0.01 M HCl, 140 mM NaCl at 25 °C and gently mixed at 25 °C for one hour. The suspension was split in 3 samples (A, B and C) of 300 μL each and added of: A. 50 μL of the same medium; B. 50 μL of 140 mM NaCl, 0.2 M sodium phosphate, pH 5.3; C. 50 μL of 140 mM NaCl, 0.2 M sodium phosphate, pH 7.4. The resulting suspensions were centrifuged at 13,000 rpm for 3 minutes and concentration was assessed by HPLC using a Jasco AS-1555 System (Tokyo, Japan), equipped with two PU-2080plus pumps, a detector UV-2075plus, one FP-2020plus and an analytic column Jupiter (C18, 5 μm, 300 Å, 250 x 4.6 mm) from Phenomenex (Torrence, USA), eluted with water (eluent A) and acetonitrile (eluent B), both containing 0.05% of TFA, in a gradient mode: 16 min from 10% to 95% eluent B. The UV detector was set at 280 nm and the flow rate at 1 mL/min. The insulin concentration in each sample was derived from a calibration line obtained from insulin dilutions in 0.01 M HCl from 0.01 mg/mL (1.74 μM) to 0.9 mg/mL (156.97 μM). The dilutions were generated from an insulin stock solution in 10 mM HCl whose concentration was assessed by spectrophotometric analysis.[34,35]

Insulin solubility with amphiphilic polymers. 150 μL of 10 mg/mL insulin solution in 0.01 M HCl, 140 mM NaCl was mixed with 120 μL of $mPEG_{5kDa}$-Cholane or a $mPEG_{5kDa}$-Cholesterol solutions in 0.01 M HCl, 140 mM NaCl at different concentrations (range 0-12.5 mM) and 30 μL of a 140 mM NaCl, 0.2 M sodium phosphate, pH 5.3. The samples were gently mixed for 1 minute and then

centrifuged at 13,000 rpm for 3 minutes and insulin concentration was quantified by RP-HPLC as reported above.

Microcalorimetric studies.

Isothermal titration calorimetry (ITC) was carried out to assess insulin/polymer association using a MSC-ITC equipment (Microcal Inc. Northampton, MA). A 1.5 mL volume of 15 μM insulin solution in 140 mM NaCl, 20 mM sodium phosphate, pH 7.4 was transferred into the sample cell while the reference cell was loaded with 1.5 mL of the same buffer. Insulin was titrated by injection of a 2.77 mM solutions of either $mPEG_{5kDa}$-Cholane, or $mPEG_{5kDa}$-Cholesterol or $mPEG_{5kDa}$-OH in the same buffer. The analyses were performed at 25 °C, with a stirring speed syringe of 351 rpm, by 33 sequential injections of 7 μL polymers solutions of 10 s at 5 min intervals in the sample cell. All measurements were replicated three times and data processing was performed with the Microcal Origin 3.5 software. "Blank titrations" were performed by injecting the polymer solutions into the sample cell containing plain buffer without protein and by injecting plain buffer volumes (without polymers) into the sample cell containing the protein solution. The thermograms obtained by polymer titration of the protein were subtracted of "blank titration" thermograms. The calorimetric raw data were elaborated using the software ORIGIN 7 (MicroCal, LCC ITC) to calculate the stoichiometry of binding interaction, the association constant, the enthalpy and entropy associated to the polymer/insulin association.

Dynamic light scattering

A 113 μL volume of 15 mg/mL polymer ($mPEG_{5kDa}$-Cholane or $mPEG_{5kDa}$-Cholesterol) solution in 0.01 M HCl, 140 mM NaCl were mixed with 40 μL of 1 mg/mL solution insulin in 0.01 M HCl, 140 mM NaCl in order to obtain a 1:45 protein/polymer molar ratio (m.r.) and the pH was shifted to 7.4 by adding 20 μL of 0.2 M phosphate, 140 mM NaCl, pH 7.4. The volume was brought to 200 μL with the same buffer. Solutions of the amphiphilic polymers without insulin were prepared in buffer as controls. The samples were analysed by dynamic light scattering (DLS) using a Malvern Zetasizer Nano S photon correlation spectrometer. The samples were equilibrated at 25 °C for 300 s before analyses. The size of the assemblies was derived as intensity.

Release studies

1.6 mL samples containing 43 μM insulin and 1.94 mM of $mPEG_{5kDa}$-Cholane or $mPEG_{5kDa}$-Cholesterol were prepared from a 0.5 mg/mL (86 μM) stock solution of insulin and 30 mg/mL stock solutions of the polymers in 0.01 M HCl, 140 mM NaCl and adding 160 μL of 0.2 M phosphate and

140 mM NaCl pH 7.4. Control solution of insulin at the same concentration was generated by adding the volume of 0.01 M HCl, 140 mM NaCl instead of the polymer stock solution. The solutions were transferred into a 20 kDa cut-off dialysis Float-A-Lyzer (Spectrum Laboratories, Los Angeles, CA, USA) and dialyzed against 2 L of 20 mM phosphate, 140 mM NaCl, pH 7.4 at room temperature. At scheduled times, 30 μL of the samples inside the dialysis device were withdrawn and analysed by RP-HPLC to quantify the residual insulin according to the method reported above.

Insulin conformational studies

Circular dichroism of insulin and insulin/polymer assemblies at different pHs. A 1 mg/mL insulin stock solution in 0.01 M HCl, 140 mM NaCl was prepared. 400 μL insulin/polymer mixtures at 15 μM insulin concentration were prepared at different pHs by adding 35 μL of the insulin stock solution to appropriate volumes of 10 mg/mL $mPEG_{5kDa}$-Cholane or $mPEG_{5kDa}$-Cholesterol solutions in 0.01 M HCl, 140 mM NaCl, and 40 μL of 200 mM phosphate, 140 mM NaCl, pH 5.3 or pH 7.4 to obtain final polymer concentrations of 15 μM (1:1 insulin/polymer m.r.) or 0.675 mM (1:45 insulin/polymer m.r.). Control solution of insulin was prepared as reported above without polymers. Solutions of polymers at same concentrations without insulin were also prepared to quantify the contribution of polymer alone to the circular dicrographic (CD) profile. Samples were analysed in triplicate using a quartz cuvette with optical path of 1 cm and CD spectra were recorded in the 195-260 nm range at 25 °C. The CDNN deconvolution software developed by Gerald Bohm of University of Halle (Germany) was used to process the data.

Melting point of insulin and insulin/polymer assemblies. A 1 mL volume of 1:45 insulin/polymer MR assemblies at 15 μM insulin concentration in 0.01 M HCl, 140 mM NaCl were prepared as reported above. Each sample was analysed by circular dichroism under 1 degree/min stepwise increase of temperature from 25 to 90 °C. Circular dicrographic spectra were recorded every 5 °C increase in the 195-260 nm UV spectrum range using a quartz cuvette with optical path of 1 cm. The CD spectra were recorded in triplicate as reported above. Insulin alone (no polymer) was tested as control.

Circular dichrographic analysis after prolonged heating stress. A 1 mL volume of 1:45 insulin/polymer molar ratio assemblies at 15 μM insulin concentration in 0.01 M HCl, 140 mM NaCl were prepared as reported above and incubated in a Thermoblock (Eppendorf Thermomixer R, New York, NY-USA) at 50 °C for 72 hours. CD spectra of samples were recorded in triplicate as reported above. Insulin alone (no polymer) was tested as control.

Fibrillation studies

Effect of pH, temperature and concentration on insulin fibrillation. A 90 μL volume of a 2 mg/mL insulin stock solution in 0.01 M HCl, 140 mM NaCl was added of 198 μL of 0.01 M HCl, 140 mM NaCl followed by 36 μL of 0.01 M HCl, 140 mM NaCl or 0.2 M phosphate, 140 mM NaCl at pH 5.3 or 7.4 and 36 μL of a 500 mM ThT in 0.01 M HCl, 140 mM NaCl. 0.01 M HCl, 140 mM NaCl. The samples were transferred on wells of a Corning 96-well half bottom plate (Corning 3381, Flintshire, U.K.), sealed with a Costar aluminum foil, and incubated at 25, 37 and 50 °C, without agitation. Insulin fibrillation was monitored by a Vicktor X3 plate reader by recording the fluorescence at 480 nm every 35 minutes for 92 hours. The analyses were done in triplicate.

Volumes of 90, 135, 180 μL of a 2 mg/mL insulin solution in 0.01 M HCl, 140 mM NaCl were brought to 324 μL with the same medium resulting in 0.5, 0.75 and 1 mg/mL insulin. Samples were added of 36 μL of a 500 μM ThT solution in 0.01 M HCl, 140 mM NaCl and transferred on wells of a Corning 96-well half bottom plate (Corning 3381, Flintshire, U.K.), sealed with a Costar aluminium foil, and incubated at 25, 37 and 50 °C, without agitation. Every 35 min for 92 h, the samples underwent fluorescence analysis using a Viktor X3 (PerkinElmer Italia - MI) plate reader (λ_{ex} = 440 nm, λ_{em} = 480 nm). The analyses were done in triplicate. The data were analysed using WorkOut 2.5 and GraphPad Prism 4.0 software, applying the least-squares fitting method and the following equation to derive the fibrillation "lag time":

$$Y = y_0 + y_f/(1 + exp(- (t-t_0)K))$$

where Y is the detected fluorescence, y_0 is the fluorescence at time 0, and K is the apparent rate constant for the growth of the fibril corresponding to $1/\tau$.[36] The lag time was derived from t_0 and K as follows:

$$\text{Lag time} = t_0 - (2/K) = t_0 - 2\tau$$

Fibrillation of insulin with mPEG$_{5kDa}$-Cholane and mPEG$_{5kDa}$-Cholesterol. A 2 mg/mL insulin solution was prepared as described previously in 0.01 HCl, 140 mM NaCl. 50 mg/mL mPEG$_{5kDa}$-Cholane and mPEG$_{5kDa}$-Cholesterol solutions were prepared in the same medium. Volumes of polymer stock solutions were added to 90 μL of insulin stock solution followed by 36 μL of 0.01 M HCl, 140 mM NaCl or 0.2 M phosphate, 140 mM NaCl at pH 5.3 or 7.4 and 36 μL of a 500 mM ThT in 0.01 M HCl, 140 mM NaCl to obtain a final insulin concentration of 0.5 mg/mL (87 μM) and 87 μM, 0.44 mM and 0.87 mM polymer concentration yielding 1:1, 1:5 and 1:10 insulin/polymer m.r.,

respectively, and a final volume of 360 μL. The samples were transferred on wells of a Corning 96-well half bottom plate (Corning 3381, Flintshire, U.K.), sealed with a Costar aluminium foil, and incubated at 37 and 50 °C, without agitation. Insulin fibrillation was monitored as described above.

Transmission electron microscopy (TEM)

A 2 mg/mL insulin solution was prepared as described previously in 0.01 HCl, 140 mM NaCl. Solutions of $mPEG_{5kDa}$-Cholane and $mPEG_{5kDa}$-Cholesterol at 50 mg/mL were prepared in the same medium. Volumes of $mPEG_{5kDa}$-Cholane or $mPEG_{5kDa}$-Cholesterol solution were added to 90 μL of insulin stock solution in order to achieve a 0.5 mg/mL (87 μM) insulin concentration and 87 μM (1:1 insulin/polymer m.r.) or 0.87 mM (1:10 insulin/polymer m.r.) polymer concentration and total volume of 360 μL. Control samples containing 0.5 mg/mL insulin were prepared with the same procedure without polymers. Samples at pH 7.4 were prepare with the same procedure by using the same stock solutions of insulin and polymers and adding 36 μL of 0.2 M phosphate, 140 mM NaCl, pH 7.4 for pH-shift.

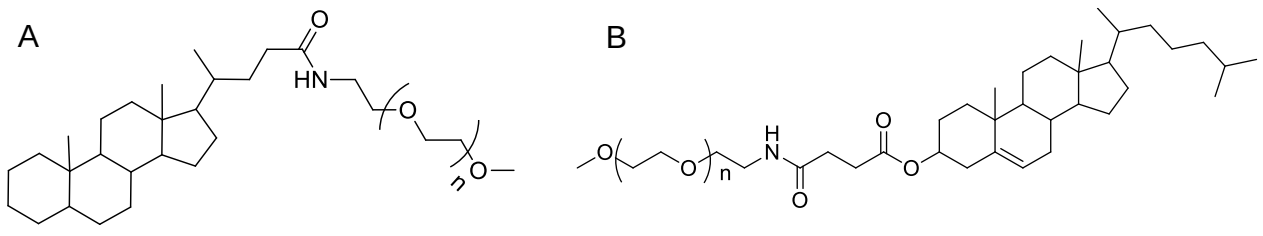
Samples were imaged immediately after their formulation or after incubation at 50 °C for 72 hours. For TEM imaging, samples were transferred on copper grids (400 mesh), stained with a 1% (w/v) uranyl acetate solution, and covered with a punctate carbon layer. The samples were analysed by TEM in negative staining mode using a Tecnai G2 microscope (FEI).

Results and discussion

Insulin fibrillation represents one of the main problems in the development of sustained delivery systems including polymeric matrices and infusion pumps[37] and it can also take place in vivo after insulin infusion or injection.[38,39] Insulin fibrillation is detrimental for its biological activity since this protein displays its activity by monomer binding to surface cell receptors. In addition, pre-fibrillar oligomers and fibrils are reported to be cytotoxic or immunogenic.[40] In order to increase the protein stability and minimize the fibrillation of insulin in solution for application in infusion pumps, we have explored its combination with two amphiphilic PEG based polymers, namely $mPEG_{5kDa}$-Cholane and $mPEG_{5kDa}$-Cholesterol.

Amphiphilic polymer synthesis

Scheme 1 reports the structure of the two mPEG derivatives, $mPEG_{5kDa}$-Cholane and $mPEG_{5kDa}$-Cholesterol used for the physical PEGylation of insulin.



Scheme 1. Structure of $mPEG_{5kDa}$-Cholane (A) and $mPEG_{5kDa}$-Cholesterol (B).

$mPEG_{5kDa}$-Cholane was synthesised according to a simple and reproducible protocol reported in the literature.[26] The ^{1}H-NMR, the Snyder's assay used to quantify non reacted amino group of $mPEG_{5kDa}$-NH_2, and mass spectrometry analyses (Figure SI-1A, Supporting Information) showed that the conjugation yield of the cholane moiety to the terminal amino group of $mPEG_{5kDa}$-NH_2 was 97% and the final product had bell shape typical of the poly-disperse PEG with signal centered at 5400 Da m/z (Figure SI-1B).

$mPEG_{5kDa}$-Cholesterol was synthesised according to a three step-procedure: (i) synthesis of cholesteryl hemisuccinate by reacting cholesterol with succinic anhydride; (ii) conversion of the terminal carboxylic acid of cholesteryl hemisuccinate to acyl-chloride; (iii) conjugation of the acyl chloride to $mPEG_{5kDa}$-NH_2. The cholesterol hemisuccinylation yield calculated by the ratio of the ^{1}H-NMR signal of cholesterol and hemisuccinate moiety was found to be 98%. The ESI-TOF mass analysis (Figure SI-2) performed in negative mode provided a 485.37 Da m/z signal, which is in agreement with the calculated molecular weight of 486.37 Da of this derivative. The cholesteryl hemisuccinate conversion to acyl chloride (II) determined by ^{1}H-NMR analysis (Figure SI-3), after reaction with methanol, was found to be 96.7%. The ^{1}H-NMR analysis of the conjugate $mPEG_{5kDa}$-Cholesterol (Figure SI-4A) obtained by cholesteryl hemisuccinate chloride with $mPEG_{5kDa}$-NH_2 showed that the conjugation yield was 98%, which was in fair agreement with the results obtained by the Snyder's assay (94.7%). The mass analysis of the $mPEG_{5kDa}$-Cholesterol conjugate (Figure SI-4B) showed a bell-shaped m/z dispersion cantered at 5560 Da m/z confirming the expected molecular weight of the conjugate. The dynamic light scattering analysis (Figure SI-5) showed that $mPEG_{5kDa}$-Cholesterol self-assembles into colloidal structures with critical micelle concentration (CMC) of 40.7±5.0 μM, which is higher than CMC of $mPEG_{5kDa}$-Cholane (11.2 ± 5.0) previously calculated.[32] In this regard, it is important to note that $mPEG_{5kDa}$-Cholane terminates with the polycyclic unit [tetradecahydro-1*H*-cyclopenta(a)phenanthrene] of the hydrophobic moiety while in $mPEG_{5kDa}$-Cholesterol terminates with the linear chain of cholesterol [dimethyl-(2*R*)-6-methylheptan]. These

differences seem to have a strong impact on the association processes. The different CMC values seem to indicate that in the case of $mPEG_{5kDa}$-Cholesterol the polycyclic moieties strongly associate while the terminal alkyl fraction of $mPEG_{5kDa}$-Cholesterol slightly reduces the association of the polycyclic units.

Insulin solubility studies.

A solubility study was performed to investigate the capacity of $mPEG_{5kDa}$-Cholesterol and $mPEG_{5kDa}$-Cholane to interact with insulin. Preliminary studies showed that insulin solubility was 13.5, 0.87 and 5 mg/mL at pH 2.0, pH 5.3 (corresponding to its isoelectric point) and pH 7.4 (physiological pH), respectively, which is in fair agreement with the pH dependent solubility reported in the literature.[41] Accordingly, the study was performed at pH 5.3, at which the protein has the lowest solubility.

The results described in

Figure 1 show that both polymers efficiently increase the insulin solubility. Notably, insulin dissolution profiles with the two polymers are fairly overlapping, which indicates that the two polymers possess comparable solubilizing properties for this biomolecule.

At a $mPEG_{5kDa}$-Cholesterol and $mPEG_{5kDa}$-Cholane concentration of 5 mM, the insulin solubility increased of 76% and 86%, respectively. By elaborating the solubility profiles, the of lowest number of polymer chains required to solubilize one molecule of insulin was found to be 45.

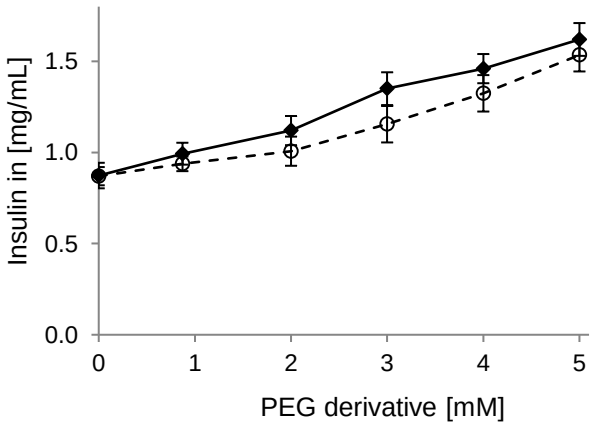


Figure 1. Solubility increase of insulin in the presence of $mPEG_{5kDa}$-Cholane (♦) and $mPEG_{5kDa}$-Cholesterol (○) with respect to insulin alone at pH 5.3.

Microcalorimetric studies.

Microcalorimetric analyses were performed to derive quantitative information of the binding of the two amphiphilic polymers with insulin. The analyses were performed at pH 7.4 to avoid technical limitations due to the low solubility of insulin, which may result in precipitation in the titration chamber, and to determine the polymer/protein association under physiological conditions.

The isothermal titration calorimetry (ITC) diagrams reported in Figure 2 show that $mPEG_{5kDa}$-OH does not associate with the protein while both mPEG derivatives associate with insulin indicating that associations are mediated by the terminal hydrophobic moiety (cholane or cholestrol). Under the analytical conditions, the protein/polymer interaction occurred according to a monomodal exothermal process where the injection of the polymer into the cell containing the protein induced heat release (siΔH), which constantly decreased throughout the titration.

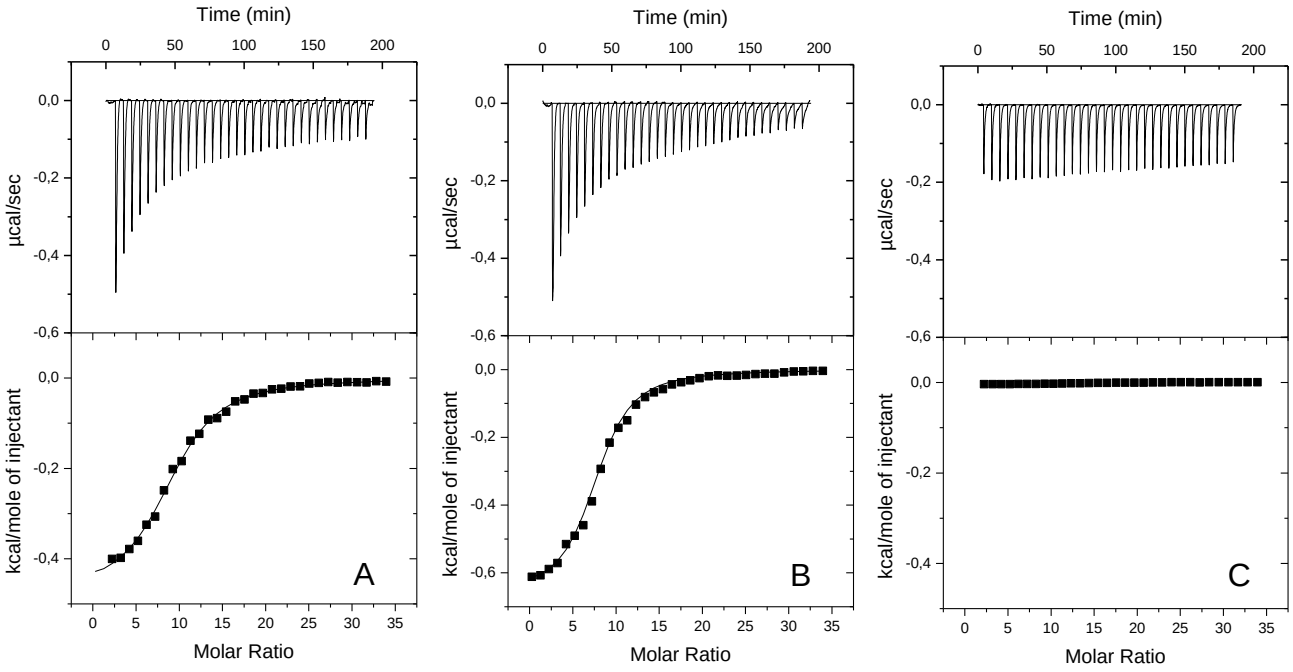


Figure 2. Isothermal titration calorimetric profiles of insulin/polymer association. The microcalorimeter cell was loaded with a 14 µM insulin solution and the syringe with a 2.77 mM $mPEG_{5kDa}$-Cholane (Figure A), or $mPEG_{5kDa}$-Cholesterol (Figure B) or $mPEG_{5kDa}$-OH (Figure C) in 20 mM phosphate, 140 mM NaCl, pH 7.4. The upper panels show the profile of calories involved at

each injection; the bottom panels show the profile of the normalized molar heat subtracted of the polymer dilution contribution.

The calorimetric data were found to fit a one set binding site elaboration model. The association parameters reported in Table I show that in both cases the association was driven by overall exothermic (-ΔH) and entropic (+ΔS) processes, which resulted in similar Gibbs free energy change (ΔG).

The number of association sites obtained by ITC was similar for $mPEG_{5kDa}$-Cholane and $mPEG_{5kDa}$-Cholesterol.

The association constant (Ka) values show that $mPEG_{5kDa}$-Cholesterol has higher affinity for the protein with respect to $mPEG_{5kDa}$-Cholane indicating that cholesterol has structure and conformation that better fit the binding sites of insulin for association. Considering the CMC values of the two polymers we hypothesised that the cholane orientation in the $mPEG_{5kDa}$-Cholane favours the interaction with cyclic structures on protein surface while the cholesterol moiety orientation in the $mPEG_{5kDa}$-Cholesterol that exposes the alkyl chain favours the interaction with planar non-polar surfaces such as the β-sheet structures. However, it should be noted that the Ka obtained with the polymers is similar to the Ka of insulin dimerization ($2.22 \ 10^5 \ M^{-1}$) indicating that the polymer can compete with this process.[42]

Table I. Association parameters of insulin with $mPEG_{5kDa}$-Cholane and $mPEG_{5kDa}$-Cholesterol derived from ITC measurements: number of association sites of insulin for the polymers (N), association constant (Ka), association enthalpy (ΔH), association entropy (ΔS), Gibbs free energy (ΔG).

	$mPEG_{5kDa}$-Cholane	$mPEG_{5kDa}$-Cholesterol
N	7.41 ± 0.15	7.75 ± 0.12
K [M^{-1}]	$3.98 \pm 0.30 \ 10^4$	$1.14 \pm 0.13 \ 10^5$
ΔH [cal / mol]	-606.0 ± 16.7	-664.4 ± 14.1
ΔS [cal / (mol K)]	19.0	20.9
ΔG [cal/mol]	-5058.85 cal/mol	-5566.94 cal/mol

Size of insulin/polymer assemblies

Dynamic light scattering (DLS) analyses summarized in Figure 3 show that the hydrodynamic size of the insulin/polymer assemblies are similar to the size of micelles obtained with polymer alone. Insulin/mPEG$_{5kDa}$-Cholesterol assemblies and mPEG$_{5kDa}$-Cholesterol micelles have sizes of 18.15±0.26 nm and 18.23±0.26 nm, respectively, which are slightly bigger than the insulin/mPEG$_{5kDa}$-Cholane assemblies (16.05±0.63 nm) mPEG$_{5kDa}$-Cholane micelles (16.29±0.57 nm). These differences seem to confirm that by virtue of the different orientation of cholane and cholesterol attached to mPEG-NH_2, the two hydrophobic moieties interact with the protein in a different way.

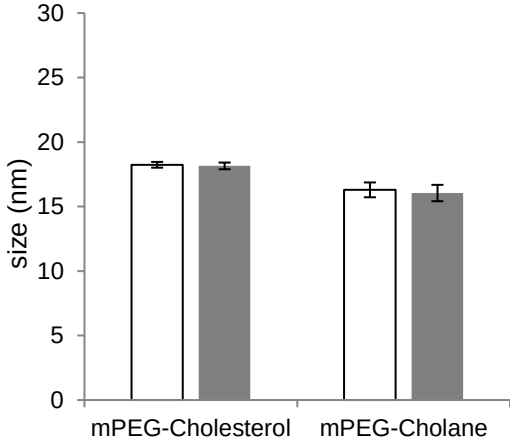


Figure 3. Hydrodynamic diameters ("size") of mPEG$_{5kDa}$-Cholesterol and mPEG$_{5kDa}$-Cholane micelles without (□) and with (■) insulin in 20 mM phosphate, 140 mM NaCl, pH 7.4. 1:45 insulin/polymer molar ratios were used.

Release studies.

A release study under physiologic mimicking conditions (pH 7.4) was performed using a dialysis membrane with 20 kDa cut-off to investigate whether the polymer association controls the protein availability.

Figure 4 shows that insulin formulated with the amphiphilic polymers is completely released from the dialysis device in 16 hours, despite the calorimetric and light scattering analyses showed that the polymers interact with the protein to yield large assemblies with size above the dialysis membrane cut-off. Unexpectedly, non-formulated insulin (polymer-free formulation) was released in 32 hours.

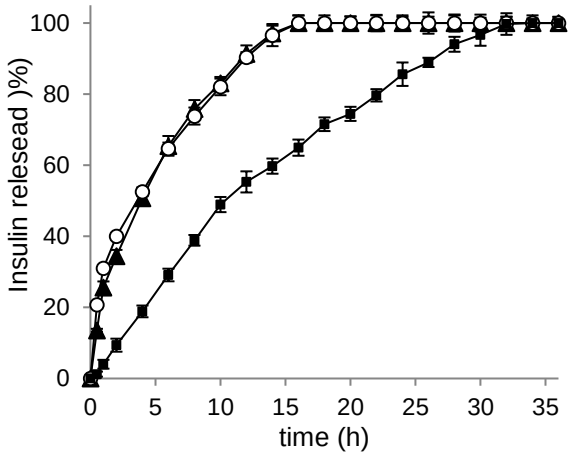


Figure 4. Insulin release profile in the presence of mPEG$_{5kDa}$-Cholane associate (▲) and mPEG$_{5kDa}$-Cholesterol (○) and in the absence of polymers (■), in 20 mM phosphate, 140 mM NaCl, pH 7.4. A 20 kDa membrane MWCO cut-off was used.

The faster release of insulin formulated with mPEG$_{5kDa}$-Cholane and mPEG$_{5kDa}$-Cholesterol with respect to non-formulated insulin may be ascribed to the ability of the amphiphilic polymers to maintain the monomeric state of the protein (hydrodynamic size 11.6 Å) and prevent the multimeric association up to hexameric assemblies (hydrodynamic size of 19.8 Å), which spontaneously occurs in buffer at pH 7.4.[37] Therefore, insulin formulated with the polymers rapidly diffuses through the dialysis membrane meanwhile it is released from the assemblies; on the contrary, non-formulated insulin undergoes self-association, which increases the size and consequently reduces the dialysis process.

Conformational studies

The effect of the insulin/polymer association on the structural conformation of insulin was investigated by near UV circular dichroism. The structural content of the protein was obtained by the elaboration of the dichroic spectra registered at the three pHs used in the solubility studies (pH 2.0, 5.3 and 7.4; Figure SI-6) and 1:0, 1:1 and 1:45 insulin/polymer molar ratio (Figure SI-7).

The results reported in Figure 5 show that insulin in buffer possesses mainly α-helix and random coil conformation with slight differences at the various pH, which is in agreement with the data reported in the literature.[37,43] The slight decrease of the α-helix content as the pH increases (41.7%, to 38.4% and to 36.7% at pH 2, pH 5.3 and pH 7.4, respectively) is compensated by an increase of the random coil content (45.9%, 47.7%, 48.1 % at pH 2.0, 5.3, 7.4, respectively) and the β-sheet content (12.4%, 13.8%, 15.2 % at pH 2.0, 5.3 and 7.4, respectively).

Notably, at all the investigated pHs, both PEG derivatives induce an increase of α-helix content of insulin. The α-helix content increase in the 1:45 protein/polymer assemblies was in the range of 15-18% for both polymers, which was accompanied by a slight decrease of random coil conformation (about 2-6%) and a remarkable decrease of β-sheet conformation (32-43%).

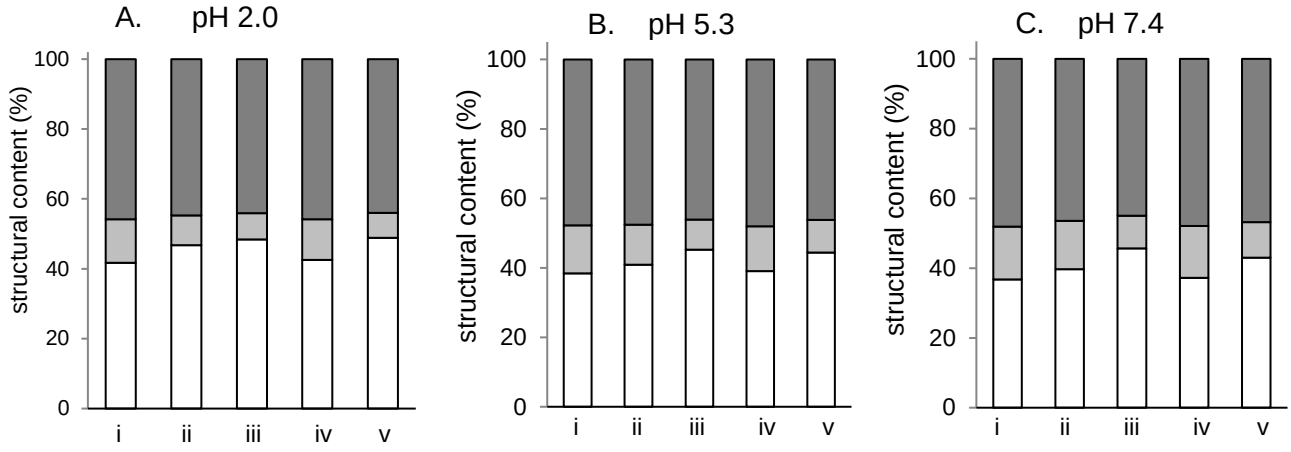


Figure 5. Structural content of α-helix (white), β-sheet (light grey) and random coil (dark grey) at pH 2.0 (A), 5.3 (B) and 7.4 (C) of free insulin (i), 1:1 (ii) and 1:45 (iii) insulin/mPEG$_{5kDa}$-Cholane molar ratio, and 1:1 (iv) and 1:45 (v) insulin/mPEG$_{5kDa}$-Cholesterol molar ratio.

The prevention of β-sheet formation and the increase of the α-helix content in the presence of the two amphiphilic polymers can be beneficial for the colloidal stability of insulin, and is in agreement with the evidences reported in the literature that β-sheet breaking agents decrease the fibrillation of peptides such as synuclein.[44] According to the study reported by Goldman et al., the $[\theta]_{208}/[\theta]_{222}$ ratio obtained from the CD spectrum of insulin can be used to estimate the self-association state of the protein. Indeed, the band at 222 nm is mainly attributable to β conformation, which is predominant in the dimer, while the band at 208 nm is correlated to the α-helix conformation, which is a characteristic of the monomer. The results reported in Table II show that under all pH conditions the $[\theta]_{208}/[\theta]_{223}$ ratio is higher in the presence of the polymers with respect to the non-formulated insulin indicating that insulin is prevalently in the monomeric state. Thus, we can conclude that the increase of the α-helix content promoted by the amphiphilic polymers may reduce the insulin dimerization and hexamerisation.

Table II. $[\theta]_{208}/[\theta]_{223}$ molar ellipticity ratio of 15 μM insulin solution, insulin/$mPEG_{5kDa}$-Cholane and insulin/$mPEG_{5kDa}$-Cholesterol assemblies at equimolar protein concentration at 1:1 and 1:45 insulin/polymer molar ratios at different pHs.

	$[\theta]_{208}/[\theta]_{223}$		
	pH 2	pH 5.3	pH 7.4
Insulin	1.35	1.24	1.16
Insulin/$mPEG_{5kDa}$-Cholane (m.r. 1:1)	1.53	1.33	1.24
Insulin/$mPEG_{5kDa}$-Cholane (m.r. 1:45)	1.60	1.57	1.50
Insulin/PEG_{5kDa} -Cholesterol (m.r. 1:1)	1.47	1.26	1.20
Insulin/PEG_{5kDa}-Cholesterol (m.r. 1:45)	1.67	1.58	1.54

The thermal stability of insulin was assessed by circular dichroism at pH 2.0 (Figure SI-8A), which is the worst condition for insulin stability as it favours the fibrillation process.[8,37,45]

Figure 6A shows that as the temperature rises from 25 °C to 90 °C the α-helix content of non-formulated insulin constantly decreases from 41.7 % to 22.6 % while the β-sheet and random coil content increases from 12.4 % to 22.4 % and from 45.9% to 55.1%, respectively. Notably, in the presence of $mPEG_{5kDa}$-Cholane and $mPEG_{5kDa}$-Cholesterol, the structural alterations of insulin under temperature increase are remarkably reduced (Figure SI-8B and C). Figure 6B shows that in the presence of $mPEG_{5kDa}$-Cholane, the insulin secondary structure remains almost unchanged up to 40 °C. As the temperature increases from 25 °C to 90 °C the α-helix content decreases from 48.4% to 29.3% and the β structure and random coil contents increase from 7.4% to 19.7% and from 44.2% to 51.6%, respectively. Figure 6C shows that $mPEG_{5kDa}$-Cholesterol has higher stabilizing effect on insulin than $mPEG_{5kDa}$-Cholane. Indeed, insulin conformation is unchanged up to 50 °C and as the temperature rises from 25 °C to 90 °C the α-helix content decreases from the 48.8 to 32.2 % while β structure and random coil increase from 7.1% to 15.5% and from 43.9 % to 52.3%, respectively.

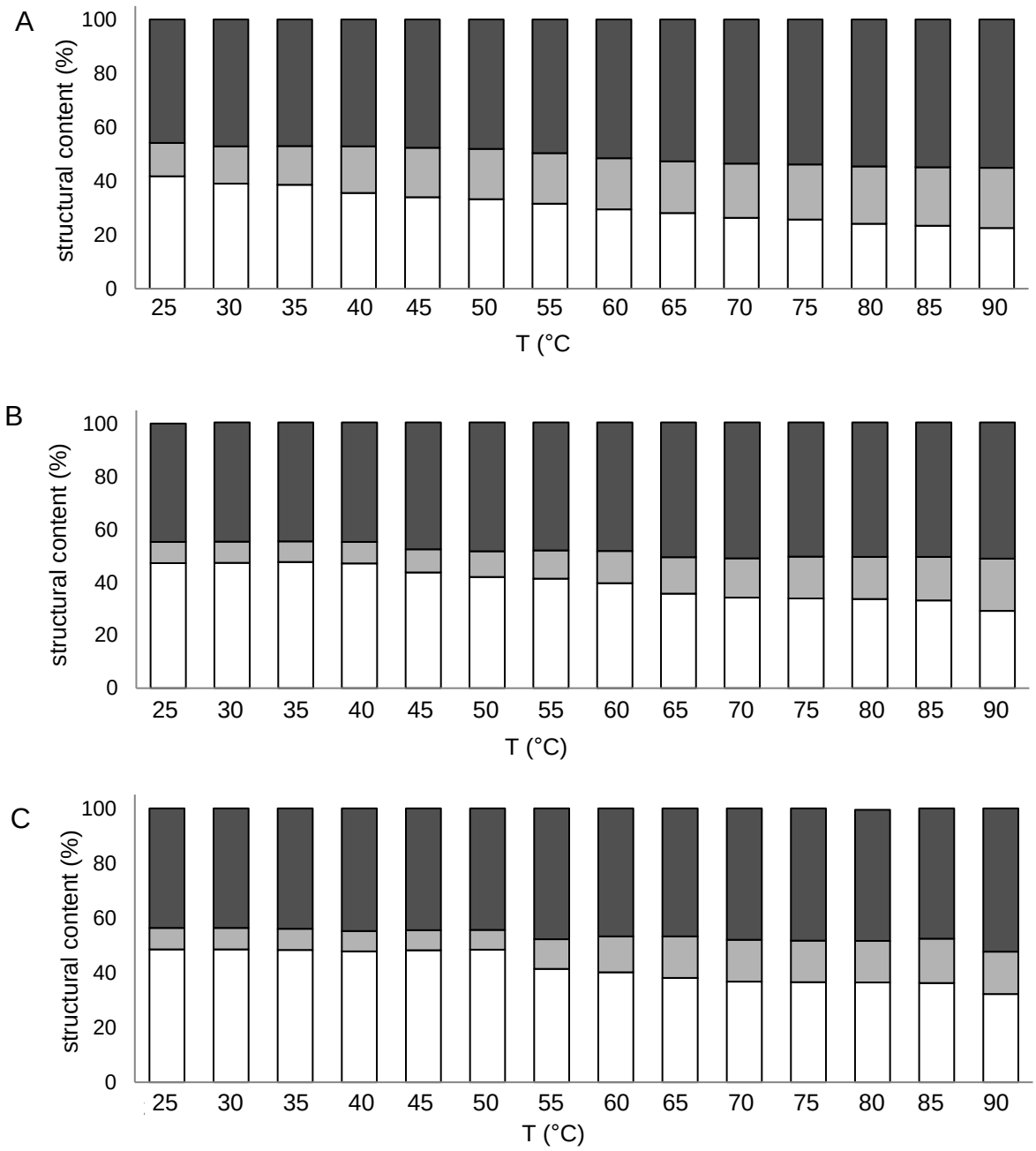


Figure 6. Structural content (%) of α-helix (white), β-sheet (light grey) and random coil (dark grey) of insulin at pH 2.0 exposed to stepwise temperature increase from 25 to 90 °C: A. non-formulated insulin; B. 1:45 insulin/PEG_{5kDa}-Cholane assemblies; C. 1:45 insulin/PEG_{5kDa}-Cholesterol.

The beneficial effect of protein/polymer association on insulin stability was further confirmed by long term stability studies. Figure 7 shows that after 72 h incubation at 50 °C the α-helix content of non-formulated insulin decreases from 41.7% to 11.3 %, and the β-structure and the random conformation increase from 12.4% to 28.4% and from 45.8% to 60.3%, respectively. On the contrary, the insulin conformation is substantially unaltered when associated with $mPEG_{5kDa}$-Cholesterol while negligible alterations have been observed with $mPEG_{5kDa}$-Cholane.

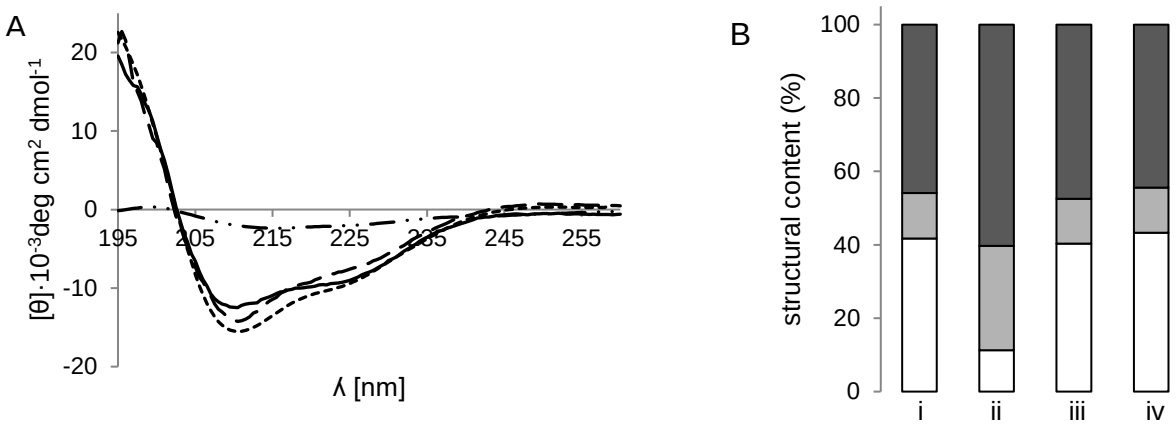


Figure 7. Circular dichroism spectra (A) and α-helix (white), β-sheet (light grey) and random coil (dark grey) content (B) of: non-formulated insulin at pH 2.0 (—, i); non-formulated insulin after 72 h incubation at pH 2.0 and 50 °C (· · — · ·, ii); 1:45 insulin/PEG_{5kDa}-Cholane molar ratio after 72 h incubation at pH 2.0 and 50 °C (– – – , iii); 1:45 insulin/$mPEG_{5kDa}$-Cholesterol molar ratio after 72 h incubation at pH 2.0 and 50 °C (-------, iv).

Fibrillation studies

ThT fibrillation assays were performed under stressing conditions, pH 2.0 and 5.3 at 50 °C, in order to accelerate this process (Figure SI-10-13). Indeed, acidic pH and high temperature are two major triggers for insulin fibrillation.

The fibrillation profiles reported in 8 show that both amphiphilic polymers significantly reduce the extent of insulin fibrillation and increase the lag time compared to non-formulated insulin. The results reported in the figure show that the stabilizing effect depends on the protein/polymer ratio. However, a beneficial effect on protein stability was observed also with high protein/polymer molar ratio (1:1), indicating that even little amount of these polymers in the formulation may stabilize the protein. The fibrillation lag times reported in Table III show that at a 1:1 and 1:5 insulin/polymer molar ratio, $mPEG_{5kDa}$-Cholesterol possesses higher capacity to delay fibrillation of insulin with respect to $mPEG_{5kDa}$-Cholane.

At 1:10 protein/polymer molar ratio, both $mPEG_{5kDa}$-Cholane and $mPEG_{5kDa}$-Cholesterol were found to suppress the protein fibrillation even under the conditions that trigger faster fibrillation (pH 2.0) suggesting that differences of the two polymers could be minimized as the polymer to insulin ratio increases.

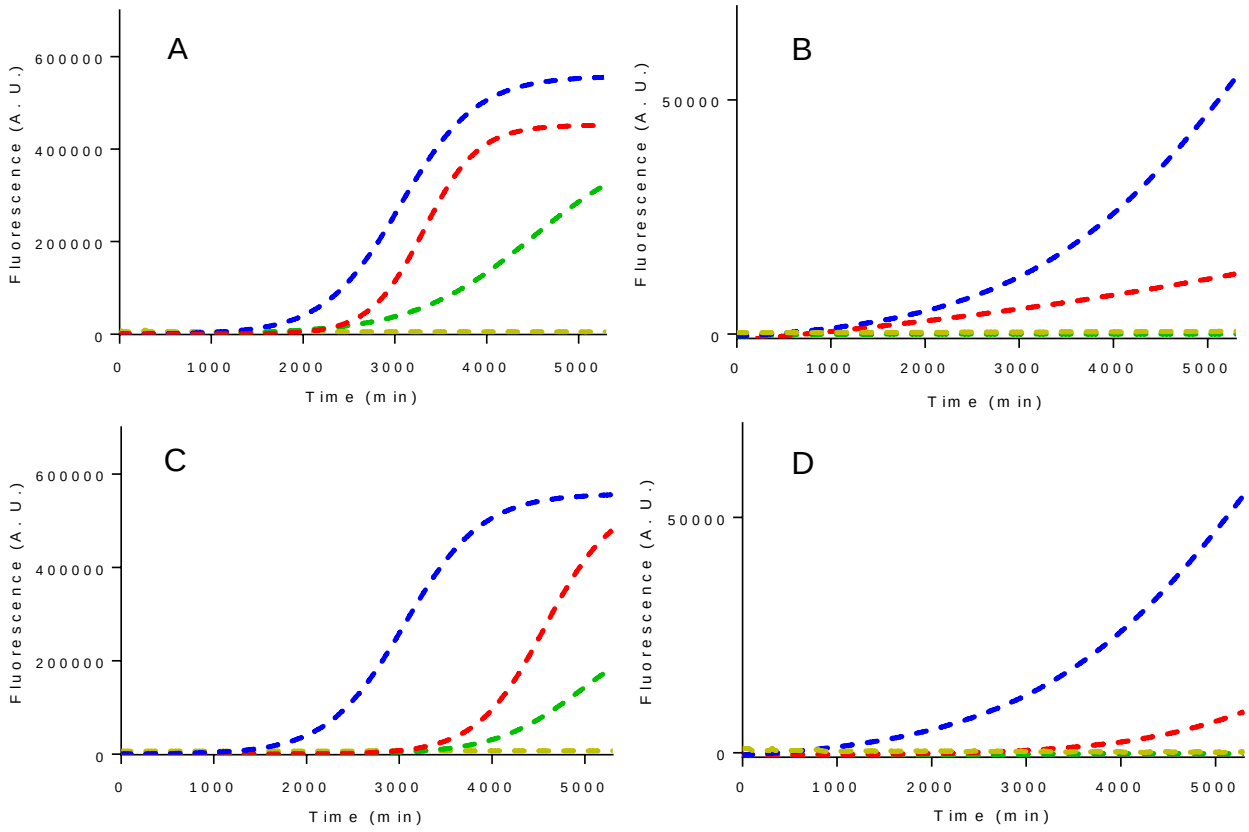


Figure 8. ThT assay fibrillation profiles at 50 °C of free insulin (—) and 1:1 (—), 1:5 (—) and 1:10 (—) insulin/mPEG$_{5kDa}$-Cholane (A and B) or insulin/mPEG$_{5kDa}$-Cholesterol (C and D) molar ratio assemblies in 0.01 M HCl, 140 mM NaCl, pH 2.0 (A and C) and in 20 mM phosphate, 10 mM NaCl, pH 5.3 (B and D). All samples contain insulin at 0.5 mg/mL.

However, even in this case, mPEG$_{5kDa}$-Cholesterol was slightly superior than mPEG$_{5kDa}$-Cholane in enhancing the protein stability, indicating that the affinity strength is relevant in determining the stabilization properties of the polymer. Indeed, the higher capacity of mPEG$_{5kDa}$-Cholesterol to inhibit insulin fibrillation may be ascribable to the tighter association of the polymer with the protein as already observed by the microcalorimetric titration.

Table III. Fibrillation lag times of insulin at pH 2 alone or in the presence of $mPEG_{5kDa}$-Cholane or $mPEG_{5kDa}$-Cholesterol. ND= non detectable; m.r.= molar ratio.

	Fibrillation lag times (min)
insulin	2230
1:1 insulin/mPEG-Cholane m.r.	2729
1:5 insulin/mPEG-Cholane m.r.	3192
1:10 insulin/mPEG-Cholane m.r.	ND
1:1 insulin/mPEG-Cholesterol m.r.	3835
1:5 insulin/mPEG-Cholesterol m.r.	4030
1:10 insulin/mPEG-Cholesterol m.r.	ND

Studies reported in the literature showed that the insulin fibrillation is due to the misfolding of the monomeric polypeptide, while dimers and hexamers generate from the native folded protein. Accordingly, conditions that favour the insulin dimerization and hexamerization delay the fibrillation process.[37,46,47] Therefore, insulin fibrillation is accelerated by processes that interfere with the formation of stable dimers and hexamers and favour the partial misfolding of the monomeric species. The results reported above seem to show that, despite the monomeric insulin is the form that can undergo partial unfolding and fibrillation, the amphiphilic polymers forestall either dimerization and hexamerization or fibrillation by stabilization of the insulin monomer conformation (Table II end Figure 5) and surface shielding.

TEM imaging

The effect of the amphiphilic polymers on protein fibrillation was verified by TEM analysis of the samples incubated at 50 °C for 72 h at pH 2.0, using the same protein/polymer ratios used in the ThT studies.

Figure 9 shows high amounts of macroscopic aggregates and fibrils of non-formulated insulin after incubation at 50 °C, with broad size dispersity, which is in fair agreement with the ThT results reported above. The 1:1 insulin/$mPEG_{5kDa}$-Cholane formulation shows fibrils even though at less extent than the non-formulated insulin. The 1:1 insulin/$mPEG_{5kDa}$-Cholesterol formulation shows a very low number of fibrils confirming the higher capacity of this polymer to prevent the insulin fibrillation. According to the ThT results, TEM images show that the 1:10 insulin/polymer formulations does not yield fibrils.

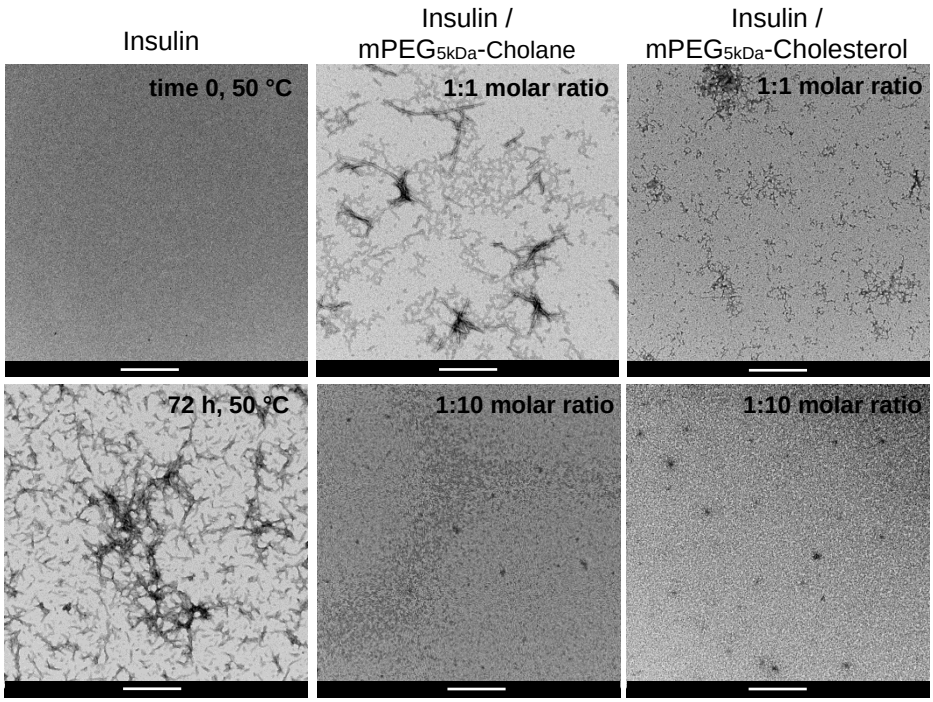


Figure 9. TEM images of insulin in 0.01 M HCl, 140 mM NaCl, pH 2.0 after dissolution and after incubation at 50 °C for 72 hours (left column), and of 1:1 and 1:10 insulin/mPEG$_{5kDa}$-Cholane molar ratio assemblies (central column) and 1:1 and 1:10 insulin/mPEG$_{5kDa}$-Cholesterol molar ratio assemblies (right column) in 0.01 M HCl, 140 mM NaCl, pH 2.0 after incubation at 50 °C for 72 hours. Size bars: 500 nm.

We also imaged insulin and insulin/polymer assemblies at pH 7.4 in freshly prepared solutions in order to verify the appearance of macroscopic structures under physiologic conditions. Figure 10 shows that under this condition non-formulated insulin forms large amorphous aggregates while, in the presence of the amphiphilic polymers, insulin does not fibrillate, which confirms the preliminary ThT studies at pH 7.4 (Figure SI-12) and the literature reports.[48]

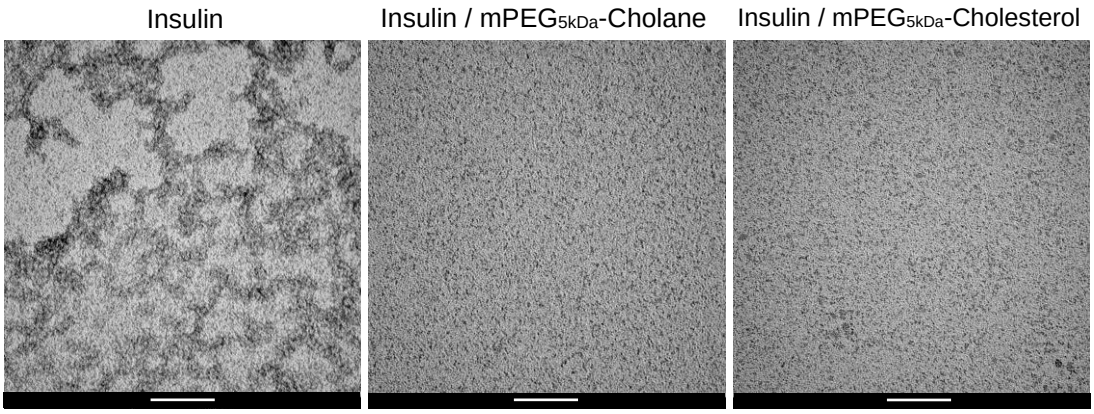


Figure 10. TEM images of insulin, 1:10 insulin/mPEG$_{5kDa}$-Cholane and insulin/mPEG$_{5kDa}$-Cholesterol molar ratio assemblies at pH 7.4. Size bar: 100 nm

Conclusions.

Physical PEGylation can be successfully used to enhance the biopharmaceutical features of therapeutic proteins that possess poor biophysical features, namely solubility, chemical and structural stability, limited pharmacokinetic profiles.[32,33] In this paper we have shown for the first time that two PEGylating platforms containing polycyclic anchoring groups, namely cholane and cholesterol, can stabilize the monomeric form of insulin thus preventing either dimerization and hexamerization, which occur when the monomer is in the native conformation, or fibrillation which occurs by partial monomer unfolding. The experimental evidences show that the monomer stabilization results from a combination of conformational stabilization and protein surface shielding provided by the two PEG derivatives. However, the chemical structure and orientation of the hydrophobic moiety, which is responsible of the polymer interaction, is paramount to dictate the stabilization of the protein. Indeed, mPEG chain conjugated to a different side of two polycyclic steroids (namely cholanic acid and cholesterol) with similar structure, can yield different stability suggesting that a different docking to the hydrophobic interfaces of insulin is obtained.

This study opens new perspectives for the development of infusion pumps and portable delivery systems for insulin administration where new excipients can be exploited for the long-term stabilization of the protein.

References.

1. Murphy MP, LeVine H, 3rd 2010. Alzheimer's disease and the amyloid-beta peptide. J Alzheimers Dis 19(1):311-323.
2. Knowles TPJ, Vendruscolo M, Dobson CM 2014. The amyloid state and its association with protein misfolding diseases. Nature Reviews Molecular Cell Biology 15:384.
3. Manning MC, Chou DK, Murphy BM, Payne RW, Katayama DS 2010. Stability of Protein Pharmaceuticals: An Update. Pharmaceutical Research 27(4):544-575.
4. Patel J, Kothari R, Tunga R, Ritter N, Tunga B 2011. Stability considerations for biopharmaceuticals: overview of protein and peptide degradation pathways. BioProcess International 9:2-11.
5. Mahler H-C, Friess W, Grauschopf U, Kiese S 2009. Protein aggregation: pathways, induction factors and analysis. Journal of pharmaceutical sciences 98(9):2909-2934.
6. Dobson CM, Karplus M 1999. The fundamentals of protein folding: bringing together theory and experiment. Current opinion in structural biology 9(1):92-101.
7. Murphy RM 2002. Peptide aggregation in neurodegenerative disease. Annual review of biomedical engineering 4(1):155-174.
8. Brange J 1992. Chemical stability of insulin. 4. Mechanisms and kinetics of chemical transformations in pharmaceutical formulation. Acta pharmaceutica nordica 4(4):209-222.
9. Howey DC, Bowsher RR, Brunelle R, Woodworth JR 1994. A Rapidly absorbed analogue of human insulin. Diabetes 43(3):396-402.
10. Brange J 1997. The new era of biotech insulin analogues. Diabetologia 40(2):S48-S53.
11. Brange J, Vølund A 1999. Insulin analogs with improved pharmacokinetic profiles. Advanced drug delivery reviews 35(2-3):307-335.
12. DeFelippis MR, Chance RE, Frank BH 2001. Insulin self-association and the relationship to pharmacokinetics and pharmacodynamics. Critical Reviews™ in Therapeutic Drug Carrier Systems 18(2):201-264.
13. Raskin P, Holcombe JH, Tamborlane WV, Malone JI, Strowig S, Ahern JA, Lavent F 2001. A comparison of insulin lispro and buffered regular human insulin administered via continuous subcutaneous insulin infusion pump. Journal of Diabetes and its Complications 15(6):295-300.
14. Vajo Z, Fawcett J, Duckworth WC 2001. Recombinant DNA technology in the treatment of diabetes: insulin analogs. Endocrine reviews 22(5):706-717.
15. Lougheed WD, Zinman B, Strack TR, Janis LJ, Weymouth AB, Bernstein EA, Korbas AM, Frank BH 1997. Stability of insulin lispro in insulin infusion systems. Diabetes care 20(7):1061-1065.
16. Wright AWD, Little JA 1998. Cannula occlusion with use of insulin lispro and insulin infusion system. Diabetes care 21(5):874-874.
17. Wolpert HA, Faradji RN, Bonner-Weir S, Lipes MA 2002. Metabolic decompensation in pump users due to lispro insulin precipitation. Bmj 324(7348):1252-1253.
18. Baudyš M, Uchio T, Mix D, Kim SW, Wilson D 1995. Physical stabilization of insulin by glycosylation. Journal of pharmaceutical sciences 84(1):28-33.
19. Calceti P, Salmaso S, Walker G, Bernkop-Schnürch A 2004. Development and in vivo evaluation of an oral insulin–PEG delivery system. European Journal of Pharmaceutical Sciences 22(4):315-323.
20. Hinds KD, Kim SW 2002. Effects of PEG conjugation on insulin properties. Advanced drug delivery reviews 54(4):505-530.
21. Hinds K, Koh JJ, Joss L, Liu F, Baudyš M, Kim SW 2000. Synthesis and characterization of poly (ethylene glycol)− insulin conjugates. Bioconjugate Chemistry 11(2):195-201.
22. Choudhary S, Kishore N, Hosur RV 2015. Inhibition of insulin fibrillation by osmolytes: Mechanistic Insights. Scientific reports 5:17599.
23. Chi EY, Krishnan S, Randolph TW, Carpenter JF 2003. Physical stability of proteins in aqueous solution: mechanism and driving forces in nonnative protein aggregation. Pharmaceutical research 20(9):1325-1336.
24. Kurinomaru T, Shiraki K 2015. Noncovalent PEGylation of L-asparaginase using PEGylated polyelectrolyte. Journal of pharmaceutical sciences 104(2):587-592.

25. Luengo-Alonso C, Torrado JJ, Ballesteros MP, Malfanti A, Bersani S, Salmaso S, Caliceti P 2015. A novel performing PEG-cholane nanoformulation for amphotericin B delivery. International journal of pharmaceutics 495(1):41-51.
26. Salmaso S, Bersani S, Mastrotto F, Tonon G, Schrepfer R, Genovese S, Caliceti P 2012. Self-assembling nanocomposites for protein delivery: Supramolecular interactions between PEG-cholane and rh-G-CSF. Journal of controlled release 162(1):176-184.
27. Salmaso S, Bersani S, Scomparin A, Balasso A, Brazzale C, Barattin M, Caliceti P 2014. A novel soluble supramolecular system for sustained rh-GH delivery. Journal of controlled release 194:168-177.
28. Mueller C, Capelle MAH, Arvinte T, Seyrek E, Borchard G 2011. Noncovalent pegylation by dansyl-poly (ethylene glycol) s as a new means against aggregation of salmon calcitonin. Journal of pharmaceutical sciences 100(5):1648-1662.
29. Youngster S, Wang Y-S, Grace M, Bausch J, Bordens R, Wyss DF 2002. Structure, biology, and therapeutic implications of pegylated interferon alpha-2b. Current pharmaceutical design 8(24):2139-2157.
30. Li Y, Wang Y, Wei Q, Zheng X, Tang L, Kong D, Gong M 2015. Variant fatty acid-like molecules Conjugation, novel approaches for extending the stability of therapeutic peptides. Scientific reports 5:18039.
31. Knudsen LB 2010. Liraglutide: the therapeutic promise from animal models. International journal of clinical practice 64:4-11.
32. Ambrosio E, Barattin M, Bersani S, Shubber S, Uddin S, van der Walle CF, Caliceti P, Salmaso S 2016. A novel combined strategy for the physical PEGylation of polypeptides. Journal of Controlled Release 226:35-46.
33. Ambrosio E, Podmore A, Gomes dos Santos AL, Magarkar A, Bunker A, Caliceti P, Mastrotto F, van der Walle CF, Salmaso S 2018. Control of peptide aggregation and fibrillation by physical PEGylation. Biomacromolecules 19(10):3958-3969.
34. Kavimandan NJ, Losi E, Wilson JJ, Brodbelt JS, Peppas NA 2006. Synthesis and Characterization of Insulin− Transferrin Conjugates. Bioconjugate chemistry 17(6):1376-1384.
35. Gill SC, Von Hippel PH 1989. Calculation of protein extinction coefficients from amino acid sequence data. Analytical biochemistry 182(2):319-326.
36. Lee C-C, Nayak A, Sethuraman A, Belfort G, McRae GJ 2007. A three-stage kinetic model of amyloid fibrillation. Biophysical journal 92(10):3448-3458.
37. Nielsen L, Khurana R, Coats A, Frokjaer S, Brange J, Vyas S, Uversky VN, Fink AL 2001. Effect of environmental factors on the kinetics of insulin fibril formation: elucidation of the molecular mechanism. Biochemistry 40(20):6036-6046.
38. Störkel S, Schneider HM, Müntefering H, Kashiwagi S 1983. Iatrogenic, insulin-dependent, local amyloidosis. Laboratory investigation; a journal of technical methods and pathology 48(1):108-111.
39. Dische FE, Wernstedt C, Westermark GT, Westermark P, Pepys MB, Rennie JA, Gilbey SG, Watkins PJ 1988. Insulin as an amyloid-fibril protein at sites of repeated insulin injections in a diabetic patient. Diabetologia 31(3):158-161.
40. Noormägi A, Gavrilova J, Smirnova J, Tõugu V, Palumaa P 2010. Zn (II) ions co-secreted with insulin suppress inherent amyloidogenic properties of monomeric insulin. Biochemical Journal 430(3):511-518.
41. Fischel-Ghodsian F, Brown L, Mathiowitz E, Brandenburg D, Langer R 1988. Enzymatically controlled drug delivery. Proceedings of the National Academy of Sciences 85(7):2403-2406.
42. Uversky VN, Garriques LN, Millett IS, Frokjaer S, Brange J, Doniach S, Fink AL 2003. Prediction of the association state of insulin using spectral parameters. Journal of pharmaceutical sciences 92(4):847-858.
43. Hua Q-x, Weiss MA 2004. Mechanism of insulin fibrillation the structure of insulin under amyloidogenic conditions resembles a protein-folding intermediate. Journal of Biological Chemistry 279(20):21449-21460.
44. Kim YS, Lim D, Kim JY, Kang SJ, Kim Y-H, Im H 2009. β-Sheet-breaking peptides inhibit the fibrillation of human α-synuclein. Biochemical and biophysical research communications 387(4):682-687.
45. Dunkelberger EB, Buchanan LE, Marek P, Cao P, Raleigh DP, Zanni MT 2012. Deamidation accelerates amyloid formation and alters amylin fiber structure. Journal of the American Chemical Society 134(30):12658-12667.

46. Phillips NB, Whittaker J, Ismail-Beigi F, Weiss MA 2012. Insulin fibrillation and protein design: topological resistance of single-chain analogs to thermal degradation with application to a pump reservoir. Journal of diabetes science and technology 6(2):277-288.
47. Vinther TN, Norrman M, Strauss HM, Huus K, Schlein M, Pedersen TÅ, Kjeldsen T, Jensen KJ, Hubálek F 2012. Novel covalently linked insulin dimer engineered to investigate the function of insulin dimerization. PLoS One 7(2):e30882.
48. Gong H, He Z, Peng A, Zhang X, Cheng B, Sun Y, Zheng L, Huang K 2014. Effects of several quinones on insulin aggregation. Scientific reports 4:5648.

Graphical abstract