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Oxidation, aggregation and immunogenicity of therapeutic proteins

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Chapter 3

Plain and mono-PEGylated recombinant human insulin exhibit similar stress-induced aggregation profiles

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Abstract

PEGylation has been suggested to improve the stability of insulin, but evidence for that is scarce. Here we compared the forced aggregation behavior of insulin and mono-PEGylated insulin (PEG-insulin). Therefore, recombinant human insulin was conjugated on Lys B29 with 5 kDa PEG. PEG-insulin was purified by size exclusion chromatography (SEC) and characterized by mass spectrometry. Next, insulin and PEG-insulin were subjected to heating at 75 °C, metal-catalyzed oxidation and glutaraldehyde cross-linking. The products were characterized physicochemically by complementary analytical methods. Mono-PEGylation of insulin was confirmed by SEC and mass spectrometry. Under each of the applied stress conditions, insulin and PEG-insulin showed comparable degradation profiles. All the stressed samples showed submicron aggregates in the size range between 50-500 nm. Covalent aggregates and conformational changes were found for both oxidized products. Insulin and its PEGylated counterpart also exhibited similar characteristics when exposed to heat stress, i.e. slightly changed secondary and tertiary structures, covalent aggregates with partially intact epitopes and separation of chain A from chain B. Glutaraldehyde-treated insulin and PEG-insulin both contained covalent and noncovalent aggregates with intact epitopes, showed partially perturbed secondary structure and substantial loss of tertiary structure. From these results we conclude that PEGylation does not protect insulin against forced aggregation.

Introduction

Throughout almost one century, different production platforms, purification protocols and formulations have been investigated in order to produce highly purified insulin with high physico-chemical stability [1]. Still, none of the current insulin formulations is capable of fully protecting this small polypeptide hormone against degradation. In particular, insulin aggregation is hard to avoid and the presence of covalent aggregates in insulin products has been associated with the induction of anti-insulin antibodies from which systemic hypersensitivity and insulin resistance may arise [2-4].

With the progress made in organic and amino acid chemistry, the chemical modification of proteins has become one of the methods to improve the half-life of a therapeutic protein. In particular, PEGylation has been widely used to achieve this aim and PEGylation has been proposed also as a way to increase the stability of biopharmaceutical products [5-6]. A great number of procedures and chemicals have been developed to achieve site-specific modifications [7].

The influence of PEG conjugation to insulin on its stability with respect to the formation of fibrils has been investigated by several authors [8-10]. Briefly, it was found that PEGylation on lysine (Lys) B29 or phenylalanine (Phe) B1 improved insulin's long-term physical stability, as evaluated by a shaking testing done at 100 strokes/min and 37 °C, parallel to an enhancement of its plasma half-life. The higher the molecular weight of PEG (ranging between 750 Da and 2 kDa), the more stable the conjugated protein was found to be. However, when conjugating 5 kDa PEG on Phe B1, a slight decrease in the biological activity has been found [6]. A similar decrease in the activity of the hormone was observed when two 600 Da or 2 kDa PEG chains were covalently bound to the protein [6]. However, no literature is available concerning the behavior of PEGylated-insulin when exposed to other stress factors.

The aim of this work was to investigate the effect of three different stress methods on the stability of PEGylated-insulin in comparison to unmodified insulin, with a special emphasis on the formation of amorphous, non-fibrillar aggregates. To this end, mono-PEGylated insulin (PEG-insulin) was prepared by site-specific linkage of 5 kDa PEG onto Lys B29 of recombinant human insulin. Both proteins, insulin and PEG-insulin, were exposed to three stress conditions, i.e. thermal stress, oxidation by Cu²⁺/ascorbate, and cross-linking with glutaraldehyde. By using complementary physicochemical analytical

methods, we show that the sensitivity of PEG-insulin to these stress factors is comparable to that of its unmodified counterpart.

Materials and Methods

Materials

Recombinant human insulin containing 0.4% (w/w) zinc ions was provided by Schering Plough, Oss, The Netherlands. Glutaraldehyde, sodium borohydride, L-ascorbic acid, copper (II) chloride, arginine, disodium hydrogen phosphate, boric acid and ammonium bicarbonate were purchased from Sigma-Aldrich, Germany. Alpha-methoxy omega-carboxylic acid succinimidyl ester poly(ethylene glycol) (MeO-PEG-NHS, 5 kDa) was purchased from Iris Biotech GmbH, Germany. Glacial acetic acid and acetonitrile were purchased from Boom, The Netherlands. 2,5-dihydroxybenzoic acid (DHB) and sinapinic acid (SA) peptide/protein calibrations standards for MALDI-TOF MS were purchased from Bruker Daltonik GmbH, Bremen, Germany. Deionized water for MS measurements was obtained from a Milli-Q water purification system (Millipore, USA). All chemicals were of analytical grade and used without further purification.

Synthesis and purification of PEG-insulin

Ten mg of insulin were dissolved in 0.1 M hydrochloric acid and brought to a final volume of 1 mL with 60% acetonitrile/40% H_3BO_3 0.1 M, pH 10. Under these alkaline conditions the nucleophilicity of the ϵ -amino group of the Lys B29 is higher than that of the α -amino groups of Gly A1 and Phe B1, so the conjugation should preferentially occur in position B29 [9].

A 3-fold molar excess of MEO-PEG-NHS, 5 kDa, was added in three times, 1 equivalent every 15 minutes, to the insulin solution. After 45 minutes the reaction mixture was purified by SEC, using an Insulin HMWP Column, 7.8 x 300 mm (Waters) and an Agilent 1200 HPLC system (Agilent Technologies, Palo Alto, CA, USA) coupled to a UV detector set at 276 nm. The mobile phase was composed of a mixture of 1 g/L L-arginine aqueous solution:acetonitrile:glacial acetic acid 65:20:15 (v/v/v) as reported in the United States and European pharmacopeias [11-12]. The elution buffer was freshly prepared, filtered and degassed prior to use.

After purification, PEG-insulin was concentrated to about 0.5 mg/mL using centrifugal filter units (Ultra 15 mL, 10 kDa, Amicon, Millipore, USA) and dialyzed against 10 mM sodium phosphate, pH 7.4 (PB), using Slide-A-Lyzer dialysis cassettes with 10 kDa cut-off (Biorad, The Netherlands).

Reduction and alkylation of PEG-insulin

To confirm that PEG was conjugated to chain B, first sodium phosphate buffer was exchanged with 0.1 M ammonium bicarbonate (ABI), pH 8, using centrifugal filter units. Next PEG-insulin was reduced by a 50-fold excess of DTT and alkylated by a 100-fold excess of iodoacetamide with respect to the moles of disulfides contained in the insulin molecule, as reported by Tuesca et al [13].

Digestion of PEG-insulin and insulin

To confirm the site of conjugation, 0.5 mg/mL of PEG-insulin and insulin in 0.1 M ABI, pH 8, were treated with a 0.25 mL of gel immobilized trypsin (Pierce Chemical Company, Rockville, IL). The mixture was incubated overnight at 37 °C. The sample was shaken for ten seconds at 300 rpm every five minute using a Thermomixer (Eppendorf, USA). The immobilized trypsin was then removed from the solution by centrifugation and the supernatant was recovered, concentrated with centrifugal filter units (Amicon, Ultra 15 mL, 3 kDa) and kept at 4 °C until analysis. MS results were eventually compared.

Forced degradation of insulin and PEG-insulin

All the formulations and the dilutions were made in PB. PEG-insulin was obtained as described above. Insulin (not PEGylated) was first dissolved in 0.1 M hydrochloric acid and diluted in PB. The pH was adjusted to 7.4 using 0.1 M sodium hydroxide and the insulin concentration was determined by UV spectroscopy, using a molecular weight of 5.8 kDa and an extinction coefficient of $6200 \text{ M}^{-1} \text{ cm}^{-1}$ at 276 nm [14]. Further dilution in PB was done to obtain a final insulin concentration of 0.5 mg/mL.

Heat treatment was done by heating solutions of 0.5 mg/mL insulin and PEG-insulin, 1 mL in 1.5-mL safe-lock eppendorf tubes, at 75 °C for 1 hour using a water bath [15].

Metal-catalyzed oxidation was performed by adding to solutions of 0.5 mg/mL insulin and PEG-insulin, 2.5 mL in 5-mL glass light-resistant vials, 0.4 mM CuCl₂ in PB, to a final concentration of 40 μM. The reaction was allowed to proceed for 10 minutes and subsequently 40 mM L-ascorbic acid in PB was added to a final concentration of 4 mM. After three hours of incubation at room temperature the reaction was quenched by adding 100 mM EDTA in PB, to a final concentration of 1 mM [16]. The oxidized samples were dialyzed at 4 °C against PB for 24 hours.

Cross-linking with glutaraldehyde was achieved by incubating for 2 minutes 2.5 mL (in 5-mL glass light-resistant vials) of 0.5 mg/mL insulin and PEG-insulin solutions, with glutaraldehyde (final concentration of 4.23 mM). After 2 minutes, 11 μL of 12% (w/w) NaBH₄ in 14 M NaOH, diluted 30 times in water, was added to a final concentration of 646 μM and the mixtures were left in the dark at room temperature for 20 hours [17]. Next, the solutions were dialyzed at 4 °C against PB for 24 hours.

UV spectroscopy

UV/VIS measurements were performed with an Agilent 8453 UV/VIS spectrophotometer (Waldbronn, Germany), which included a Peltier element for temperature control. Quartz cells with a path length of 1 cm were used for all measurements. Scans were taken from 200-900 nm with 1 nm intervals at 25 °C.

Near-UV and far-UV circular dichroism (CD) spectroscopy

Near-UV CD and far-UV CD spectra were recorded from 250 to 320 nm and 190 to 250 nm, respectively, using a Jasco J-815 CD spectrometer (Jasco International, Tokyo, Japan). Analyses were performed in a 1-cm (near-UV CD) and a 1-mm (far-UV CD) path length quartz cuvette at 20 °C using a scan rate of 100 nm/min, a response time of 2 s, and a bandwidth of 1 nm. Each spectrum was the result of an averaging of 6 repeated scans and background

corrected with the corresponding buffer spectrum. The CD signals were converted to molar ellipticity per amino acid residue.

Nanoparticle tracking analysis (NTA)

NTA measurements were performed at room temperature with a NanoSight LM20 (NanoSight, Amesbury, United Kingdom), equipped with a sample chamber with a 640-nm laser and a Viton fluoroelastomer O-ring, as described previously by Filipe et al ^[18]. Briefly, the samples were injected in the sample chamber with sterile syringes (BD Discardit II, New Jersey, USA) until the liquid reached the tip of the nozzle. The software used for capturing and analyzing the data was the NTA 2.0 Build 127. The samples were measured for 60 s with manual shutter and gain adjustments. The “single shutter and gain mode” was used to capture the protein aggregates. The mean size was calculated by using the NTA software.

Light obscuration analysis

Light obscuration analysis was performed using a PAMAS SVSS-C (PAMAS GmbH, Bad Salzuflen, Germany). The pre-run volume was 0.3 mL and for each sample, 3 measurements were done, using a volume of 0.2 mL. Between each measurement, the instrument was washed with Milli-Q water, followed by 30% (v) ethanol in water if necessary. After cleaning, water was measured to confirm that the instrument was cleaned (max. 50 particles/mL, 1µm). For the measurement the samples were diluted 100-fold in PB and the blank was subtracted.

Intrinsic and extrinsic steady state fluorescence spectroscopy

Intrinsic fluorescence was measured in 96-well plates using the plate reader unit of the FS920 fluorescence spectrometer (Edinburgh Instruments). For the measurement, all the formulations were diluted to a concentration of 0.1 mg/mL to avoid inner filter effects. Tyrosine was selectively excited at 275 nm. The emission spectra were recorded from 290 to 400 nm using a step size of 2 nm, gain of 145, Z-position of 20 mm, number of flashes 50 with a frequency of 400 Hz. The extrinsic fluorescent dyes 4,4'-dianilino-1,1'-

binaphthyl-5,5'-disulfonic acid (Bis-ANS) and thioflavin T (ThT) were employed to characterize the aggregates [19].

For measurements of Bis-ANS fluorescence, 10 μL of an aqueous solution of 42 μM Bis-ANS were added to 200 μL of 0.1 mg/mL (PEGylated) insulin sample. The concentration of the stock solution was determined by using an extinction coefficient of 16760 $\text{M}^{-1} \text{cm}^{-1}$ in water at 385 nm. Each sample was excited at 385 nm and the emission spectra were recorded between 400 and 550 nm, using a step size of 2 nm, gain of 150, Z-position of 20 mm, number of flashes 50 with a frequency of 400 Hz.

For measurements of ThT fluorescence, 10 μL of a 210 μM aqueous solution of ThT were added to 200 μL of 0.1 mg/mL (PEGylated) insulin sample. The determination of the concentration of the stock solution was done using an extinction coefficient of 36000 $\text{M}^{-1} \text{cm}^{-1}$ in water at 412 nm. Each sample was excited at 450 nm and the emission spectra were recorded between 465 and 600 nm, using a step size of 2 nm, gain of 130, Z-position of 20 mm, number of flashes 50 with a frequency of 400 Hz.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Acrylamide gradient gels (10-20% tris-tricine) were purchased from Bio-Rad and run under reducing (sample buffer containing 5% (v/v) β -mercaptoethanol) and nonreducing (sample buffer without β -mercaptoethanol) conditions at 100 V at room temperature. The cathode electrophoresis buffer was 0.1 M tris(hydroxymethyl) aminomethane, 0.1 M tricine, and 3 mM SDS, pH 8.3. The anode electrophoresis buffer was 0.1 M Tris pH 8.9. Gel electrophoresis was performed with a Biorad Protean III system (Biorad, Veenendaal, The Netherlands). Samples were boiled at 95 $^{\circ}\text{C}$ for 2 minutes and then centrifuged for 1 minute at 13000 rpm, before application to the gel. A polypeptide marker solution (Biorad, The Netherlands) was included on the gel for apparent molecular weight determination.

Western blotting

Proteins run on SDS-PAGE gels were blotted onto a polyvinylidene difluoride (PVDF) immuno blotting membrane, overnight at 4 $^{\circ}\text{C}$ using a mini trans-blot

system with a voltage applied of 30 V (Biorad, The Netherlands). Blots were blocked for 2 hours at room temperature with 0.1% (w/v) nonfat milk powder in 0.1% (v/v) Tween 20 in phosphate-buffered saline (PBS) with constant orbital shaking. After washing with 0.1% (v/v) Tween 20 in PBS and with water, the blots were incubated with polyclonal guinea pig anti-human insulin antibody (Abcam) in 0.1% (w/v) nonfat milk powder in 0.1% (v/v) Tween 20 in PBS overnight at 4 °C with constant orbital shaking. Blots were washed with 0.1% (v/v) Tween 20 in PBS and with water. Blots were incubated with peroxidase labeled goat polyclonal anti-guinea pig IgG (Abcam) in 0.1% (w/v) nonfat milk powder in 0.1% (v/v) Tween 20 in PBS overnight at 4 °C with constant orbital shaking. Blots were washed with 0.1% (v/v) Tween 20 in PBS and with water and incubated in a solution of 4-chloro-1-naphthol (Sigma-Aldrich) in methanol (20% (v/v)), water, and H₂O₂ (0.015% (v/v)). After color development the blots were stored in water overnight in the dark to increase the intensity of the bands.

Mass spectrometry

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) was performed on BIFLEX MALDI-TOF MS (Bruker Daltonics, Bremen, Germany). Specifically, 1 µL of sample solution and 1 µL of matrix solution were mixed in an Eppendorf tube and applied onto the MALDI sample plate and let to dry at room temperature. Saturated DHB was prepared in a mixture of 50% acetonitrile, 50% deionized water, and 0.1% trifluoroacetic acid as matrix solutions. Analysis was performed in the linear positive mode with delayed extraction of 200 ns. The samples were irradiated by a 337 nm pulsed nitrogen laser. The laser intensity was optimized to give the best signal-to-noise ratio for each sample. The acceleration voltage was 19 kV. Each mass spectrum was generated by averaging ~300 laser shots. MALDI-TOF MS evaluation was performed with FlexAnalysis Software (Bruker Daltonics) by a centroid peak detection algorithm with a signal-to-noise threshold of 3, a peak width of 1 m/z, a height of 80%. External calibration was performed by using a solution of peptide and protein calibration standards from Bruker Daltonics.

Results and Discussion

For the preparation of PEG-insulin we used PEG with a chain length of 5 kDa, because shorter PEGs are likely less stabilizing ^[10] and PEG with a longer chain length would compromise insulin's specific activity ^[8]. In this section are reported the results related with the synthesis, purification and characterization of PEG-insulin, followed by the characterization of the aggregates. SEC, NTA, light obscuration and SDS-PAGE were used for investigating the size and (non)covalent nature of the aggregates; UV, CD, and fluorescence spectroscopy for obtaining information about protein conformation and Western blotting for studying the integrity of epitopes.

Synthesis and purification of mono-PEGylated insulin

As can be seen from Figure 1, the yield of PEG-insulin, as determined by SEC and based on integrated peak area, progressively increased with the addition of MeO-PEG-NHS, while the percentage of unmodified insulin decreased. Beyond the addition of 3 equivalents of PEG, only a substantial increase of bi-PEGylated insulin, but not PEG-insulin, was achieved (results not shown).

The crude reaction mixture was purified by collecting the SEC fraction eluting between 15 and 17 minutes. The same SEC procedure was used for preparative and quantitative purposes. By re-analyzing the purified fraction on SEC, the final percentage of PEG-insulin was found to be $99.0 \pm 0.4\%$ (Figure 1, Table I).

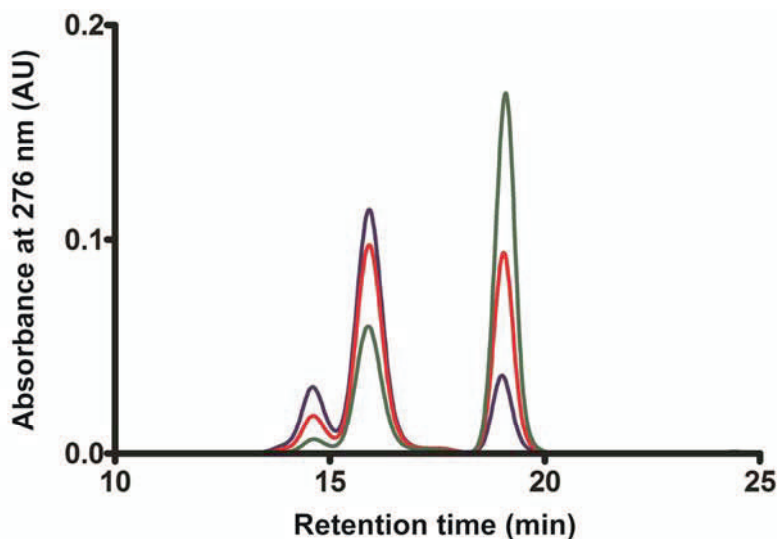


Figure 1. Synthesis of PEG-insulin as monitored by SEC: chromatograms of the reaction mixture after adding 1 eq. (green), 2 eq. (red), 3 eq. (blue) of PEG 5 kDa. After adding each equivalent, the reaction mixture was allowed to equilibrate for 15 minutes.

Table I. Summary of SEC analysis of unstressed insulin and PEG-insulin* (in %).

Sample	Monomer	Dimer	Trimer	Larger aggregates	Fragments	Insoluble
Insulin	99.3 ± 0.1	0.7 ± 0.1	nd	nd	nd	nd
PEG-insulin	99.0 ± 0.4	0.8 ± 0.3	nd	nd	0.2 ± 0.0	nd
HI	89.5 ± 0.3	5.1 ± 0.2	nd	nd	2.6 ± 0.1	2.9 ± 0.3
HPI	85.4 ± 2.5	2.6 ± 0.4	nd	nd	5.3 ± 0.8	6.7 ± 2.1
MI	73.6 ± 0.2	20.3 ± 0.2	6.1 ± 0.3	nd	nd	nd
MPI	64.2 ± 10.3	13.3 ± 1.5	nd	nd	22.5 ± 11.7	nd
CRI**	70.7 ± 0.3	28.2 ± 0.5	29.1 ± 0.1	51.4 ± 1.8	2.5 ± 0.0	nd
CRPI**	133.3 ± 4.8	16.3 ± 1.1	nd	nd	1.7 ± 1.0	nd

* Percentages are based on the AUC of the peaks. Errors are the deviation from the average between two batches. nd = not detectable.

** For glutaraldehyde-treated samples, the total AUC was higher when compared with the native protein, probably due to changes in the molar extinction coefficient or extensive light scattering.

Mass spectrometric analysis of mono-PEGylated insulin

MALDI-TOF MS analysis revealed the presence of mono-PEGylated insulin, showing a peak centered at $m/z \sim 10600$ (Figure 2, red spectrum). The typical distribution of peaks 44 Da apart from each other is related to the polydispersity of the PEG, i.e. the varying number of ethylene oxide monomer units in the polymer chain.

Confirmation of the site of conjugation was achieved in two steps. First, PEG-insulin was reduced, alkylated, purified by SEC and analyzed with MALDI-TOF MS to prove that PEG is indeed bound to chain B but not chain A. The charge distribution centered at $m/z \sim 8400$ matches well the calculated mass centered around 8359 Da for a fully alkylated insulin B chain conjugated with PEG 5 kDa (Figure 2, black spectrum).

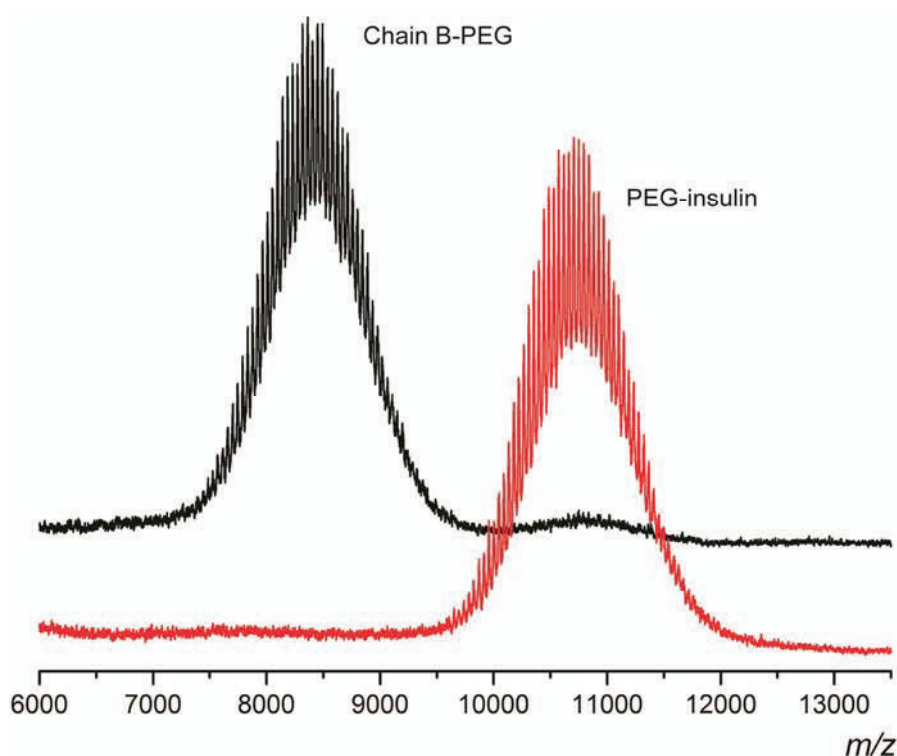


Figure 2. Matrix-assisted laser desorption/ionization time of flight mass spectrometry spectra of PEG-insulin (red) and chain B of reduced/alkylated PEG-insulin purified by size-exclusion chromatography (black).

There was no evidence of any PEG conjugated to chain A (results not shown).

Second, plain insulin as well as the conjugate was subjected to trypsin digestion to confirm that PEGylation had occurred at the C-terminal end of the B-chain, more precisely on Lys B-29. Trypsin is a common enzyme used for the selective cleavage of peptides/proteins at the carboxyl side of the amino acid residues Lys (K) and arginine (R). As can be seen in Figure 3, only chain B of insulin has two sites that can readily be cleaved by trypsin.

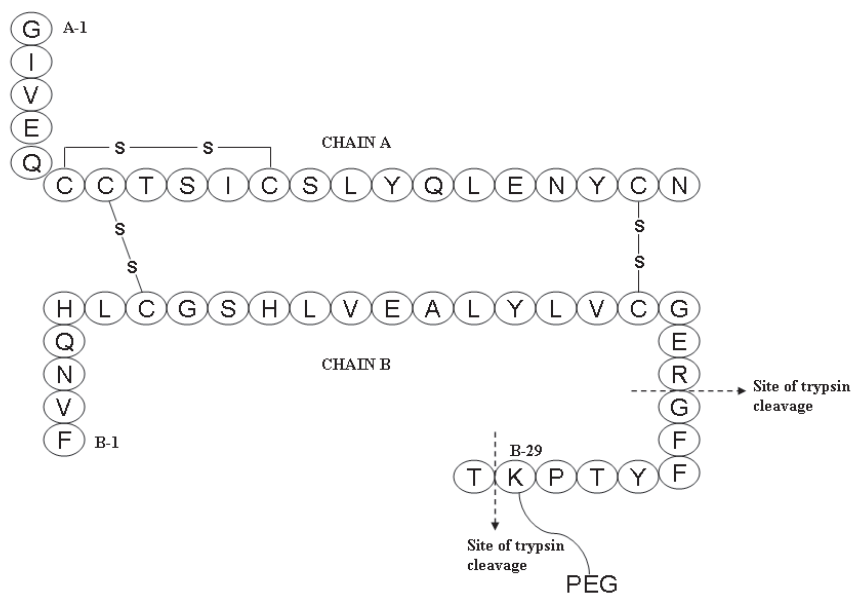


Figure 3. Primary structure of human insulin, including the theoretical cleavage sites of trypsin and the site of PEG conjugation.

The site of the conjugation could be confirmed indirectly by detecting the peptidic fragment (A₁-A₂₁)-(B₁-B₂₂) for both the unmodified insulin (Figure 4, panel A) and PEG-insulin (Figure 4, panel B).

For unmodified insulin, the peak at 4869.4 Da depicted in Figure 4, panel A, corresponds well to the calculated molecular weight of 4866.2 Da for chain A-(B₁-B₂₂) fragment. The peaks at 5710.5 Da and 5812.1 Da correspond to chain A-(B₁-B₂₉) and undigested insulin, respectively. In the spectrum of plain insulin also masses of 959.4 Da and 858.4 Da corresponding to B₂₃-B₃₀ and B₂₃-B₂₉, respectively, could be discerned (data not shown). The spectrum of

digested PEG-insulin (without reduction/alkylation) showed a main peak at 4869.7 Da (Figure 4, panel B) belonging to chain A-(B₁-B₂₂), indicating that the PEG conjugation was definitely not on chain A. Moreover, beneath the main peak a low-signal peak distribution with the PEG profile (44-Da spacing) can be seen, most likely corresponding to the peptide fragments (B₂₃-B₃₀)-PEG and (B₂₃-B₂₉)-PEG. For comparison, the reduced and alkylated PEG-insulin was also digested by trypsin under the same conditions.

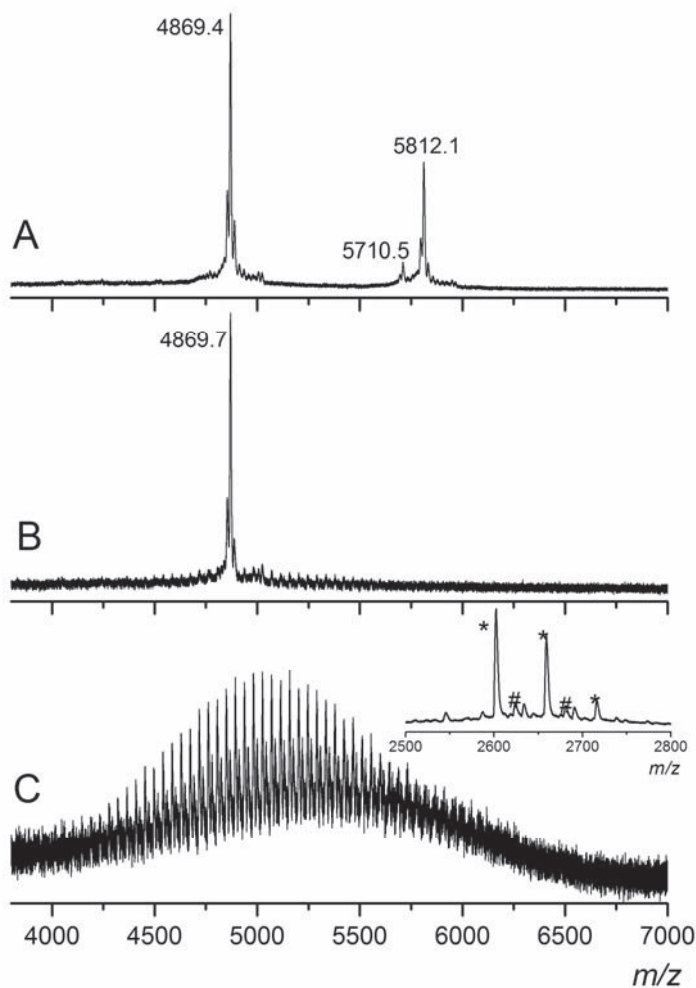


Figure 4. Comparison of MALDI-TOF MS spectra of A) digested insulin, B) digested PEG-insulin and C) digested PEG-insulin after reduction/alkylation. The inset in panel C shows the fully and over-alkylated chain B₁-B₂₂ (*) and chain A (#) peaks.

As can be seen in Figure 4, panel C, the intense 4869 Da peak belonging to chain A-(B₁-B₂₂) had disappeared. Instead, a series of overlaying Gaussian peak distributions were detected. In Figure 4, panel C, the overlapping charge distributions can be attributed to the (B₂₃-B₃₀)-PEG and (B₂₃-B₂₉)-PEG fragments and free PEG, with theoretical masses centered around 5777 Da, 5676 and 4933 Da, respectively. The inset in panel C of Figure 4, indicates that there is no PEG attached to chain (B₁-B₂₂) (*) and chain A (#) fragments.

Altogether, these results show that we successfully synthesized insulin that is mono-PEGylated at Lys (B₂₉).

Characterization of insulin and PEG-insulin

SEC of native insulin and PEG-insulin showed a main peak of monomeric protein which in both cases represented more than 99% of the protein (Table I and Figure 5, panel A).

SDS-PAGE under nonreducing conditions showed a monomer band with an apparent molecular weight of approximately 6 kDa and 12.5 kDa for plain and PEGylated insulin, respectively (Figure 5, panel B, left). The broad band for PEG-insulin can be ascribed to the heterogeneous molecular weight of the PEG (cf. the MS results, Figure 2). The higher apparent molecular weight for the PEGylated insulin in SDS-PAGE as compared to the MS data is due to the fact that PEG hardly interacts with SDS, resulting in a lower electrophoretic mobility of PEG-insulin as compared to a non-PEGylated protein with the same molecular weight. Western blotting showed that both plain and PEG-insulin reacted with polyclonal anti-human insulin antibody (Figure 5, panel B, right).

NTA is an emerging technique which is particularly suitable to determine the particle size in polydisperse samples ^[18]. Since insulin monomers and small oligomers do not scatter enough light to be detected by this method, only larger nano-sized aggregates are selectively detected. For the native proteins, relatively low counts between 0.5-1.0*10⁸ particles per mL, with a size distribution centered at ~100 nm, were detected (Figure 5, panel C, Table II).

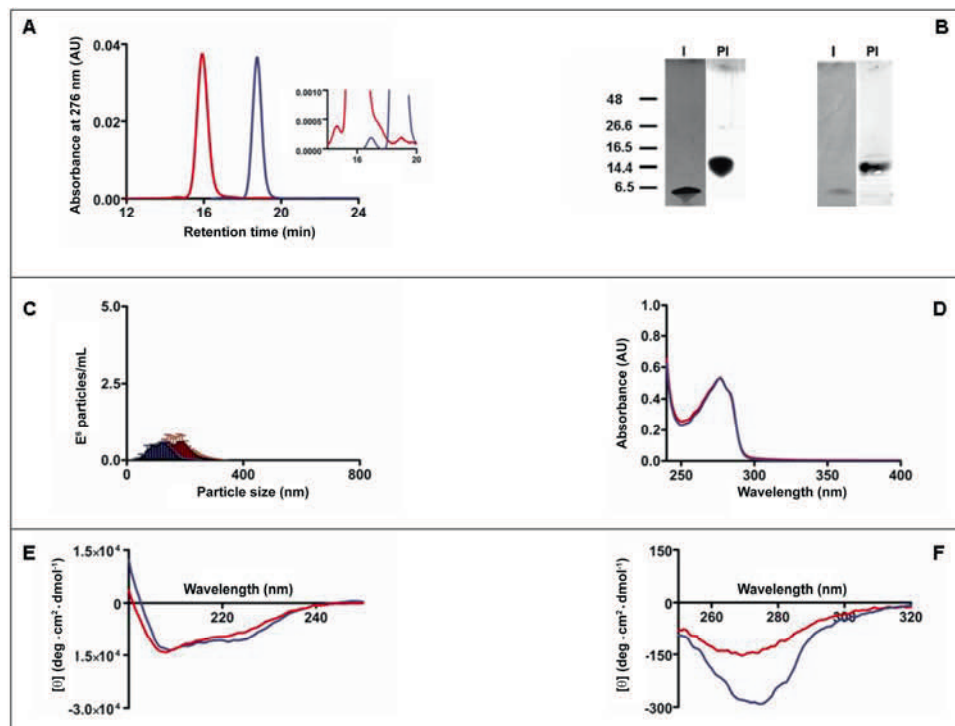


Figure 5. Main characteristics of insulin (I) (blue color) and PEG-insulin (PI) (red color) samples: (A) SEC (the inset shows the zoom in of insulin and PEG-insulin for a better correlation with the % reported in table I), (B, left side) nonreducing SDS-PAGE (numbers indicate M.W. in kDa) and (B, right side) corresponding Western blot, (C) NTA, (D) UV, (E) far-UV CD, (F) near-UV CD.

Subvisible particles in the range between 1 and 200 μm were analyzed by light obscuration. For both insulin and PEG-insulin, approximately $100\text{--}200 \times 10^3$ particles per mL were found (Table II), mainly in the size range 1–3 μm .

The UV spectra of proteins can give important information regarding the presence of aggregates in the solution. The decrease in A_{280}/A_{260} ratio in particular indicates the presence of aggregates [20]. The ratio found for insulin and PEG-insulin obtained from the UV spectra (Figure 5, panel D) was similar (Table III).

Far-UV CD was employed to investigate the secondary structure. PEG-insulin showed a slightly higher $\theta_{208\text{nm}}/\theta_{223\text{nm}}$ ratio (Figure 5, panel E and Table III) as compared to plain insulin. This change has been observed by other authors

and has been assigned to a slight increase in antiparallel β -structure content [9, 21].

Table II. Concentration and mean size of the particles detected with NTA and light obscuration*.

Sample	Nanoparticle Tracking Analysis		Light Obscuration (10 ³ /mL)		
	10 ⁸ /mL	Mean size (nm)	1-10 μ m	10-25 μ m	> 25 μ m
Insulin	0.52 $\pm$ 0.04	116.5 $\pm$ 5.5	155 $\pm$ 38	3.35 $\pm$ 1.66	0.83 $\pm$ 0.83
PEG-insulin	0.78 $\pm$ 0.26	168.0 $\pm$ 3.0	286 $\pm$ 35	-	0.41 $\pm$ 0.41
HI	1.14 $\pm$ 0.12	197.5 $\pm$ 22.5	172 $\pm$ 37	-	-
HPI	1.96 $\pm$ 0.5	242.5 $\pm$ 48.5	215 $\pm$ 19	0.41 $\pm$ 0.41	-
MI	1.05 $\pm$ 0.1	193.0 $\pm$ 1.0	138 $\pm$ 3	2.50 $\pm$ 0.83	2.91 $\pm$ 1.25
MPI	4.65 $\pm$ 1.39	160.0 $\pm$ 20.0	381 $\pm$ 68	0.42 $\pm$ 0.41	-
CRI	7.00 $\pm$ 1.92	248.0 $\pm$ 10.0	415 $\pm$ 95	4.58 $\pm$ 0.41	3.74 $\pm$ 0.41
CRPI	5.50 $\pm$ 0.50	185.5 $\pm$ 18.5	403 $\pm$ 3	2.92 $\pm$ 2.08	0.41 $\pm$ 0.41

* Concentration obtained by NTA is cumulative and is reported as 10⁸ (E⁸ instead of E⁶ used in Figures 5-8, panel C). Errors are the deviation from the average between two batches. - = no particles detected.

Additional information concerning insulin's structure was obtained by analyzing its near-UV CD spectrum in the region 260–320 nm.

The signal in this region is due to the absorbance of insulin's tyrosine, phenylalanine and disulfides, and is substantially affected by its self association state [22-23]. In particular, reduction of the 273 nm CD-band is observed when insulin is dissociated [24-25]. Plain insulin presented a higher negative molar ellipticity band when compared with PEG-insulin, which can be explained by the association state of plain insulin differing from that of PEG-insulin, since in the free protein hexamer prevails [26] (Figure 5, panel F and Table III), whereas the conjugated protein appeared to be in the dimeric state, as reported by Hinds et al [9].

Intrinsic tyrosine fluorescence spectroscopy was used to further analyze the tertiary structure of the proteins. Upon excitation at 275 nm, native insulin showed an emission maximum at 299 $\pm$ 1 nm. PEG-insulin showed a similar spectrum concerning the position of the emission maximum and the intensity (Table III).

These results indicate that both plain insulin and PEG-insulin have structural properties that are consistent with earlier reports, PEG-insulin exhibiting a lower association state than plain insulin.

Table III. Summary of spectroscopic measurements of unstressed and stressed insulin and PEG-insulin*.

Sample	UV	Circular Dichroism			Intrinsic Fluorescence	Bis-ANS Fluorescence
	A 280/260	[θ]208/223	% alpha helix	[θ]273	Intensity (a.u.)	Intensity (a.u.)
Insulin	1.56 $\pm$ 0.00	1.23 $\pm$ 0.00	39.2 $\pm$ 3.7	-314.7 $\pm$ 29.4	12.7 $\pm$ 0.3	2.25 $\pm$ 0.05
PEG-insulin	1.52 $\pm$ 0.06	1.57 $\pm$ 0.05	33.5 $\pm$ 1.5	-148.3 $\pm$ 2.9	12.9 $\pm$ 0.5	3.97 $\pm$ 0.92
HI	1.42 $\pm$ 0.01	1.31 $\pm$ 0.02	33.7 $\pm$ 0.3	-250.6 $\pm$ 0.4	10.9 $\pm$ 0.0	15.76 $\pm$ 1.48
HPI	1.45 $\pm$ 0.02	1.63 $\pm$ 0.02	30.9 $\pm$ 1.9	-122.4 $\pm$ 8.4	12.7 $\pm$ 1.9	6.85 $\pm$ 1.49
MI	1.43 $\pm$ 0.01	1.37 $\pm$ 0.04	26.8 $\pm$ 3.2	-113.4 $\pm$ 10.4	7.4 $\pm$ 1.0	5.91 $\pm$ 0.04
MPI	1.26 $\pm$ 0.00	1.87 $\pm$ 0.10	10.2 $\pm$ 0.0	-55.5 $\pm$ 19.8	6.3 $\pm$ 1.9	5.88 $\pm$ 0.06
CRI	1.03 $\pm$ 0.00	1.54 $\pm$ 0.01	31.6 $\pm$ 0.9	15.5 $\pm$ 7.4	7.3 $\pm$ 0.4	5.76 $\pm$ 0.31
CRPI	1.08 $\pm$ 0.00	1.58 $\pm$ 0.07	30.7 $\pm$ 0.5	50.2 $\pm$ 11.3	12.2 $\pm$ 0.0	5.58 $\pm$ 0.62

* Molar ellipticity in (deg* cm²* dmol⁻¹), % of alpha helix calculated as reported by Pocker et al [21]. Errors are the deviation from the average between two batches.

Characterization of heat-stressed insulin and PEG-insulin

Analysis by SEC showed that heat-stressed insulin (HI) and PEG-insulin (HPI) samples contained small amounts of dimers (Figure 6, panel A, Table I). Whereas the dimer content was slightly higher for heated insulin, heated PEG-insulin showed a larger fraction of unrecovered material (Table I), likely insoluble aggregates. Moreover, in both samples a fragment eluting at ~21 min was observed (Figure 6, panel A), which was identified as chain A by MALDI-TOF MS (not shown).

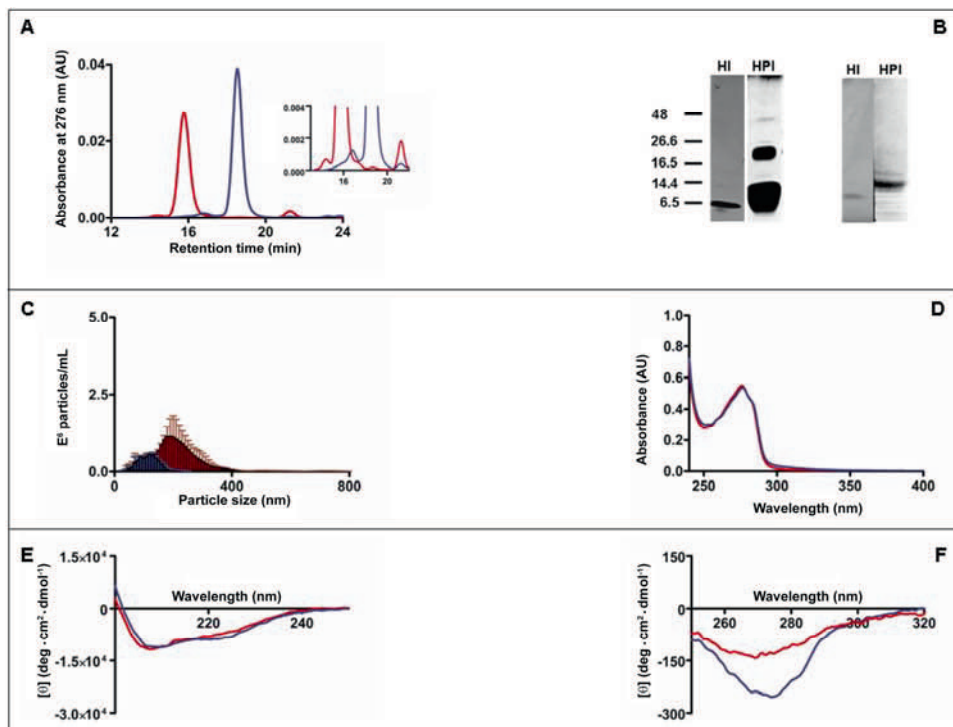


Figure 6. Main characteristics of heated insulin (HI) (blue color) and heated PEG-insulin (HPI) (red color) samples: (A) SEC (the inset shows the zoom in of insulin and PEG-insulin for a better correlation with the % reported in table I), (B, left side) nonreducing SDS-PAGE (numbers indicate M.W. in kDa) and (B, right side) corresponding Western blot, (C) NTA, (D) UV, (E) far-UV CD, (F) near-UV CD.

SDS-PAGE of HI showed a clear monomer band under nonreducing conditions, indicating the absence of covalent aggregates. For HPI, besides the monomer, a band with an apparent molecular weight of approximately 25 kDa and less intense bands with higher molecular weight were detected (Figure 6, panel B, left), indicating the presence of covalent aggregates. In Western blotting analysis only the monomer was detected, both for HI and HPI (Figure 6, panel B, right).

In NTA both HI and HPI showed an increase in particle concentration compared to the proteins before heat treatment (Table II). The size distribution of HPI was centered at about 200 nm, slightly larger than for HI (Figure 6, panel C). Light obscuration did not present major differences in subvisible particle counts with respect to the native proteins (Table II).

The UV spectra practically overlapped with those of the native proteins, although a small decrease in A280/A260 ratio was observed (Figure 6, panel D and Table III), consistent with the presence of small amount of aggregates in both HI and HPI.

Far-UV CD spectra of HI and HPI (Figure 6, panel E) were characterized by a slight increase in the 208/223 nm ratio, a slightly reduced intensity and consequently a decrease in the calculated alpha helix (Table III). The CD signals in the near-UV region (Figure 6, panel F) also were slightly reduced (Table III), indicating that HI and HPI also underwent small changes in the tertiary and quaternary structures.

The fluorescence emission spectrum was not shifted, but the intensity of HI (but not HPI) had slightly decreased (Table III).

Bis-ANS fluorescence was measured as a sensitive probe for the formation of new hydrophobic pockets in heat-stressed proteins [19, 27]. For HI and HPI Bis-ANS fluorescence was increased by approximately 5 and 2 fold, respectively (Table III), indicating a moderate increase in hydrophobicity after heating.

Thioflavin T fluorescence of the stressed samples was as low as that of native insulin and PEG-insulin (data not shown), indicating that the samples were free from fibrils.

In conclusion, the heated samples, insulin and PEG-insulin, showed similar degradation profiles.

Characterization of oxidized insulin and PEG-insulin

Metal catalyzed oxidized insulin (MI) and PEG-insulin (MPI) showed similar loss of monomer, as analyzed by SEC (Figure 7, panel A, Table I). Both MI and MPI contained substantial amounts of oligomers, mainly dimers. Besides, MPI but not MI, showed considerable fragmentation, which is likely related with the ability of radical oxidants to cleave the C-C bond of PEG [24]. Insoluble aggregates, based on the AUC of the peaks, were not found.

Nonreducing SDS-PAGE showed that both MI and MPI contained various covalent aggregates (Figure 7, panel B, left). Whereas the multimeric species present in MI reacted with the antiserum in Western blotting, the blot of MPI did not show any stained bands. This can be due to an extensive loss of the native epitopes and/or less efficient transfer to the PVDF membrane of the oligomeric PEGylated species (Figure 7, panel B, right).

Both stressed samples showed elevated levels of submicron aggregates when analyzed with NTA, mainly in the range size between 150-250 nm (Figure 7, panel C and Table II). This effect was most pronounced for MPI. Subvisible particle counts by light obscuration were similar to those for the unstressed proteins (Table II).

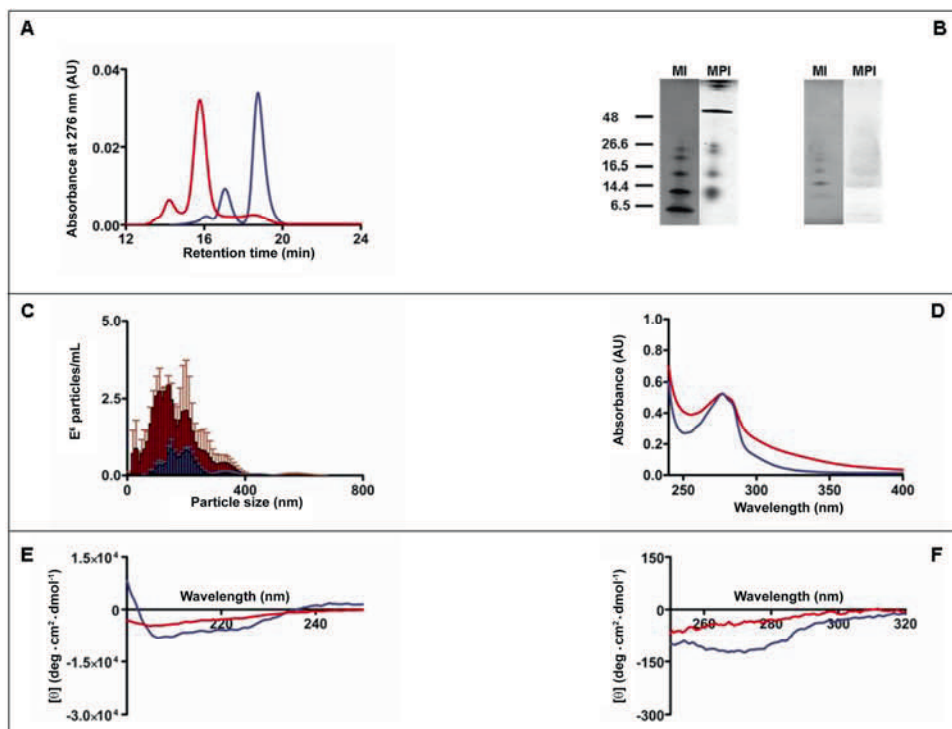


Figure 7. Main characteristics of oxidized insulin (MI) (blue color) and oxidized PEG-insulin (MPI) (red color) samples: (A) SEC, (B, left side) nonreducing SDS-PAGE (numbers indicate M.W. in kDa) and (B, right side) corresponding Western blot, (C) NTA, (D) UV, (E) far-UV CD, (F) near-UV CD.

The UV spectra of MI and MPI in Figure 7, panel D showed an increase in light scattering, indicating the presence of particles. This is clearly reflected in the reduced A280/A260 ratio compared to the unstressed compounds (Table III).

The far-UV CD spectrum of MI showed a reduction of the intensity of the entire spectrum with an increase in the 208/223 nm ratio (Figure 7, E), indicating a substantial decrease in alpha-helical content (Table III). MPI

showed an even more extensive reduction in the alpha helix content. Also the near-UV CD signals of MI and MPI were much smaller as compared to the unstressed proteins, providing evidence that both proteins had undergone considerable loss of tertiary and quaternary structure.

Bis-ANS fluorescence (Table III) was approximately 2 fold higher than for the native proteins, indicating a moderate increase in hydrophobicity. Thioflavin T fluorescence of the stressed samples was as low as that of native insulin and PEG-insulin (data not shown), indicating that the samples were free from fibrils.

Concluding, when oxidation mediated by radicals occurs, PEG might promotes rather than prevents aggregation and loss of insulin's structural features.

Characterization of glutaraldehyde-treated insulin and PEG-insulin

According to SEC, glutaraldehyde-treated insulin (CRI) showed an increase in dimer, trimer and some bigger oligomers, whereas glutaraldehyde-treated PEG-insulin (CRPI) showed a small decrease in the monomer content, parallel to the formation of dimer, (Figure 8, panel A, Table I). PEG only partially prevented the formation of bigger oligomers. Insoluble aggregates, based on the AUC of the peaks, were not found.

Nonreducing SDS-PAGE confirmed the formation of covalent oligomeric species in both CRI and CRPI (Figure 8, panel B, left). Reducing SDS-PAGE confirmed the presence of nonreducible species, in contrast with the non-stressed, heat-stressed and oxidized products (results not shown).

Western blotting revealed that most of the multimeric species in CRPI and CRI contained native epitopes (Figure 8, panel B, right), whereas the monomers in both cases react only weakly with the antiserum.

NTA showed that both CRI and CRPI contained elevated levels of submicron particles, with sizes up to ~700 nm (Figure 8, panel C, Table II). Only a minimal increase in the concentration of subvisible particle content, but not size, was observed by light obscuration analysis (Table II).

The UV spectra (Figure 8, panel D) showed a substantial reduction of the A280/A260 ratio for both CRI and CRPI (Table III), which is consistent with extensive aggregation observed with other methods.

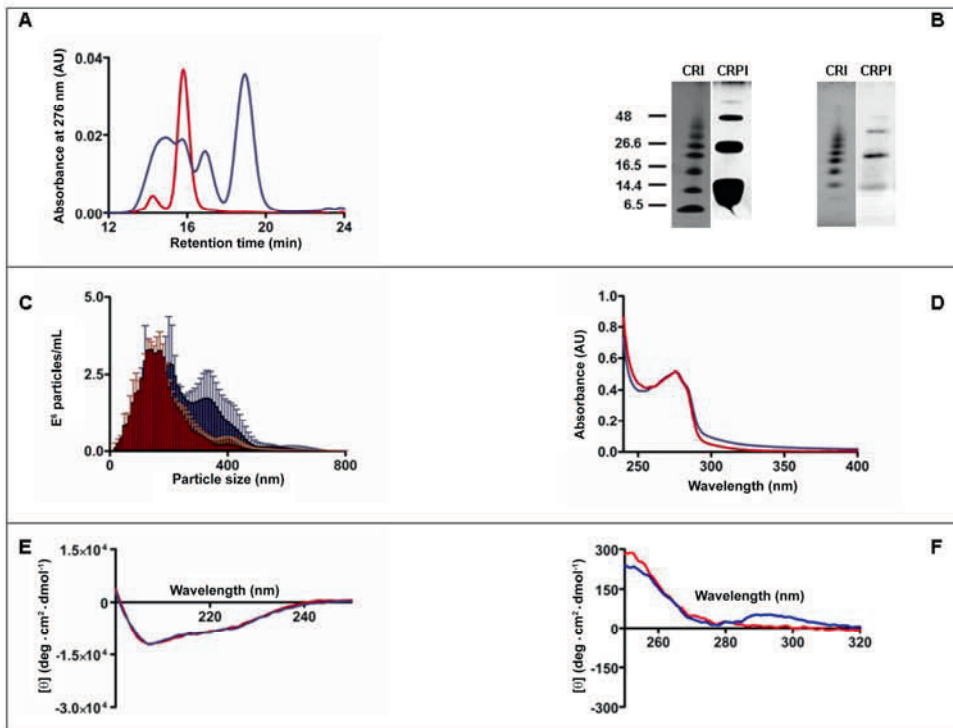


Figure 8. Main characteristics of cross-linked insulin (CRI) (blue color) and cross-linked PEG-insulin (CRPI) (red color) samples: (A) SEC, (B, left side) nonreducing SDS-PAGE (numbers indicate M.W. in kDa) and (B, right side) corresponding Western blot, (C) NTA, (D) UV, (E) far-UV CD, (F) near-UV CD.

Far-UV CD (Figure 8, panel E, Table III) indicated a loss of alpha helix content for both CRI and CRPI. The near-UV region of the spectrum showed major changes (Figure 8, panel F), indicating major perturbations of the tertiary/quaternary structures.

The extrinsic fluorescence measured with Bis-ANS (Table III) indicates that new hydrophobic surfaces were created in the cross-linked samples.

Thioflavin T fluorescence was not enhanced (not shown), indicating the absence of fibrillar species.

In conclusion, PEG is not capable to preserve insulin's structural features when the protein is treated with cross-linking agent as glutaraldehyde.

Conclusion

With this study, we demonstrated that exposure of insulin and PEG-insulin to three different stress factors, i.e. short heat exposure, metal catalyzed oxidation and cross-linking with glutaraldehyde, results in afibrillar aggregates with characteristics that strongly depend on the stress factor. In contrast, the quality of the aggregates, as shown by SEC, SDS-PAGE, Western blotting and NTA, and the altered structural properties of the proteins, as observed by CD and extrinsic fluorescence, were remarkably similar for insulin and PEGylated insulin, indicating that PEGylation neither positively nor negatively affects the sensitivity of insulin to the stress factors applied.

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