Oxidation, aggregation and immunogenicity of therapeutic proteins

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Chapter 5

Triethylenetetramine prevents insulin aggregation and fragmentation during copper catalyzed oxidation

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Abstract

Metal catalyzed oxidation via the oxidative system Cu^{2+}/ascorbate is known to induce aggregation of therapeutic proteins, resulting in enhanced immunogenicity. Hence, inclusion of anti-oxidants in protein formulations is of great interest. In this study, using recombinant human insulin (insulin) as a model, we investigated the ability of several excipients, in particular triethylenetetramine (TETA), reduced glutathione (GSH) and ethylenediamine tetraacetic acid (EDTA), for their ability to prevent protein oxidation, aggregation and fragmentation. Insulin (1 mg/ml) was oxidized with 40 μM Cu^{2+} and 4 mM ascorbate in absence or presence of excipients. Among the excipients studied, 1 mM of TETA, EDTA or GSH prevented insulin aggregation upon metal catalyzed oxidation (MCO) for 3 hours at room temperature, based on size exclusion chromatography (SEC). At lower concentration (100 μM), for 72 hours at 4 °C, TETA was the only one to inhibit almost completely oxidation-induced insulin aggregation, fragmentation and structural changes, as indicated by SEC, nanoparticle tracking analysis, light obscuration particle counting, intrinsic/extrinsic fluorescence, circular dichroism and chemical derivatization. In contrast, GSH had a slight pro-oxidant effect, as demonstrated by the higher percentage of aggregates and a more severe structural damage, whereas EDTA offered substantially less protection. TETA also protected a monoclonal IgG1 against MCO-induced aggregation, suggesting its general applicability. In conclusion, TETA is a potential candidate excipient for inclusion in formulations of oxidation-sensitive proteins.

Introduction

Protein oxidation in liquid and solid formulations is a major concern in biotherapeutics development, as it can modify protein's structure, function and safety [1-2].

Bivalent copper ion (Cu^{2+}) in particular is an atmospheric pollutant that easily contaminates surfaces and experimental buffers and at sub-micromolar concentration (≤ 0.8 μM) initiates the auto-aggregation and oligomerization of Alzheimer's Aβ peptides [3].

Further, metal catalyzed oxidation (MCO) via the oxidative system Cu^{2+}/ascorbate [4] induces aggregation and alterations in protein structure [2, 5-6], and has been shown in preclinical models to increase the immunogenicity of therapeutic proteins [7-10].

Additionally, we recently showed that human insulin, exposed to the same oxidative system, forms irreversible covalent aggregates via Michael addition to 2-amino-3-(3,4-dioxocyclohexa-1,5-dien-1-yl) propanoic acid (DOCH), formed as a consequence of phenylalanine and tyrosine oxidation [11]. Hence, molecules able to inhibit the dramatic effect of copper ions and its catalyzed oxidation of protein are of great importance in protein formulation. Since the year 2000, 48 parenteral drugs containing EDTA in various salt forms have been approved [12]. Although several classes of compounds including polyphenols [13], sugars [14], amino acids [15], thiols [16-17] and chelating agents [18] have been reported to have antioxidant properties, an effective anti-oxidant protecting proteins against copper mediated MCO has not yet been identified.

The aim of this work was to protect insulin from aggregation, fragmentation and oxidative modification upon oxidation induced via Cu^{2+}/ascorbate. With the use of complementary analytical techniques, including size exclusion chromatography (SEC), nanoparticle tracking analysis (NTA), light obscuration particle counting (LOPC), chemical derivatization with benzylamine and 2,4-dinitrophenylhydrazine (DNPH), circular dichroism, and intrinsic and extrinsic fluorescence, we show that 100 μM triethylenetetramine (TETA) [19], unlike reduced glutathione (GSH) [16-17] and ethylenediamine tetraacetic acid (EDTA) [20], is capable of inhibiting aggregation and oxidation of recombinant human insulin formulated in 50 mM phosphate buffer, pH 7.4. Moreover, we show that the anti-oxidant properties

of TETA are applicable to a monoclonal IgG1 as well. Based on our results, we believe that this chelating agent, already approved in 1985 by FDA for the treatment of Wilson's disease [21] and extensively investigated for inhibiting the oxidative stress in diabetic patients [22-32], is an excellent candidate for oxidation-sensitive protein formulations.

Materials and Methods

Materials

Recombinant human insulin (further referred to as insulin) containing 0.4% (w/w) zinc ions was provided by Merck, Oss, the Netherlands. A human monoclonal antibody of the IgG1 subclass (further referred to as IgG) was kindly provided by Dr. Vasco Filipe and described in earlier studies [33]. Disodium hydrogen phosphate, sodium sulfate, trisodium citrate, sodium azide, sucrose, DNPH, benzylamine, potassium ferricyanide ($K_3Fe(CN)_6$), TETA, EDTA, GSH, chlorogenic acid, DL-methionine, melatonine, L-histidine, (±)-α-tocopherol, L-tyrosine, L-carnosine, L-carnitine hydrochloride, copper (I) chloride, copper (II) chloride, ascorbic acid were purchased in the highest purity available from Sigma–Aldrich, Schnelldorf, Germany. Dialysis cassettes (Slide-A-Lyzer 0.5 to 3 mL, 2 kDa cutoff) and 3,3′,5,5′-tetramethylbenzidine (TMB) were obtained from Thermo Fisher Scientific, Breda, The Netherlands. Deionized water was purified through a Purelab Ultra System (ELGA LabWater Global Operations, Marlow, UK) prior to use.

Insulin formulations

Insulin formulations were prepared using a stock solution of 1.14 mg/mL insulin (concentration determined by UV spectroscopy [34], prepared by dissolving 11.4 mg insulin in 0.5 mL 0.1 M HCl, followed by addition of 9.5 mL 50 mM sodium phosphate buffer, pH 7.4 (PB). All the formulations contained a final concentration of 1 mg/mL insulin in PB and, when required, 40 μM Cu^{2+} and 4 mM ascorbate (positive control, excipient free MCO insulin) and 100 μM excipients (MCO insulin containing excipients).

The negative control was prepared by adding 120 μL PB to 880 μL insulin stock solution. The positive control was obtained by adding to 880 μL insulin stock solution, 10 μL PB, followed by 100 μL $CuCl_2$ solution (10x stock in PB)

and, after 10 minutes, 10μL ascorbate solution (100x stock in PB). The same procedure was followed to prepare MCO insulin containing excipients, by adding instead of PB, 10 μL excipient solution (TETA or GSH or EDTA, 100x stock in PB).

Metal catalyzed oxidation

Insulin samples (1 mg/mL) were oxidized for 24, 48 and 72 hours at 4 °C. Pilot studies conducted with several excipients (Table I) were performed using a final excipient concentration of 1 mM (previously prepared as 10 mM stock solutions in PB). MCO was performed for 3 hours at room temperature, using the same Cu^{2+} and ascorbate concentrations as indicated above. Note that the pH of L-tyrosine stock solution was adjusted to about 10 to increase its solubility, however this did not affect the pH of the final insulin formulation. Vitamin E was prepared by previous dissolution of the oil (4.0 μL, equivalent to 4.3 mg) in 10 μL of analytical grade acetone, followed by dissolution in 990 μL PB, to obtain a stock solution of 10 mM, which was then further diluted 10 fold in the final insulin formulation. All the other excipients tested were soluble at 10 mM concentration in PB.

SEC was performed right after the desired time of incubation, without dialysis of the samples. For all other analyses described in this work (see below), samples were analyzed after 72 hours of MCO at 4 °C and extensive dialysis against PB.

The antioxidant properties of TETA were further studied with IgG, prepared at a concentration of 0.5 mg/mL in 10 mM sodium citrate buffer, 5% (w/v) sucrose, pH 6.0 (CSB). MCO was performed with Cu^{2+} and ascorbate (prepared as 10x and 100x concentrated stock solution in CSB), and used at a final concentration of 40 μM and 4 mM, respectively. TETA was prepared as 10 mM stock in the same buffer and used at a final concentration of 100 μM. MCO was conducted for 3 hours at room temperature. SEC was performed immediately after MCO without dialysis of the samples and Bis-ANS extrinsic fluorescence measurements were performed after extensive dialysis against CSB, as described below.

Size Exclusion Chromatography

Insulin and IgG were analyzed by SEC as previously reported [5, 10]. Briefly an Insulin HMWP Column, 7.8 × 300 mm (Waters, Milford, MA, US) and an Agilent 1200 high-performance liquid chromatography system (Agilent Technologies, Palo Alto, California, US) coupled to an ultraviolet (UV) detector set at 276 nm was employed for insulin. 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). A TSK Gel 4000 SW_{XL} column (300 mm x 7.8 mm) with a TSK Gel 4000 SW_{XL} pre-column (Tosoh Bioscience, Montgomeryville, PA, USA) was used for IgG, employing the same system and UV detector, set at 280 nm. The mobile phase was composed of 100 mM sodium phosphate, 100 mM sodium sulfate, 0.05 % (w/v) sodium azide, pH 7.1. A flow rate of 0.5 ml/min was applied for both analyses.

Nanoparticle tracking analysis

Nanoparticle tracking analysis (NTA) measurements were performed at room temperature with a NanoSight LM20 (NanoSight Ltd., Minton Park, Amesbury, Wiltshire, UK), equipped with a sample chamber with a 640 nm laser. Sample, at a concentration of approximately 0.5 mg/mL, were injected into the chamber automatically using sterile syringes (BD Discardit II, New Jersey) connected to an automatic pump (NanoSight Ltd., Minton Park, Amesbury, Wiltshire, UK). The software used for capturing and analyzing the data was the NTA 2.0 Build 127 (NanoSight Ltd., Minton Park, Amesbury, Wiltshire, UK). Samples were measured for 90 s with manual shutter and gain adjustments. The "single shutter and gain mode" was used to capture the protein aggregates. The mean arithmetic size was calculated by using the NTA software. Each sample was measured in duplicate. Placebo formulations showed a particle concentration that was too low to allow accurate measurements, hence subtraction of the buffer solution counts from the sample counts was not performed.

Light obscuration particle counting

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, three measurements were performed using a volume of 0.2 mL per measurement. Between each measurement, the instrument was washed with Millipore Q water, followed by 30% (v/v) ethanol in water, if necessary, until the background signal was less than 10 particles/mL. Placebo formulation signals were subtracted from the corresponding protein sample signals.

Intrinsic and Bis-ANS extrinsic steady state fluorescence spectroscopy

To measure the intrinsic tyrosine fluorescence, dialyzed insulin samples were diluted in PB to 0.1 mg/mL. Duplicates of 200 µL of each sample, pipette into wells of a flat black 96 well plate (Greiner Bio-One B.V., Alphen a/d Rijn, NL) were measured with a Tecan infinite M1000 fluorometer (Tecan group Ltd., Männedorf, Switzerland). The excitation wavelength was set at 275 nm and the emission spectrum was recorded between 290-400 nm.

Extrinsic Bis-ANS fluorescence was used to monitor the formation of exposed hydrophobic regions. To 198 µL (n=2) of 0.1 mg/mL dialyzed insulin or IgG sample, 2 µL Bis-ANS (stock 100 µM in water), was added to a final concentration of 1 µM. Samples were excited at 385 nm using the instrument described above and the emission spectra were recorded from 400 nm to 600 nm. A step size of 2 nm, 50 flashes with a frequency of 400 Hz, a gain of 189 for IgG and 150 for insulin and, a Z-position of 21.5 mm for IgG and 20.0 mm for insulin, were used.

Benzylamine derivatization

Benzylamine derivatization was performed to determine the relative amount of DOPA (3,4-dihydroxyphenylalanine) and DOCH (2-amino-3-(3,4-dioxocyclohexa-1,5-dien-1-yl) propanoic acid) in each insulin sample [35]. The dialyzed samples in 50 mM ammonium bicarbonate (ABI) pH 9.0 were diluted with the same buffer to 0.1 mg/mL. To 500 µL of these solutions, 1 µL of pure benzylamine was added to a final concentration of 20 mM. Next, 5 µL of 50 mM $K_3Fe(CN)_6$ dissolved in ABI pH 9.0, were added to a final concentration of 0.5 mM. The reaction was conducted for 2h at room temperature before performing a fluorescence emission scan with an excitation wavelength of 360 nm and an emission range of 400-550 nm. Controls for non-specific

fluorescence included derivatization reagents (without protein) and non-derivatized protein incubated under the same conditions.

Carbonyl analysis with DNPH

Free carbonyl groups, which can arise from oxidation of lysine, proline and isoleucine, were measured using a sensitive ELISA method described by Buss et al. [36], which requires less protein than the conventional colorimetric carbonyl assay [37], besides being more accurate [38]. Briefly, 45 µL of dialyzed sample in PB were mixed with 45 µL 10 mM DNPH in 2 M HCl to allow DNPH to react (via Schiff base formation) with potential carbonyl groups generated on the insulin molecule during MCO. After 45 min. of incubation at room temperature, 10 µL of this solution were added to 1 mL PB. Next, 200 µL aliquots were added in triplicate into wells of a Greiner Microlon 96 well ELISA plate and incubated overnight. Subsequently the wells were washed 5 times with 300 µL washing buffer (PB with 0.05 % (w/v) Tween 20) and blocked for 2 hours at room temperature with 300 µL blocking buffer (0.1% w/v BSA, 0.1% Tween 20 in PB). Next, plates were washed three times with washing buffer and 200 µL of antibody solution (anti-DNP-antibody 1:1000, 0.1% BSA, 0.1% Tween 20 in PB) were added to the wells for 1 h at 37 °C. After a final washing step, the ELISA was developed by the addition of 100 µL TMB. Plates were incubated until a blue color became clearly visible (~10 min) and the colorimetric reaction was then stopped by the addition of 100 µL 0.18 M sulfuric acid. Absorbance at 450 nm was measured using Tecan's infinite M1000 plate reader controlled by the i-control 1.5 software (Tecan group Ltd., Männedorf, Switzerland). In order to roughly estimate the nmol of carbonyl groups formed per mg of insulin, MCO insulin excipients free and non oxidized insulin, were analyzed via the classical colorimetric assay, as previously described [37].

Circular dichroism spectroscopy

Near- and far-UV circular dichroism (CD) measurements were performed on a J 815 CD spectrometer (Jasco International, Tokyo, Japan) connected to a Jasco PTC 423S temperature controller set to 25 °C. For near-UV CD analysis, 800 µL sample diluted to 0.8 mg/mL in PB was measured in a 1-cm path

length quartz cuvette while for far-UV CD, 300 μL sample diluted to a concentration of 0.2 mg/mL in PB was measured in a 1-mm path length quartz cuvette. Spectra were recorded from 250-320 nm and 190-250 nm for near- and far-UV CD measurements, respectively, at a scan rate of 100 nm/min, a bandwidth of 1 nm and a response time of 2 s. CD spectra of six sequential measurements were averaged and corrected for the blank (PB). The CD signals were converted to molar ellipticity per amino acid residue ([θ]). Alpha helix content was estimated based on [θ] at 223 nm ($[\theta]_{223}$), according to Pocker et al [39].

Results

Aggregate and particle content: SEC, NTA, LOPC

At the beginning of this work, extensive pilot studies were conducted to test the potential antioxidant activity of several excipients at 1 mM concentration: L-carnitine, L-carnosine, chlorogenic acid, etylendiaminotetraacetic aicd (EDTA), melatonine, DL-methionine, reduced L-glutathione (GSH), triethylenetetramine (TETA), histidine, (±)-α-tocopherol (vitamin E) and L-tyrosine. After confirming via SEC that none of the excipient employed affected insulin's aggregation state after 3 hours of incubation at room temperature (data not shown), oxidative studies were performed as previously reported [5]. Results (Table I) indicate that insulin oxidized in presence of 1 mM of one of the excipients reported above, shows similar or even higher amounts of aggregates compared with insulin oxidized in absence of excipients, except for TETA, GSH and EDTA, where no differences with the negative control were detected (Table I). Based on the results of the initial screening, TETA, GSH and EDTA were selected to be studied in more detail for their putative protective action against MCO of insulin.

Table I. Aggregate content in MCO insulin samples from a screening study.

MCO [a]	Excipient (1 mM)	Aggregate content (%) [b]
No	None (negative control)	0.5 ± 0.2
Yes	None (positive control)	21.5 ± 4.2
Yes	L-carnitine	24.2 ± 5.4
Yes	L-carnosine	22.7 ± 0.2
Yes	Chlorogenic acid	39.3 ± 1.2
Yes	Melatonine	24.1 ± 6.9
Yes	DL-methionine	41.6 ± 7.1
Yes	L-histidine	29.2 ± 6.1
Yes	(±)-α-Tocopherol (vitamin E)	32.9 ± 4.0
Yes	L-tyrosine	25.8 ± 1.3
Yes	TETA	0.7 ± 0.2
Yes	GSH	0.8 ± 0.2
Yes	EDTA	0.6 ± 0.1

[a] MCO was performed by incubation with 40 μM Cu^{2+} and 4 mM ascorbate for 3 hours at room temperature.

[b] Calculated from SEC data as previously reported [8].

The experimental procedure to test TETA, GSH and EDTA, was established based on the typical storage temperature of liquid protein formulations and on using a minimal required amount of excipients based on previous work on the anti-oxidant activity of EDTA [20]. Additionally, at 1 mM concentration, GSH still presents some reducing activity with respect to insulin disulfide bridges, while EDTA and, to a lower extent TETA, appear to sequester zinc ions, as indicated by near-UV CD which showed a decrease in [θ] at 273 nm ($[\theta]_{273}$; data not shown). MCO studies were therefore conducted with 100 μM excipient, at 4 °C for 72 hours.

SEC was used to demonstrate that none of the excipients used affected insulin's aggregation state during the applied incubation time (data not shown). Following MCO, the chromatographic behavior of each sample was investigated (Figure 1A). Native insulin shows a single peak with a retention time of 17.3 minutes, representing monomeric insulin. Note that the mobile phase employed contains acetonitrile and acetic acid, which dissociate native non-covalent oligomeric insulin species (i.e. dimer, tetramer, hexamer) normally present in PB at pH 7.4 in presence of zinc ions [44], while it does not dissociate the covalent aggregates obtained by MCO [11]. The elution behavior of MCO insulin, formulated without excipients, presents several peaks eluted earlier than 17.3 minutes as well as a broad main peak eluting at a slightly longer retention time. This indicates that MCO induced extensive aggregation and fragmentation. The amount of insulin aggregates was calculated as previously reported [5], using the area under the curve (AUC) obtained by SEC (i.e. $AUC_{aggregate\ peak}/AUC_{total\ native}$ x 100%) (Figure 1A).

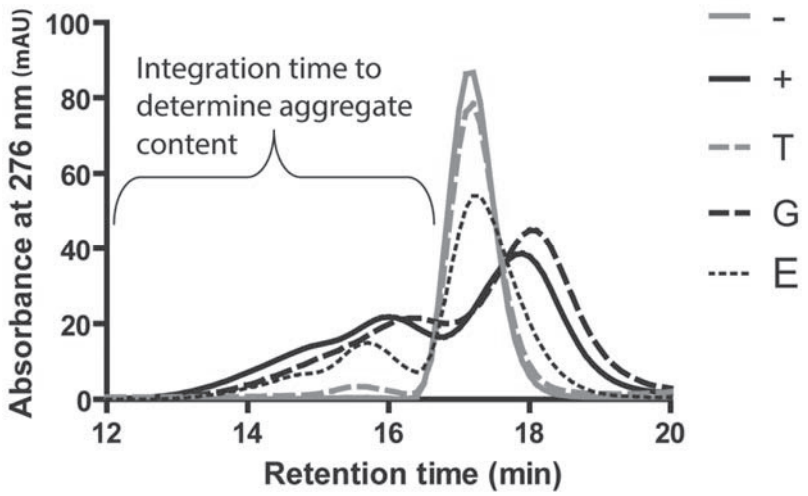

Figure 1A. SEC profiles of insulin samples incubated for 72 h. Brackets delineate the time region for AUC integration used for calculation of the aggregate content. Negative control (-) was insulin incubated in PB, other samples were incubated with 40 μM Cu^{2+} and 4 mM ascorbate in absence (+, positive control) or presence of (TETA (T), GSH (G), EDTA (E)) 100 μM excipients as indicated in the graph.

The chromatogram of MCO insulin formulated with 100 µM TETA almost completely overlapped with that of native insulin, suggesting TETA's protective effect towards aggregation and fragmentation. GSH (100 µM) formulated MCO insulin showed a similar behavior as excipient free MCO insulin, indicating absence of any beneficial activity of GSH at this concentration. Finally, in EDTA (100 µM) formulated MCO insulin high amounts of aggregates were measured, but fragmentation seemed to be partially inhibited, as indicated by the absence of species eluting after the monomeric peak. Figure 1B illustrates the strong time-dependent increase in aggregate content of MCO insulin formulated without excipients or with GSH or EDTA. In contrast, after 24 and 48 hours the aggregate content of MCO insulin formulated with TETA was almost as low as that in unmodified insulin (negative control), and only after 72 hours the amount of aggregates calculated had increased to a few percent. In conclusion, 100 µM TETA is highly effective in preventing MCO induced insulin aggregation and fragmentation while the other two candidates had hardly any protective effect under our experimental conditions.

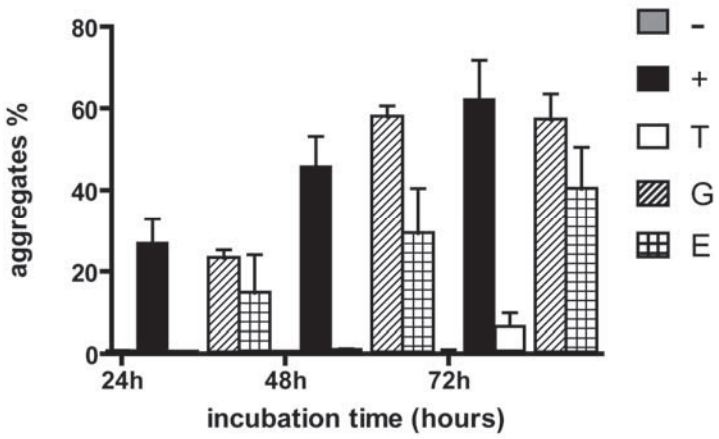

Figure 1B. Percentages of aggregated insulin calculated from SEC profiles after 24 h, 48 h and 72 h of incubation time at 4 °C. Negative control (-) was insulin incubated in PB, other samples were incubated with 40 µM Cu^{2+} and 4 mM ascorbate in absence (+, positive control) or presence of (TETA (T), GSH (G), EDTA (E)) 100 µM excipients as indicated in the graph. Error bars represent the deviation from the average of two batches.

Interestingly, the same concentration of TETA appeared to be a valuable antioxidant also in presence of monovalent copper ions (40 μM Cu^{+}) as indicated by the absence of aggregates (based on SEC, data not shown) during MCO performed for 3 hours at room temperature using the oxidative system Cu^{+}/ascorbate.

NTA was used to monitor the formation of submicron-sized particles during MCO and the results are presented in Figure 2 and Table II. For native insulin relatively low particle counts were obtained. For the positive control, an approximately seven fold higher particle concentration was measured. MCO insulin formulated with TETA showed low particle counts comparable to those in the negative control, demonstrating a protective effect of TETA, whereas samples containing GSH or EDTA contained substantially higher particle concentrations.

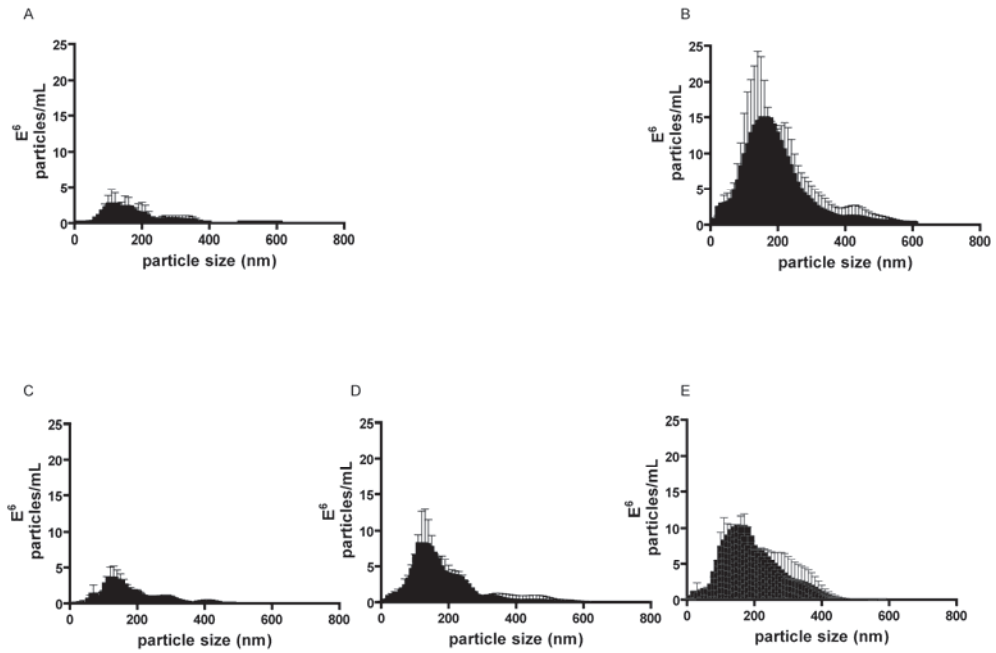

Figure 2. NTA analysis of insulin samples incubated for 72 h: Negative control (insulin incubated in PB) (**A**), other samples were incubated with 40 μM Cu^{2+} and 4 mM ascorbate in absence of excipient (**B**) (positive control) or in presence of 100 μM TETA (**C**), GSH (**D**), or EDTA (**E**). Error bars represent the deviation from the average of two batches.

LOPC was employed to estimate micron-sized aggregates. Non oxidized insulin contained a low background of 0.79 ± 0.27*10^3 particles/mL. In the positive control, the particle concentration in the range 1-10 μm was about ten fold higher than in the negative control (see Table II). MCO of insulin formulated with GSH presented a two fold increase in particle concentration when compared with native insulin, while the TETA- and EDTA-containing formulations showed an approximately three fold increase in particle concentration (in the range 1-10 μm), suggesting a similar inhibiting effect of the excipients on micron-sized particle formation. Micron particles in the larger size range (>10 μm) were barely detected in most of the samples, although the level for the EDTA-containing sample was slightly elevated (Table II).

Table II. Particles concentration and spectroscopic features of insulin samples formulated with and without excipients.

Sample[a]	NTA[b]		LOPC particles concentration (10^3 /mL)[c]			CD	Benzoxazole fluorescence
	Mean size (nm)	Particles concentration (10^8/mL)	1-10 µm	10-25 µm	>25 µm	% alpha helix[d]	Intensity (a.u.)[e]
-	193.7 ± 3.8	0.38 ± 0.01	0.79 ± 0.27	0.06 ± 0.05	0.02 ± 0.02	44.9 ± 1.9	5.2 ± 0.3
+	216.9 ± 27.7	2.60 ± 0.05	7.65 ± 3.31	0.08 ± 0.05	0.02 ± 0.01	34.3 ± 2.9	16.5 ± 0.7
T	182.1 ± 12.2	0.69 ± 0.10	2.44 ± 0.40	0.05 ± 0.01	0.02 ± 0.007	39.6 ± 0.9	6.3 ± 0.4
G	193.2 ± 20.4	1.14 ± 0.04	1.71 ± 0.21	0.02 ± 0.02	0.01 ± 0.005	26.9 ± 0.5	15.8 ± 2.5
E	200.1 ± 22.4	1.80 ± 0.25	2.85 ± 0.92	0.2 ± 0.1	0.1 ± 0.09	38.5 ± 0.7	15.0 ± 1.7

[a] Negative control (-), positive control (excipient free MCO insulin,+), MCO insulin formulated with 100 µM TETA (T), GSH (G), and EDTA (E).

[b] Placebo formulations showed negligible particle counts in NTA.

[c] For LOPC analysis, particle counts of placebo formulations were subtracted from the corresponding protein sample. Typically, the particle concentration of placebo formulations was between 0.19-0.40*10^3 particles/mL, between 0.01*-0.05*10^3 particles/mL and negligible in the size ranges 1-10 µm, 10-25 µm and >25 µm, respectively.

[d] Calculated according to Pocker et al [39].

[e] Calculated based on the fluorescence at the emission maximum (460 nm).

Chemical modifications: DOPA, DOCH and carbonyl content

As previously reported by Sharov et al. [40-41], benzylamine and its derivatives can selectively form a benzoxazole with characteristic fluorescence, after reaction with phenylalanine and tyrosine oxidation products like DOPA (3,4 dihydroxyphenylalanine) and DOCH (2-amino-3-(3,4-dioxocyclohexa-1,5-dien-1-yl) propanoic acid), providing a useful qualitative tool to identify the presence of specific oxidative chemical modifications. Results (Table II) clearly indicate that oxidized phenylalanine and tyrosine are barely detectable in non oxidized insulin, while in the positive control (excipient free MCO insulin), a three fold higher fluorescence was measured, indicating chemical modification of these aromatic amino acid residues. TETA formulated MCO insulin appeared not to contain these oxidized species, as revealed by its benzoxazole fluorescence spectrum, the intensity of which was comparable to that of plain (non oxidized) insulin treated with benzylamine. By contrast, oxidized insulin formulated with GSH and EDTA showed increased benzoxazole fluorescence intensity comparable to that of the positive control, pointing to the generation of DOPA and DOCH during MCO (Table II).

Other widely used markers of oxidative stress are carbonyl groups, which can be formed during reaction of lysine, proline and isoleucine with reactive oxygen species [42-43]. Figure 3A shows results of the carbonyl content measured for all samples via a sensitive ELISA method. Native insulin gave an OD 450 nm signal comparable to that of the blank (reagent incubated in buffer). The positive control, presented a much higher absorbance, caused by the hydrazone formed between DNPH and new carbonyl groups on the insulin molecule. According to a direct spectroscopic measurement of DNPH-modified MCO insulin, the carbonyl content in the positive control was estimated to be 24.8±7.9 nmol/mg of insulin, which is in the same order of magnitude as the value reported by Montes-Cortes et al. for insulin exposed to plasma from diabetic patient containing reactive oxygen species (ROS), for 4 hours at 37 °C [42]. The relatively small increase in the carbonyl content observed for TETA-containing insulin formulation exposed to MCO, relative to that of the positive control, suggests that TETA almost totally inhibits MCO induced insulin oxidation. In contrast, GSH and EDTA did not have any protecting effect (Figure 3A).

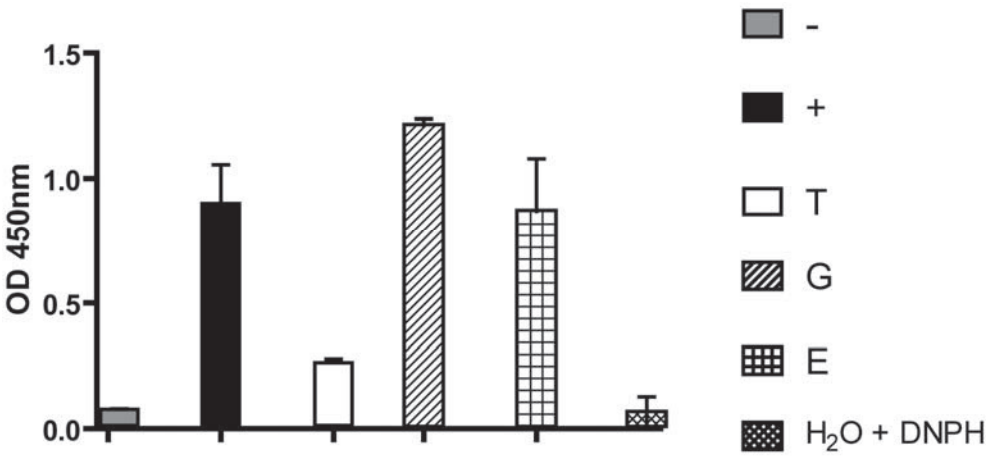

Figure 3A. Carbonyl content of insulin samples incubated for 72 h at 4 °C. Negative control (-) was insulin incubated in PB, other samples were incubated with 40 μM Cu^{2+} and 4 mM ascorbate in absence (+, positive control) or presence of 100 μM TETA (T), GSH (G), or EDTA (E), as indicated in the graph. Error bars represent the deviation from the average of two batches.

Secondary and tertiary structure: far-UV CD, intrinsic and extrinsic steady state fluorescence

Spectroscopic techniques can provide useful information about the higher order structural features of a protein. To investigate whether the excipients used would disturb insulin's quaternary structure, near-UV CD measurements were performed after incubating the formulations for 72 hours at 4 °C (without MCO). The negative magnitude of the near-UV CD signal at 273 nm ($[\theta]_{273}$) is directly proportional to the native noncovalent association state of insulin (i.e. monomer < dimer < hexamer) [44]. Our results suggest that with 100 μM of excipient, insulin remained in its tetrameric-hexameric state when formulated with TETA or GSH in PB ($[\theta]_{273}$ 289.4±0.2 and 312.5±1.5 deg cm^2 dmol^{-1}, respectively). However, in the EDTA-containing formulation insulin's quaternary structure was partially dissociated towards a dimeric-tetrameric equilibrium, as indicated by a reduced $[\theta]_{273}$ (227.1±3 deg cm^2 dmol^{-1}). This was expected because EDTA in this concentration is known to deplete zinc from insulin, resulting in a drop of the negative CD signal [44]. Therefore, near-UV CD was not further performed in the following studies on the antioxidant activity of each excipient.

For insulin, far-UV CD has been widely used to estimate the alpha helix content [39, 45-46]. Table II shows that the content in alpha helix in the negative control is about 45%, which is in line with the literature [39]. MCO of excipient free insulin induced approximately 10% loss in alpha helix content as expected based on our previous studies [39]. TETA insulin showed a minimal loss of 5%, when compared to negative control, while insulin's secondary structure in MCO insulin formulated with GSH, appeared to be seriously perturbed, leading to approximately 20% loss in alpha helix. This is likely due a pro-oxidant activity of the excipient, which in fact can reduce Cu^{2+} into Cu^{+}, which eventually generates hydroxyl radical species in aerobic environment [1]. EDTA formulated MCO insulin showed a slight decrease in alpha helix content roughly comparable to that of TETA formulated MCO insulin.

Fluorescence spectroscopy performed with an excitation wavelength of 275 nm gives information about the environment of the four tyrosine residues in insulin at positions A14, A19, B16 and B26. Non oxidized insulin showed an emission maximum at 299±1 nm (Figure 3B). MCO insulin showed a substantial decrease in the fluorescence intensity but no shift in the emission maximum, suggesting a change in tertiary structure and/or chemical modification of tyrosine residues. The fluorescence emission spectrum of MCO insulin containing TETA almost completely overlapped with that of non oxidized insulin, confirming the protective effect of TETA against MCO. GSH formulated MCO insulin showed a spectrum comparable to that of excipient free MCO insulin, while MCO of insulin in presence of EDTA appeared to result in partial loss of its structural features.

Extrinsic Bis-ANS fluorescence was used as a sensitive probe for the formation of new hydrophobic surfaces in insulin exposed to MCO. A moderate increase in Bis-ANS fluorescence of the MCO (excipient free) sample was observed, as previously reported [5], while all other formulations tested showed Bis-ANS fluorescence spectra comparable to that of the negative control (data not shown). This indicates that all the excipients used inhibit MCO-induced formation of new hydrophobic surfaces.

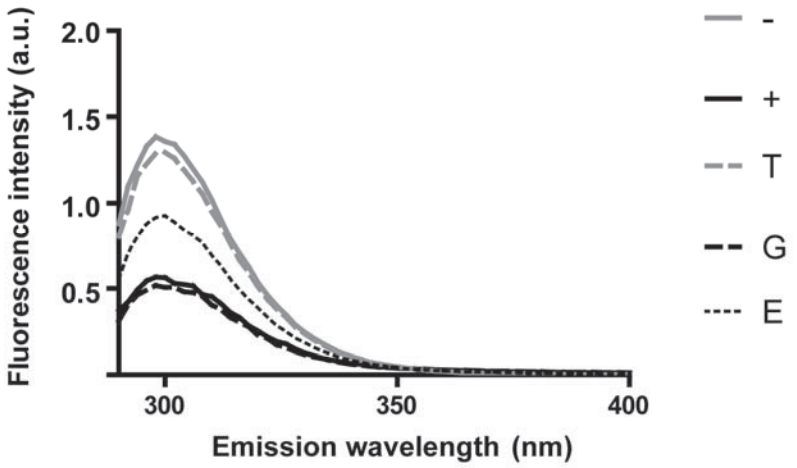

Figure 3B. Intrinsic steady-state fluorescence of insulin samples incubated for 72 h at 4 °C. Negative control (-) was insulin incubated in PB, other samples were incubated with 40 μM Cu^{2+} and 4 mM ascorbate in absence (+, positive control) or presence of 100 μM TETA (T), GSH (G), or EDTA (E), as indicated in the graph. Intrinsic steady-state fluorescence data were obtained averaging the spectra of two batches.

Inhibition by TETA of aggregation and fragmentation of a monoclonal antibody during MCO

To confirm the protective properties of TETA using another therapeutic protein, MCO was applied to IgG and the products were analyzed by SEC and extrinsic Bis-ANS fluorescence, which were previously shown to be suitable techniques to pick up oligomers, fragments and structural modification of this IgG exposed to MCO [10]. Figure 4A shows the SEC profiles of untreated IgG and MCO IgG in presence and absence of 100 μM TETA and Figure 4B the monomer, aggregate and fragment contents derived from the SEC analysis.

The results clearly illustrate the protection by TETA against aggregation and fragmentation of MCO IgG.

Moreover, the fluorescence of Bis-ANS, a dye sensitive to hydrophobic regions in proteins, indicated that TETA prevents the generation of aggregates containing new hydrophobic regions during MCO of IgG (Figure 5).

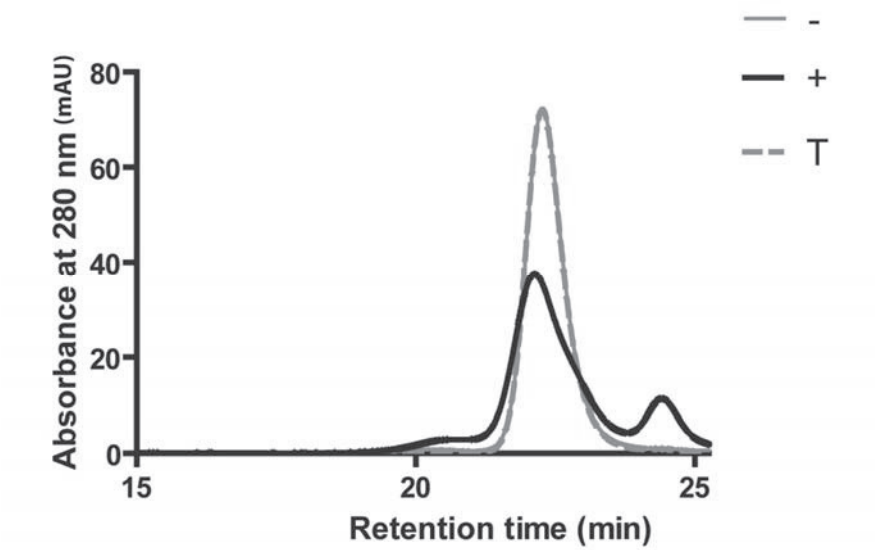

Figure 4A. SEC profiles of IgG samples. Negative control (-) was IgG incubated in CSB, other samples were incubated with 40 μM Cu^{2+} and 4 mM ascorbate in absence (+, positive control) or presence of 100 μM TETA (T), as indicated in the graph.

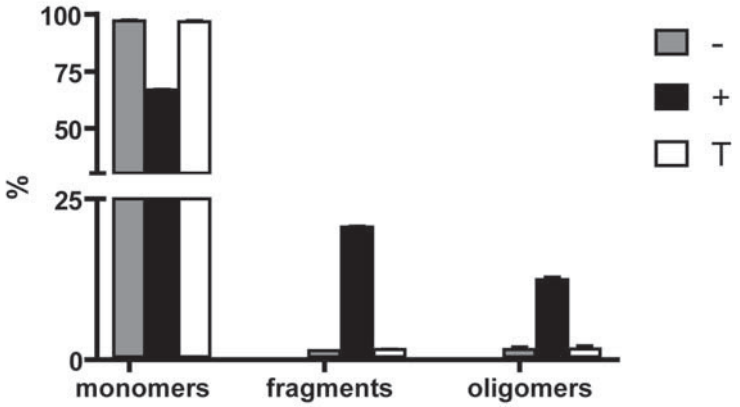

Figure 4B. Percentages of aggregated and fragmentated IgG calculated from SEC profiles after 3 h of incubation time at room temperature. Negative control (-) was IgG incubated in CSB, other samples were incubated with 40 μM Cu^{2+} and 4 mM ascorbate in absence (+, positive control) or presence of 100 μM TETA (T), as indicated in the graph**.** Error bars represent the deviation from the average of two batches.

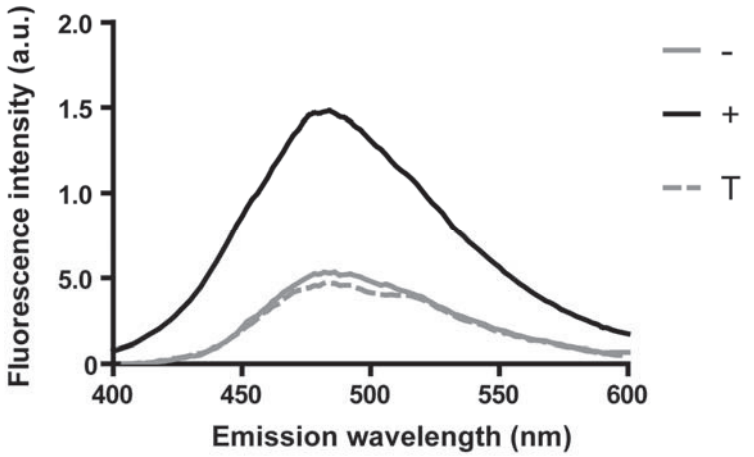

Figure 5. Extrinsic Bis-ANS fluorescence of IgG samples after 3 h incubation at room temperature. Negative control (-) was IgG incubated in CSB, other samples were incubated with 40 μM Cu^{2+} and 4 mM ascorbate in absence (+, positive control) or presence of 100 μM TETA (T), as indicated in the graph. Spectra represent the average obtained from two batches.

Discussion

MCO represents a common degradation pathway of protein and peptide [47] therapeutics and justifies the search for formulation strategies for its inhibition. Classic inhibitors of MCO are chelating agents such as EDTA. Depending on the stability constant of the coordination complex that each chelating agent can form with different metals, the choice of the chelator can depend on the metal involved in the catalyzed oxidation. Excess of EDTA, relative to Cu^{2+}, is often used to quench MCO involving Cu^{2+} but several studies involving Fe^{2+} have suggested its pro-oxidant activity [20, 48], thus favoring protein degradation.

In this work it is shown that the chelating agent TETA protects insulin and IgG from aggregation and fragmentation during MCO via Cu^{2+}/ascorbate. TETA is an FDA approved drug [21] and it has been recently widely investigated in the treatment of diabetes mellitus for its selective copper ion-chelating properties [23], therefore it was selected as antioxidant in the present studies. GSH was included in our study for its radical scavenging activity [49] and because it was reported to inhibit copper catalyzed oxidation in presence of a reducing agent such as ascorbic acid [16], besides being an excellent antioxidant in the human body [17]. EDTA is broadly used as chelating agent in pharmaceutical

formulations [20] and has been previously used to quench metal catalyzed oxidation involving copper ions [2, 4, 7, 8].

In this work we showed that TETA, among the excipients employed (100 μM, in a molar excipient:Cu^{2+} ratio of 2.5:1) almost totally prevented insulin aggregation and fragmentation, as monitored by SEC, NTA and LOPC analysis. TETA still prevented insulin aggregation at 50 μM, but to a lesser extent than at 100 μM (data not shown).

Other structural features of insulin were shown to be largely preserved in presence of 100 μM TETA, as demonstrated by far-UV CD and fluorescence studies. Chemical modifications responsible for insulin aggregation (during MCO under our applied conditions), namely DOPA and DOCH [11], appeared to be inhibited by TETA, based on a chemical derivatization assay with benzylamine. These observations, together with TETA's observed protective effect against MCO-induced aggregation of a monoclonal antibody, suggest that it is a promising candidate to be considered for oxidation-sensitive protein formulations. However, follow-up studies are needed to elucidate whether TETA also inhibits oxidation of amino acid residues like methionine and tryptophan (which are not present in insulin), the oxidation of which can have undesirable pharmacological consequences [50].

The beneficial properties of TETA are probably due the high affinity constant for the complex TETA:Cu^{2+} (log K ≈ 15.0 at pH 7.0) and the square-planar geometry in which Cu^{2+} is most stable [19]. GSH instead does not inhibit MCO, probably because at the concentration used, the amount of reduced glutathione (GSH) responsible for the antioxidant activity [16] is not enough to sequester copper ions, as part of GSH reduces Cu^{2+} into Cu^{+}, yielding GSSG which is unable to prevent MCO [16].

Increasing the concentration of GSH might better chelate copper ions, however due to its reducing properties, GSH is deleterious for the integrity of disulfide bridges, as indicated by the reduction of insulin's disulfide bridge at 5 and 1 mM within 3 hours at room temperature (data not shown).

Interestingly, EDTA can form a stable complex with Cu^{2+}, with a slightly higher stability constant than TETA (log K = 15.9 at pH 7.4) [51], but the geometry of this stable complex can be lost after interactions with buffer salts [52-53], rendering the metal ion available for catalyzing protein oxidation. Higher concentrations of EDTA might however present beneficial effects for

proteins where metal coordination is not crucial for maintaining native structural features.

Conclusion

We described that micromolar concentrations of TETA, in comparison with other potential anti-oxidants, effectively inhibits copper catalyzed protein oxidation, aggregation and fragmentation. Since metal ions are ubiquitous and often contaminate therapeutic protein formulations inducing oxidative chemical modifications, which eventually can produce inactive and immunogenic products, TETA should be taken into serious consideration as a protein formulation excipient. Follow-up studies are warranted to fine-tune the optimal protective concentration of TETA, according the protein under investigation.

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