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Research paper

Enhanced physical stability of human calcitonin after methionine oxidation

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ABSTRACT

Calcitonin is a blood-calcium-lowering peptide, present in different species, which inhibits the resorption of bone by osteoclasts. Human calcitonin (hCT) is one of the few calcitonin peptides, which contains a methionine residue; this residue is in position 8. Methionines are known to be readily oxidized to sulfoxides both *in vivo* and *in vitro*. The current work describes the effect of methionine oxidation on the physical stability of hCT. Aggregation kinetics of human calcitonin were studied at different pH values by intrinsic fluorescence spectroscopy, turbidity at 350 nm, microscopy analyses, Nile Red, and 1,8-ANS fluorescence emission. In all the experiments, methionine oxidation reduced the aggregation rate of human calcitonin. The effect of methionine oxidation was independent of pH. Fluorescence lifetime data also showed that the conformation of hCT in the aggregated state can be influenced by methionine oxidation. A hypothesis for the enhanced physical stability of oxidized hCT is presented and discussed.

1. Introduction

The calcitonins are a family of calcium-regulating peptides synthesized by parafollicular cells (C-cells). Each of these peptides has several biological activities in the body, but the main function is to inhibit osteoclasts-related bone resorption. For this reason, calcitonin peptides are used for the treatment of diseases characterized by increased rates of bone resorption, such as osteoporosis, hypercalcemia, thyrotoxicosis, and Paget's disease [1,2]. The calcitonin primary structure differs between various species; however, some common features such as sequence length, presence of a disulfide bridge between residues Cys^1 and Cys^7, and a proline amide at the C-terminal position are retained. The amino acid sequence of human calcitonin is reported in Fig. 1.

In the body, natural human calcitonin is known to aggregate and form amyloid fibrils; similar fibrils are formed *in vitro* by the synthetic hCT [3]. The fibrillation *in vitro* is affected by the pH of the medium: hCT tends to fibrillate more at physiological condition than at acidic pH [4]. These aggregation properties have strong influence on the potency of hCT. In condition where aggregation and fibrillation were avoided, hCT was found to be 2–4 orders of magnitude more active than human calcitonin handled carefully but without regard to possible aggregation or fibrillation [5]. The non-aggregated hCT was also found to be 2 order of magnitude more potent than salmon calcitonin (sCT) [5].

Knowledge of the fibrillation mechanism of human calcitonin is a prerequisite both for finding the optimal formulation conditions for hCT and for designing the best hCT analogue. It has been proposed that the aggregation mechanism of hCT proceeds via a two-step process [3,4,6]. In the first step, amphiphilic α-helices are formed between the residues 8–22, and then these helices associate to form the critical nucleus; in the second step, critical nuclei associate through β-sheet formation and form mature fibrils. Modifications in the hCT sequence may affect the first, the second, or both steps of the fibrillation mechanism. hCT analogues with higher potency and reduced aggregation tendency would be an advantage. However, some chemical modifications can be undesirable, such as the various chemical degradations that may arise from non-optimal formulation or storage conditions. Chemical degradations such as cleavage of the disulfide bridge between position Cys^1 and Cys^7, deletion of the C-terminal proline amide, and oxidation of the methionine residue in position 8 have been shown to reduce the bioactivity of the peptide [7,8]. However, the aggregation and fibrillation tendency of these chemically modified variants have not been described in detail. In the current paper, the effect of methionine oxidation on the conformation and aggregation properties of hCT was investigated at different pH values employing both spectroscopy and microscopy methods.

Methionine oxidation is a major chemical degradation pathway of proteins and peptides. In nature, oxidation of Met residues is of interest because it occurs in a variety of proteins and often reduces biological activity [9–12]. During the development of biotherapeutic drugs, oxidation of methionine is an issue because of possible

Abbreviations: hCT, human calcitonin; sCT, salmon calcitonin; MS, mass spectrometry; ESI, electrospray ionization; Da, dalton; 1,8-ANS, 1-anilinonaphthalene-8-sulfonic acid; TFA, trifluoroacetic acid.

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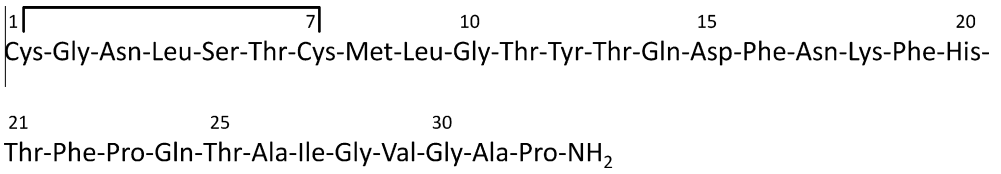

Fig. 1. Amino acid sequence of human calcitonin.

side effects [11], reduced stability, and loss of bioactivity [13–15]. Oxidation can occur due to the presence of oxygen in the drug vials. Oxidative reactions can be catalyzed by light and the presence of impurities such as hydrogen peroxide or metal traces in formulation excipients [16–19]. Methionine sulfoxide is the most common product of methionine oxidation. Compared to methionine, methionine sulfoxide has a bigger, more polar, and less flexible side chain, with increased hydrogen bonding capabilities [20].

Oxidation of a methionine thio group to a sulfoxide introduces a new chiral center in the amino acid side chain [21]. Prior to oxidation, a methionine residue has only one chiral center on the C_α; this chiral center can lead to two possible enantiomers, S-methionine and R-methionine. The two possible stereochemical configurations of methionine can be indicated as S_C or R_C, where the subscript indicates that the atom containing the chiral center is a carbon atom. Upon oxidation, formation of a new chiral center on the sulfur atom of the methionine side chain increases to four the number of possible stereoisomers: S_C, S_S, S_C, R_S, R_C, S_S, and R_C, R_S. In nature, almost all amino acids have the C_α in configuration S [22]. Therefore, it is generally accepted that the oxidation of a methionine to a sulfoxide leads to the formation of two stereoisomers S_C, S_S, S_C, R_S. In this work, it was not possible to separate the two stereoisomers of oxidized hCT. Therefore, the term oxidized hCT here indicates a mixture of hCT containing both sulfoxide stereoisomers in an unknown ratio.

2. Materials and methods

2.1. Materials

Hydrogen peroxide (H_2O_2), 7-diethylamino-3,4-benzophenoxazine-2-one (Nile Red), 1-anilino-8-naphthalene sulfonate (1,8-ANS), acetonitrile, trifluoroacetic acid (TFA), and trypsin (dimethylated, proteomics grade) were supplied by Sigma–Aldrich Chemie GmbH (Buchs, Switzerland). Anhydrous citric acid and L-Methionine were purchased from Fluka GmbH (Buchs, Switzerland). Sodium phosphate mono- and di-basic were acquired from Riedel-de Haën GmbH (Seelze, Germany). All chemicals and reagents were of analytical grade. Ultrapure water (Type I) was produced by a Millipore MilliQ Academic system (Millipore AG, Zug, Switzerland). Quartz cuvettes for fluorescence spectroscopy were purchased from Hellma Schweiz AG (Zumikon, Switzerland). UV transparent 96-well Costar® Corning (Corning Inc., New York, NY, USA) microplates were supplied by Vitaris (Vitaris SA, Baar, Switzerland). UV–Vis transparent Greiner VIEWseals™ (Greiner Bio-One GmbH, Frickenhausen, Germany) were purchased from Huber & Co. AG (Reinach, Switzerland). Slide-A-Lyzer® dialysis cassette (Pierce Biotechnology, Thermo Fisher Scientific Inc., USA) were acquired from Perbio (Perbio Science SA, Lausanne, Switzerland). The human calcitonin peptide was obtained from Novartis (Novartis AG, Basel, Switzerland).

2.2. Methods

2.2.1. Preparation of the oxidized human calcitonin

Human calcitonin was solubilized in MilliQ water to a concentration of 12 mg/mL. Hydrogen peroxide was added to hCT at a peptide:H_2O_2 molar ratio of 20:1. The sample was incubated for 2 h at 20 °C before L-methionine was added to quench the reaction. The resulting oxidized product was extensively dialyzed at 4 °C against MilliQ water using Slide-A-Lyzer dialysis cassettes with a cut-off of 2 kDa.

2.2.2. Reverse-phase chromatography and mass spectrometry analysis

To determine the efficiency of the H_2O_2-treatment in producing oxidized hCT, aliquots of both treated and untreated peptide were analyzed by reverse-phase high performance liquid chromatography (RP-HPLC). For the analysis, a Waters X-Terra MS C18 (2.1 × 250 mm, 5 μm) column (Waters AG, Baden, Switzerland) was used at 45 °C with a flow rate of 0.25 mL/min. A gradient separation with (A) water with 0.1% TFA and (B) acetonitrile with 0.1% TFA was employed. The HPLC program had the following steps: (i) time = 0: 80% mobile phase A and 20% mobile phase B; (ii) continuous decrease in% mobile phase A reaching 60% and 40% mobile phase B at t = 21.5 min; (iii) decrease in% mobile phase A from 60% (at t = 21.5 min) to 10% phase A at t = 25 min (increase to 90% mobile phase B).

For mass spectrometric analysis, the HPLC system was a Waters Alliance HT 2790 HPLC pump, a Waters UV–Vis detector 2487 in combination with a Bruker Esquire 3000 + Ion Trap mass spectrometer (Bruker Daltonics GmbH, Fällanden, Switzerland) equipped with an electrospray ionization source (ESI). The oxidation of the methionine residue in position 8 was confirmed by trypsin digestion and RP-HPLC-MS-MS analysis. The digestion products of native and oxidized hCT were separated with a Waters X-Bridge BEH300 C18 column (2.1 × 250 mm, 3.5 μm) thermostated at 40 °C and a flow rate of 0.25 mL/min. A gradient separation with (A) water with 0.1% TFA and (B) acetonitrile with 0.1% TFA was used. The HPLC program had the following steps: (i) time = 0: 90% mobile phase A and 10% mobile phase B; (ii) continuous decrease in% mobile phase A reaching 50% and 50% mobile phase B at t = 40 min; (iii) decrease in% mobile phase A from 50% (at t = 40 min) to 5% phase A at t = 45 min (increase to 95% mobile phase B). The MS/MS spectra of the peptides resulting from the trypsin digestion were acquired in Enhanced Scan Mode (FWHM 0.45) on a Bruker Esquire 3000 + Ion Trap (Bruker Daltonics GmbH, Fällanden, Switzerland) mass spectrometer in the mass range 200–2300 m/z. The mass spectrometer was calibrated with the calibration mixture provided by Bruker Daltonics. Deconvolution of the multi-charged ESI spectra was performed using the DataAnalysis software (Bruker Daltonics GmbH, Fällanden, Switzerland) and the *related-ion deconvolution* algorithm.

2.2.3. Aggregation kinetics measured by fluorescence spectroscopy and UV absorbance

The kinetics of hCT aggregation were monitored at 37 °C simultaneously by (i) UV absorbance at 350 nm, (ii) intrinsic fluorescence of Tyr^{12}, (iii) Nile Red fluorescence, and (iv) 1,8-ANS fluorescence. The concentration of native and oxidized hCT was 1 mg/mL. Aggregation rates were evaluated in 10 mM sodium citrate/phosphate buffers between pH 4 and 7. Experiments were performed with a Tecan Safire2™ (Tecan Group Ltd., Männedorf, Switzerland) microplate reader. Fluorescence emission was measured using the bottom optics. For the extrinsic fluorescence

emission experiments, concentrations of 1 μM and 50 μM of Nile Red and 1,8-ANS, respectively, were used. The following settings were used: (a) intrinsic fluorescence: λ_{ex} = 274 nm, λ_{em} = 315 nm; (b) Nile Red fluorescence: λ_{ex} = 550 nm, λ_{em} = 620 nm; (c) 1,8-ANS fluorescence: λ_{ex} = 371 nm, λ_{em} = 490 nm. Results are the average of the measurements of three independent samples.

2.2.4. Light microscopy and fluorescence microscopy with Nile Red and 1,8-ANS staining

Native and oxidized hCT samples were observed by light microscopy with enhanced phase contrast and by fluorescent microscopy after staining with Nile Red or 1,8-ANS. All samples were stained with Nile Red or 1,8-ANS immediately prior to the measurement. The concentrations of Nile Red and 1,8-ANS were 1 μM and 50 μM, respectively. The measurements were performed on an Axiovert 200 microscope (Zeiss, Göttingen, Germany) equipped with a mercury discharge lamp. A 10x A-Plan LD objective (Zeiss, Göttingen, Germany) was used. The images were acquired with a cooled Retiga 1300 C color CCD camera (QImaging, Burnaby, Canada) and processed with the Openlab software (Improvision, Coventry, UK). The concentration of hCT was 1 mg/mL in all the samples.

2.2.5. Intrinsic fluorescence lifetime

Fluorescence lifetimes of hCT were measured using time-correlated single-photon counting (TCSPC) on a IBH FluoroCube 5000 U (Horiba Jobin Yvon Inc, Stanmore, UK) equipped with a 279 nm nanoLED pulsed light excitation source and a monochromator on the emission side. Data were measured in a 1 cm pathlength cuvette. The concentration of both oxidized and non-oxidized hCT was 1 mg/mL. The lifetimes were measured at the wavelength corresponding to the maximum of emission. All the measurements were performed at 25 °C. The fluorescence lifetime decay data were fitted using the DAS6 decay analysis software, and the average lifetimes were calculated using Eq. (1):

$$\overline{\tau} = \frac{\sum_i \alpha_i \tau_i^2}{\sum_i \alpha_i \tau_i} \quad (1)$$

where τ is the fluorescence decay time and α the normalized pre-exponential factor.

3. Results

3.1. RP-HPLC and mass spectrometry (MS) analysis of H_2O_2-treated hCT

The efficacy of treatment with H_2O_2 in producing oxidized human calcitonin selectively oxidized in position 8 was evaluated by RP-HPLC-MS. Native hCT eluted at a retention time of 17.7 min, while H_2O_2-treated hCT eluted at 13.7 min (Fig. 2). The shorter retention time of H_2O_2-treated hCT is consistent with the oxidation of the peptide, which results in the formation of a more polar species which is less retained by the apolar stationary phase of the column. ESI-MS data confirmed the oxidation of the H_2O_2-treated hCT: a molecular mass increase of 16 Da was found for the H_2O_2-treated hCT sample. ESI-MS-MS analysis of the peptides

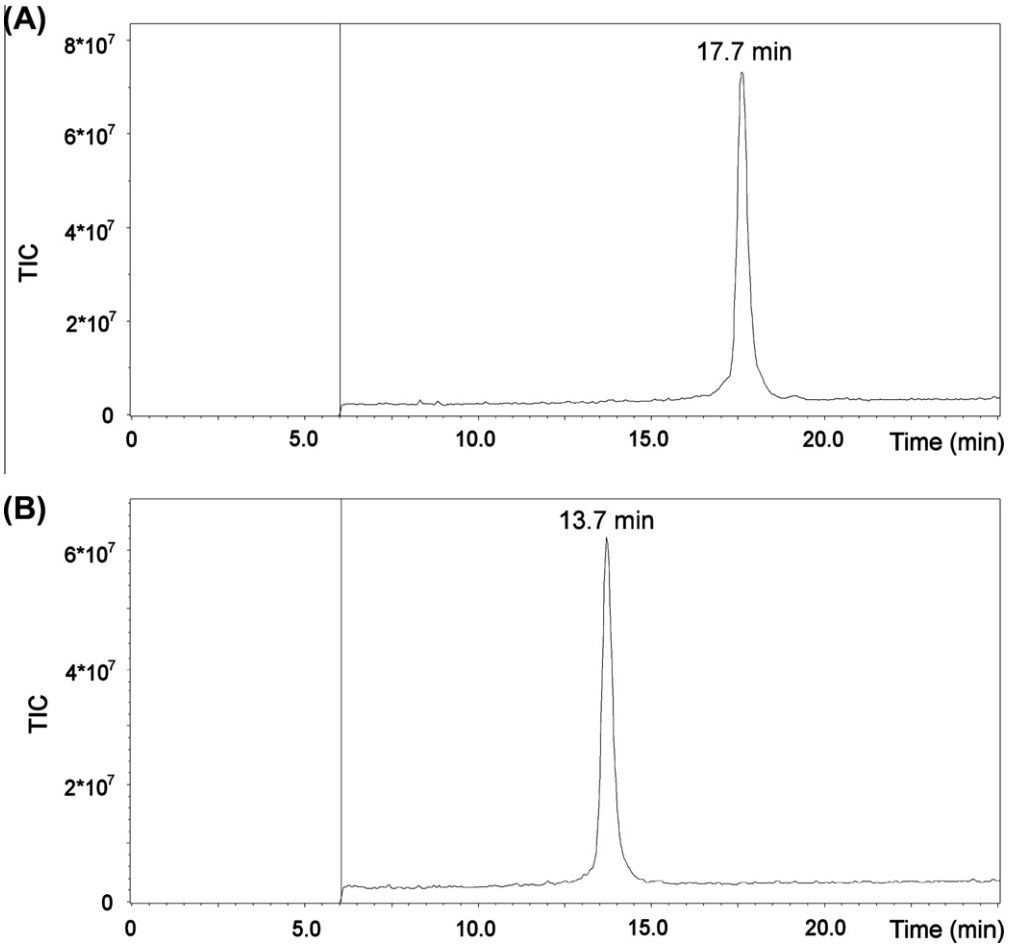

Fig. 2. Reverse-phase chromatographic separation of human calcitonin: mass spectrometry TIC trace. For the separation, the MS divert valve was open after 6 min. (A) Native hCT; (B) oxidized hCT.

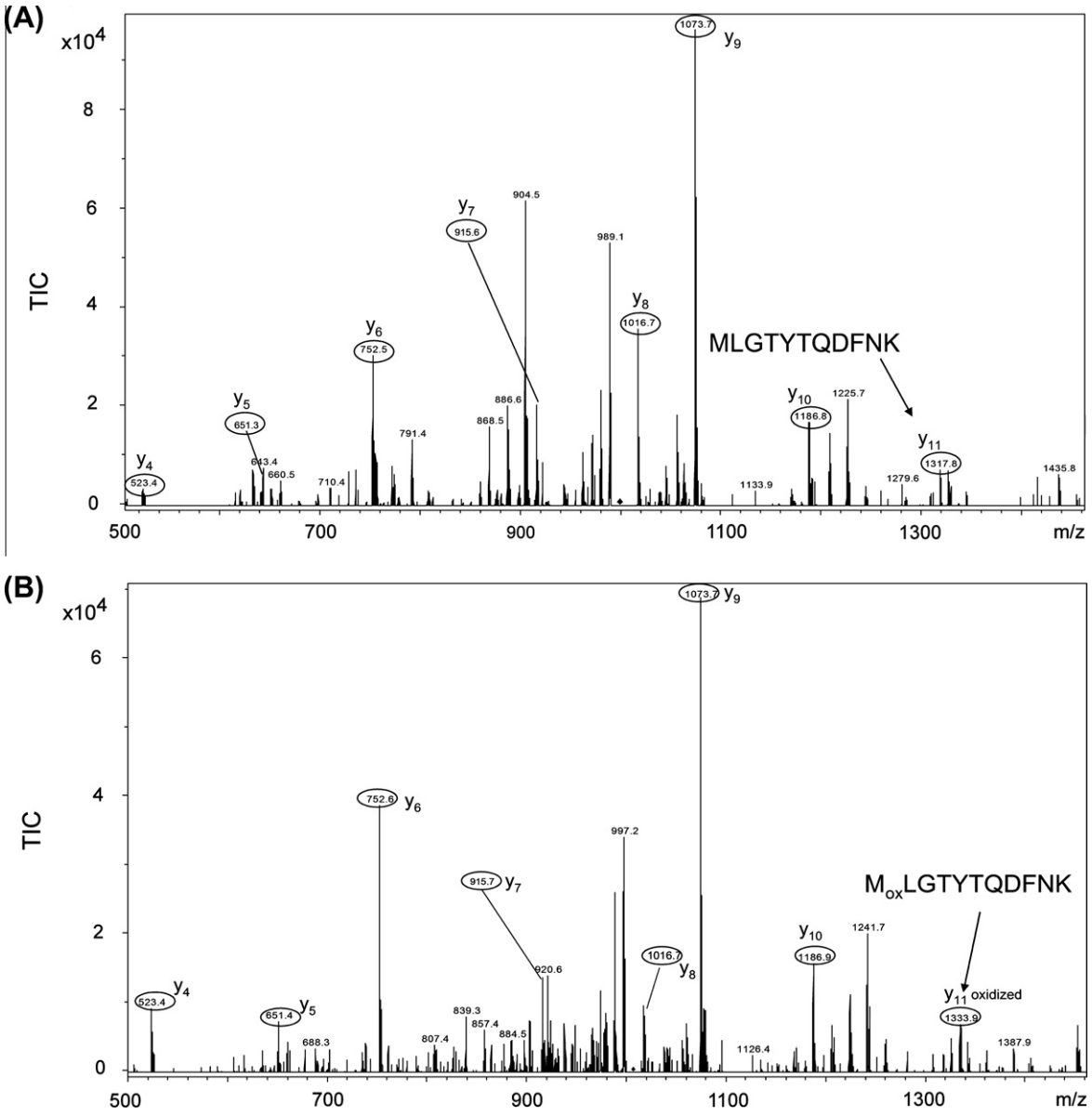

Fig. 3. ESI-MS-MS analysis of peptide (1–18)hCT obtained by trypsin digestion of human calcitonin. (A) MS^2 scan of the *m*/*z* 998.4 ion of native (1–18)hCT peptide; (B) MS^2 scan of the *m*/*z* 1006.5 ion of oxidized (1–18)hCT peptide.

Cys^1-Lys^{18} originated from the trypsin digestion of the native and oxidized hCT showed that selective oxidation of the methionine residue in position 8 was achieved (Fig. 3).

3.2. Aggregation kinetics of oxidized hCT

The kinetics of aggregation at 37 °C for both native and oxidized hCT were investigated by absorbance at 350 nm and fluorescence spectroscopy. For the measurements, sodium citrate/sodium phosphate 10 mM buffer solutions at pH values between 4 and 7 were used.

The results of intrinsic fluorescence emission, measured at the wavelength corresponding to the maximum of emission, of native and oxidized hCT samples are shown in Fig. 4. After 24 h incubation at 37 °C, neither native nor oxidized hCT showed an increase in intrinsic fluorescence emission between pH 4 and 5. Between pH 5.5 and 6.5, an increase of native hCT fluorescence emission was observed; no increase was observed for oxidized hCT. At pH 7, intrinsic fluorescence emission of both peptides increased. All increases in fluorescence emission were preceded by a lag phase; lag phases were shorter at higher pH values. Oxidized hCT samples had longer lag phases than native hCT samples.

UV absorbance kinetics at 350 nm of native and oxidized human calcitonin are shown in Fig. 5. Upon storage at 37 °C, the apparent UV absorbance at 350 nm of native human calcitonin increased more and faster than that of oxidized peptide. The faster increase of native hCT UV absorbance was observed at all pH values.

In both native and oxidized hCT, the apparent UV absorbance at 350 nm was smaller at acidic pH. A defined lag phase was observed in the native hCT sample at pH 4.5 only.

The intensities of 1,8-ANS fluorescence emission in presence of native and oxidized hCT are shown in Fig. 6. The fluorescence intensities of all samples increased upon storage at 37 °C; at acidic pH, the increases were less pronounced. Both intensities and rates of the fluorescence increases were lower upon oxidation of the peptide. Lag phases were recorded for all the samples; the length of these lag phases varied based on pH and nature of peptide.

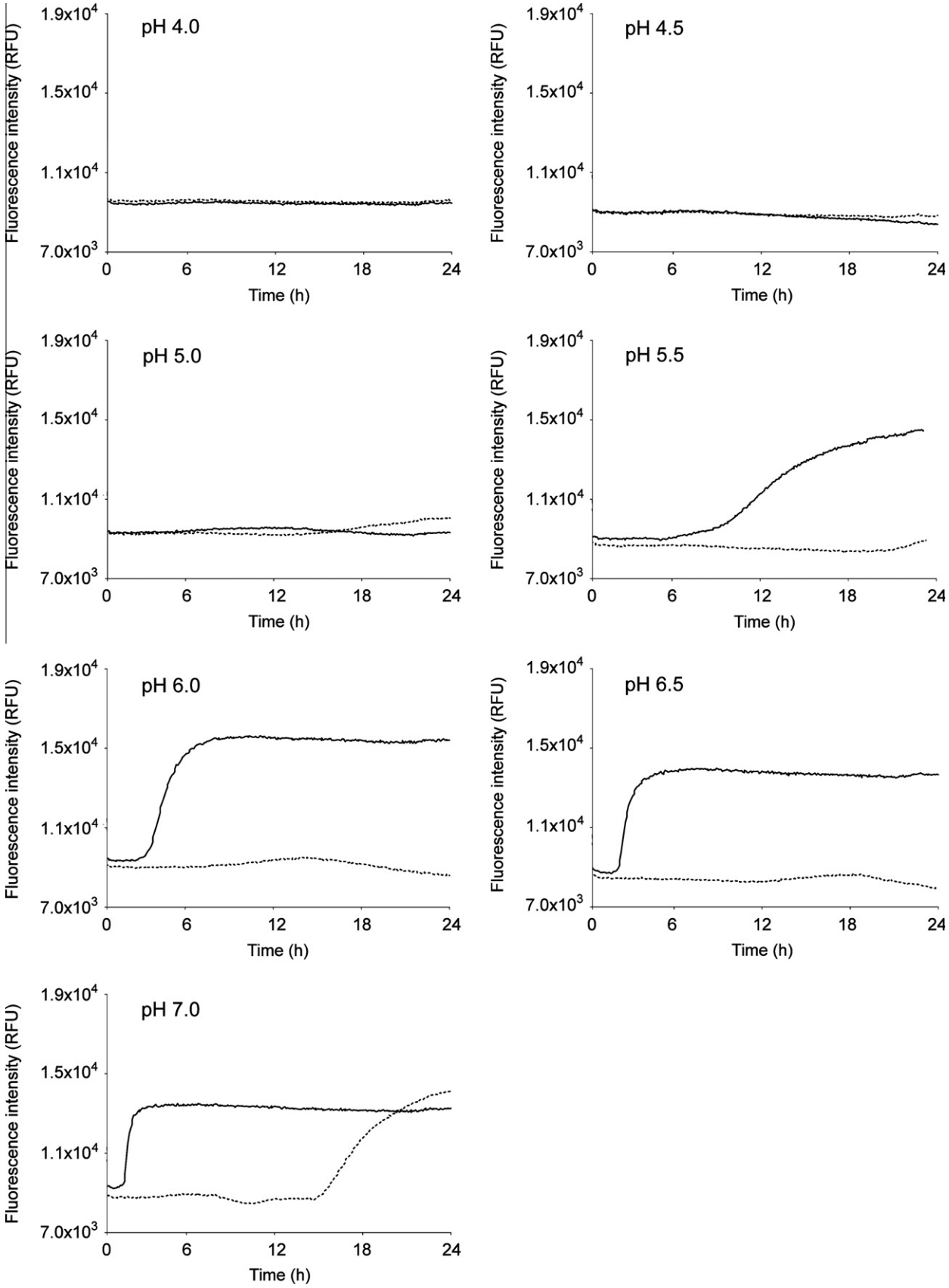

Fig. 4. Kinetics plots of Tyr^{12} fluorescence emission of native and oxidized hCT at 37 °C. Fluorescence emission of Tyr^{12} in both native and oxidized hCT was recorded at 315 nm after excitation at 274 nm. Native hCT is represented by a continuous line; oxidized hCT by a dotted line.

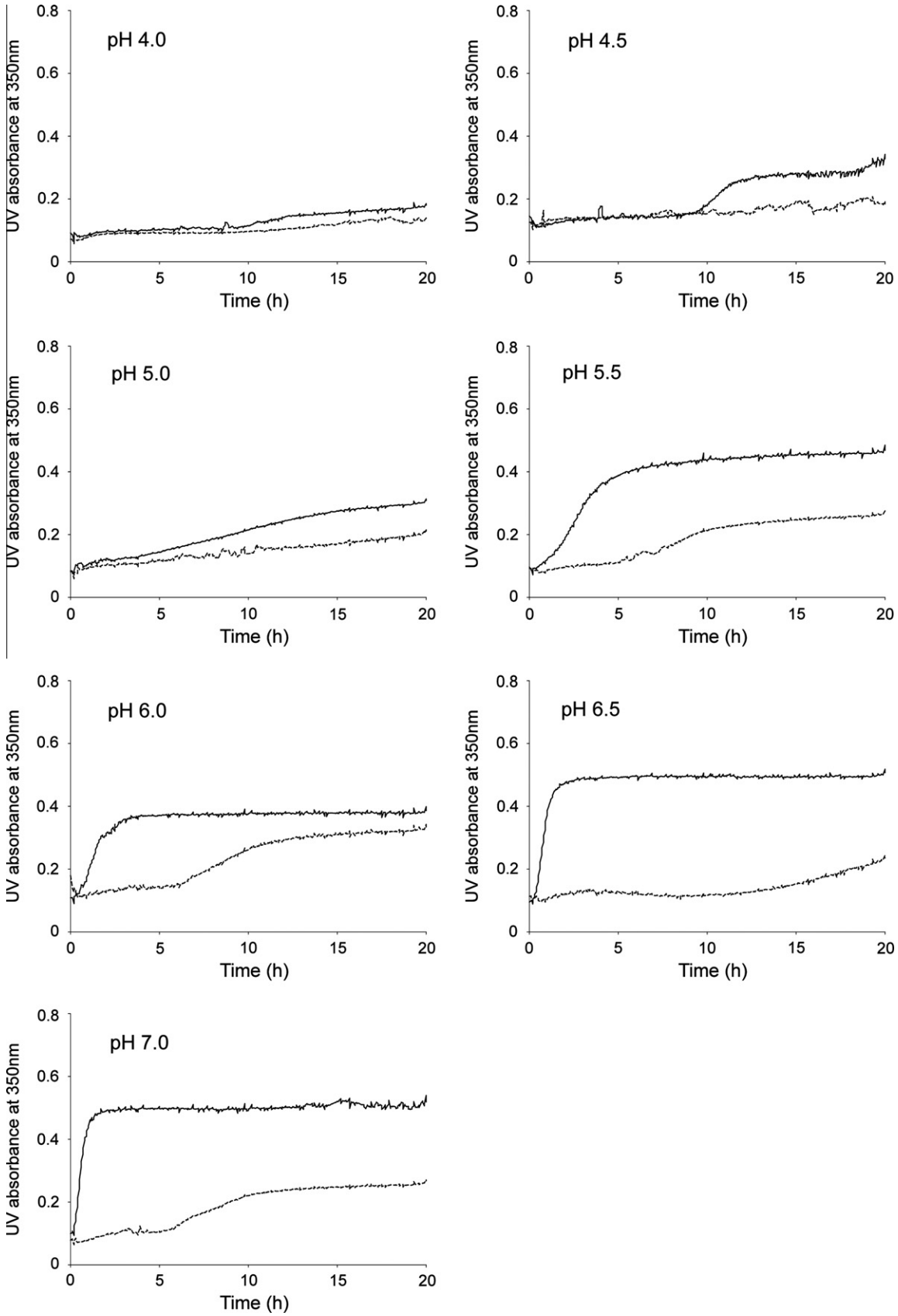

Fig. 5. Kinetics plots of UV absorbance at 350 nm of native and oxidized hCT at 37 °C. Native hCT is represented by a continuous line; oxidized hCT by a dotted line.

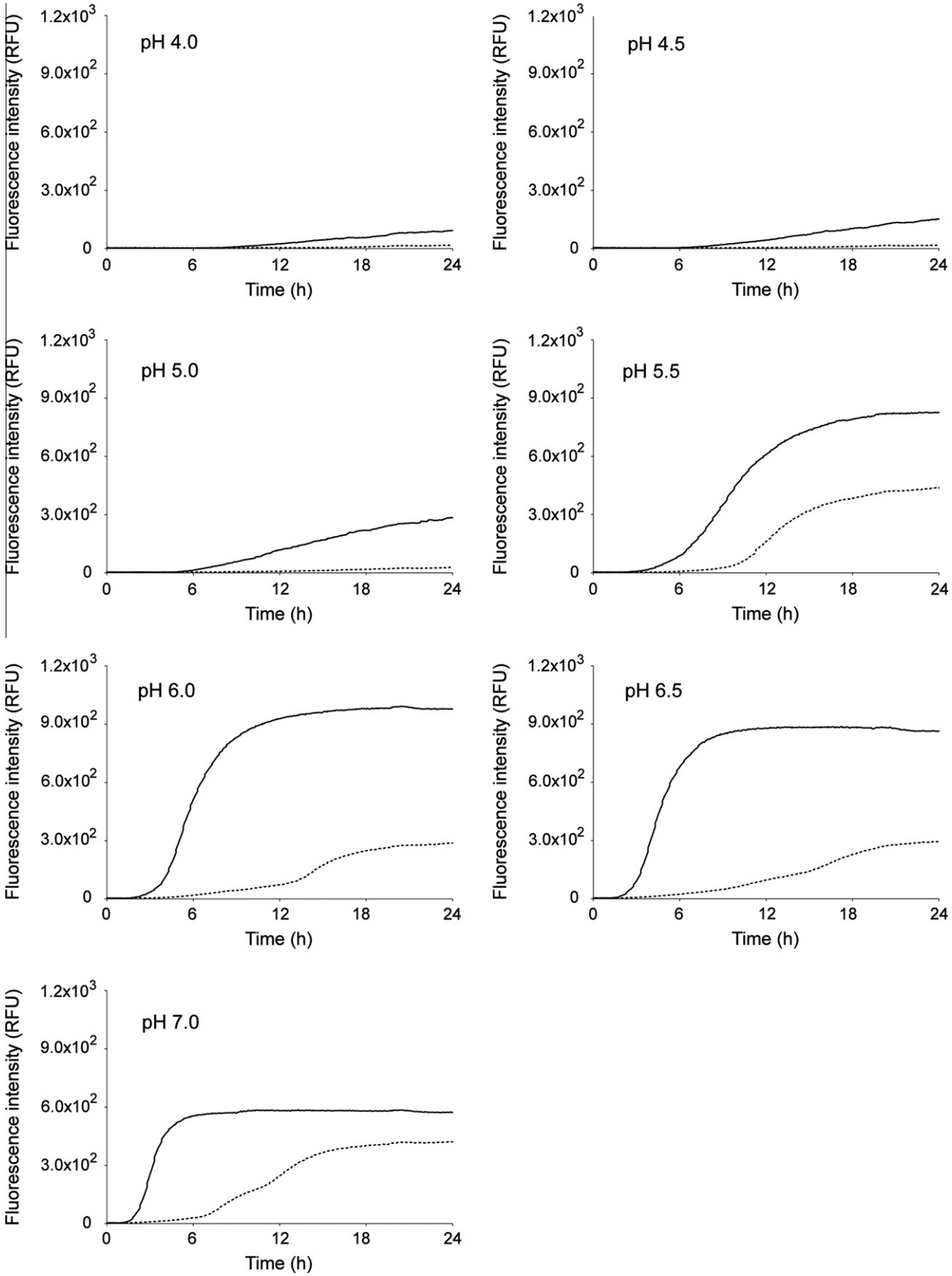

Fig. 6. Kinetics plots of 1,8-ANS fluorescence emission in presence of native and oxidized hCT at 37 °C. Fluorescence emission of 1,8-ANS was recorded at 620 nm after excitation at 550 nm. Native hCT is represented by a continuous line; oxidized hCT by a dotted line.

The longest lag phases were observed at pH 4, where native and oxidized hCT showed a lag phase of approximately 7 and 14 h, respectively. The shortest lag phases were observed at pH 7, where native and oxidized hCT showed lag phases shorter than 1 and 2 h, respectively. Results obtained in the presence of Nile Red are similar to those obtained with 1,8-ANS (data not shown).

3.3. Light microscopy and fluorescence microscopy with Nile Red and 1,8-ANS staining

Aggregation kinetics of native and oxidized hCT were monitored by light and fluorescence microscopy after incubation at 37 °C in 10 mM sodium citrate/phosphate pH 7.0. All the microscopy analyses were performed at room temperature.

Fig. 7A shows the light microscopy images of native and oxidized hCT samples after 0, 6, and 24 h of incubation at 37 °C. At the beginning of the incubation, no particles were detected in both native and oxidized hCT samples. After 6 h, native hCT showed the presence of particles with sizes between 30 and 150 μm; no particles were detected in the oxidized hCT. Samples after 24 h were not significantly different from the samples after 6 h incubation; oxidized hCT samples still did not show any particles by light microscopy.

The same samples were also observed by fluorescence microscopy; two fluorescent dyes, 1,8-ANS and Nile Red, were used. For both the native and the oxidized hCT, the two dyes were added individually to different aliquots of each of the two peptide samples. As a result, particles shown in the light microscopy images do not correspond to the particles present in the 1,8-ANS or Nile Red microscopy images.

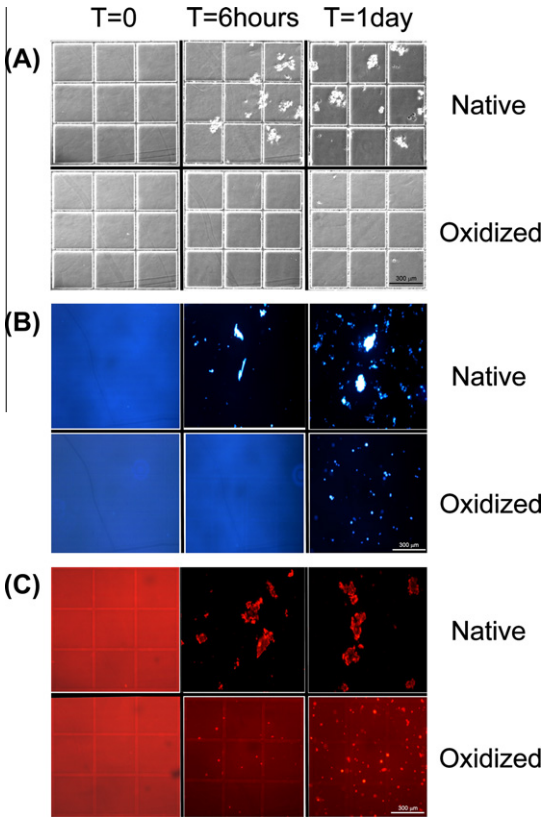

Fig. 7. Microscopy pictures of native and oxidized hCT in 10 mM sodium citrate/phosphate pH 7 at time 0 and after 6 h and 24 h incubation at 37 °C. (A) Light microscopy with enhanced phase contrast; (B) 1,8-ANS fluorescence microscopy; (C) Nile Red fluorescence microscopy. All pictures include a scale bar for rapid identification of particles size. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 7B shows the results of the 1,8-ANS fluorescence microscopy analysis. Staining with 1,8-ANS confirmed the results previously obtained by light microscopy with enhanced phase contrast. Incubation at 37 °C induced the formation of a larger number of particles in the native hCT samples. Staining with 1,8-ANS allowed a better detection of the aggregate particles; this is particularly evident for the oxidized hCT samples. Light microscopy analyses showed no particles in the oxidized hCT samples after 1-day incubation at 37 °C. On the contrary, fluorescence microscopy after staining with 1,8-ANS showed the presence of a large number of particles after 24 h. Although 1,8-ANS fluorescence microscopy showed the presence of particles in the oxidized hCT samples, these were less numerous and smaller than in the native hCT samples.

Fig. 7C shows the results of the fluorescence microscopy analysis after Nile Red staining. Compared to staining with 1,8-ANS, staining with Nile Red showed the presence of a larger number of particles in both peptides. In addition, Nile Red allowed the detection of oxidized hCT aggregates – previously undetected with other microscopy techniques – already after 6 h incubation.

The particles visualized by staining with Nile Red and 1,8-ANS were irregular in shape and heterogeneous.

3.4. Lifetime fluorescence of native and oxidized hCT after incubation at 37 °C

The mobility of Tyr^{12} was used to investigate the conformation changes of native and oxidized calcitonin upon incubation at 37 °C using intrinsic fluorescence lifetime. Fig. 8 shows the tyrosine mean weighted fluorescence lifetime of both native and oxidized hCT. During the first six hours of incubation, a decrease of tyrosine lifetime was observed for both peptides. At time 0, mean fluorescence lifetime of tyrosine residues was 1.4 ns in both peptides. After 6 h at 37 °C, tyrosine mean fluorescence lifetime values were 1.1 ns and 1.2 ns for the native and oxidized hCT, respectively. During the incubation, differences between the tyrosine mean fluorescence lifetime of native and oxidized peptides were observed. After 1 day of incubation, the tyrosine fluorescence lifetime of oxidized hCT increased to 2.1 ns; the tyrosine fluorescence lifetime of native hCT did not change. After 1 week at 37 °C, the tyrosine mean fluorescence lifetime was 1.1 ns and 2.2 ns in the native and oxidized hCT, respectively (data not shown).

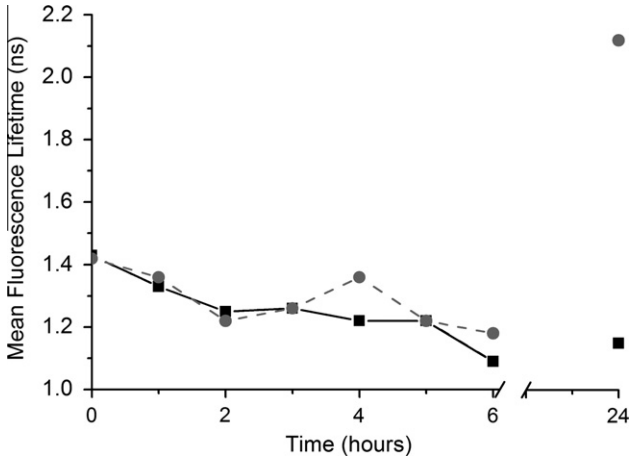

Fig. 8. Intrinsic mean fluorescence lifetime of human calcitonin in 10 mM sodium citrate/phosphate pH incubated at 37 °C for 1 day. Fluorescence lifetime of residue Tyr^{12} in native hCT is represented by a continuous line; lifetime of Tyr^{12} in oxidized hCT is indicated by a broken line.

4. Discussion

Biological macromolecules such as peptides, proteins, and antibodies have several features which can lead to instability through different degradation pathways. Among the various possible instabilities, aggregation is one often studied because of both its biological [23,24] and pharmaceutical consequences [25]. Protein aggregation is involved in neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease [24,26]. Aggregation is also a concern because of possible effects on bioactivity and safety of biotherapeutics.

Another degradation pathway is oxidation of methionine residues. Methionine oxidation is a chemical reaction that leads to formation of a sulfoxide group in the side chain of the methionine residue. The product of this chemical reaction is a more polar residue with a bigger steric hindrance. As other protein degradations, methionine oxidation can be a problem for the approval of new drugs. Authorities such as the Food and Drug Administration (FDA) and the European Medicines Agency (EMEA) demand an extensive characterization of every new drug and its related impurities. Recommended ICH guidelines have set thresholds to the maximum percentage of impurities that can be accepted in new drugs [27]. These thresholds generally vary between 0.05% and 1.0% based on the daily dose. According to guidelines, higher percentages of impurities need to be scientifically justified.

Methionine oxidation is also critical because it has been shown to influence protein conformation and aggregation. Liu et al. showed that oxidation of methionine residue Met^{33}, located in the C_H2 domain of human IgG1 Fc, increased the aggregation rate of the Fc when incubated at 45 °C; oxidation of the same residue was also responsible for an increase in the deamidation rates of the two asparagine residues, Asp^{67} and Asp^{96}, both located in the C_H2 domain [13]. Conformational changes following methionine oxidation can also be responsible for reduced potency and loss of bioactivity. Teh et al. showed that oxidation of methionine residue Met^{64} or Met^{179} greatly reduces the affinity of hCS for lactogenic receptors and its *in vitro* potency [14].

Our study on hCT is, to our knowledge, the first example of a biotherapeutic drug whose aggregation is partially inhibited as a consequence of methionine oxidation. In all our experiments, oxidized hCT was found to be more stable than the non-oxidized form. Upon incubation at 37 °C, an increase in the fluorescence emission of Tyr^{12} was observed in both oxidized and non-oxidized hCT. The increase was bigger and faster in native hCT. Previously, Arvinte et al. reported that tyrosine fluorescence emission of hCT increases in the presence of low dielectric solvents [28]. This increase was attributed to a conformation change of hCT in which the peptide undergoes a transition from an unordered to a more α-helical conformation. Arvinte et al. also showed that the formation of α-helices is one of the first steps in hCT aggregation [3]. Based on these previous results, our intrinsic fluorescence results suggest that oxidized hCT is less prone to conformation changes and aggregation.

As oxidized hCT was more stable than native hCT at all the pH values tested, the influence of methionine oxidation on hCT physical stability does not seem to be pH-dependent. The role of pH on the hCT aggregation was similar in both the oxidized and the non-oxidized peptide: acidic conditions had a beneficial effect on hCT physical stability. These findings are in agreement with previous results by Lu et al. who reported a higher stability for human calcitonin in acidic environments [29].

Using light microscopy with enhanced phase contrast, Nile Red and 1,8-ANS fluorescence microscopy showed a quicker formation of aggregates in native hCT compared to oxidized hCT. The use of fluorescent dyes allowed an earlier detection of aggregates formation. After 1-day incubation at 37 °C, no particles were observed by light microscopy, while several particles were visualized by Nile Red fluorescence microscopy. Results obtained with Nile Red fluorescence microscopy differed from those obtained with 1,8-ANS fluorescence microscopy: staining with Nile Red allowed a better detection of hCT aggregates than with 1,8-ANS (Fig. 7). This is more evident in oxidized hCT samples because of the presence of fewer particles than in native hCT samples. After 6 h of incubation at 37 °C, staining with 1,8-ANS showed no presence of particles in the oxidized hCT sample; on the contrary, staining with Nile Red allowed the detection of small aggregates inside the same sample. This difference can be attributed both to the color of these dyes or to their different binding mechanism to hCT. While for spectroscopic analyses, both dyes can be easily used, for fluorescence microscopy the blue background due to 1,8-ANS can sometime mask some particles and thus make their detection more difficult. Furthermore, Nile Red binds predominantly to hydrophobic regions, while 1,8-ANS binds with electrostatic interactions [30,31]. The different binding mechanisms of the two fluorescent dyes could explain the differences between the Nile Red and 1,8-ANS microscopy pictures. Depending on the nature of the aggregate particles, binding to one of the two dyes can be favored; the results will be a better detection of those aggregates with one of the two dyes.

The effect of methionine oxidation in slowing down hCT aggregation can be explained by considering the differences between methionine and its corresponding sulfoxide forms. In the absence of oxidation, the molecular conformation of hCT can accommodate methionine residues with non-polar thioether groups in the side chain. Upon oxidation, transformation of the thioether group to the sulfoxide stereoisomers increases the physical size of the methionine side chain as it becomes more polar in the oxidized form. The side chain size and polarity changes can influence the formation and stability of the aggregation-associated α-helix to reduce the aggregation rate.

Biopharmaceuticals are known to have the potential of inducing immune reactions. These immune reactions are divided in two classes: (i) immune reactions to neo-antigens and (ii) breakdowns of immune tolerance [32]. The first class is characterized by a rapid reaction in response to foreign antigens such as animal- or plant-derived proteins. In the second class, the breakdowns of immune tolerance, antibodies are formed against recombinant human homologues like interferon or erythropoietin. This second class of immune reactions, characterized by a slower process which can take several years before becoming clinically significant, is also believed to be induced by the presence of aggregates [33]. There is no direct relationship reported in the literature between immunogenicity and aggregation [34]. However, several examples of protein degradation products (aggregates, oxidized, or deamidated forms) could be identified as the cause of immunogenicity [34]. Different aggregates might also stimulate the immune response to a different extent; as a consequence, some protein aggregates may be more or less immunogenic than others. In our analyses, we investigated the conformation of aggregates formed in the oxidized and the non-oxidized human calcitonin using intrinsic fluorescence lifetime. Our study showed that the fluorescence lifetime of Tyr^{12} is different in native and oxidized hCT aggregates (Fig. 8). During the initial phase of aggregation, a decrease of Tyr^{12} fluorescence lifetime for both native and oxidized hCT was found; the magnitude of the decrease was similar in both samples. However, after about 1 day, once larger aggregates had been formed in both hCT samples, native hCT had a smaller fluorescence lifetime than oxidized hCT. Intrinsic fluorescence lifetime spectroscopy has been used in protein analyses to study conformation changes around the aromatic residues responsible for a fluorescence phenomenon [35]. In hCT, changes of Tyr^{12} fluorescence lifetime can indicate

conformational changes surrounding this tyrosine residue. The similar decrease in fluorescence lifetime observed in both peptides suggests that during the initial phase of aggregation both native and oxidized hCT undergo a similar conformational change. This change of conformation may be associated with the transition from unordered to α-helical structure as previously mentioned. After this initial step, hCT molecules in non-native conformations may interact with each other and form bigger aggregates in which the environment surrounding Tyr^{12} is different between oxidized and non-oxidized peptide.

In the current work, the two oxidized hCT stereoisomers associated with a sulfoxide in position Met^{8} were not separated. Although there is no evidence in the literature about the role of the stereochemistry of methionine sulfur atom on the conformation and stability of oxidized proteins, the conformations of the two stereoisomers of an oxidized peptide may be different. These different conformations of the two oxidized stereoisomers might imply different physical stabilities and could be partially responsible for the differences in the aggregation properties of oxidized and non-oxidized hCT samples.

In conclusion, our results showed an example of a biotherapeutical drug whose physical stability is enhanced, and not reduced as often reported in literature [13,36–39], as consequence of methionine oxidation. These findings clearly suggest that the effect of methionine oxidation on protein aggregation cannot be easily predicted and must be evaluated on a case-by-case basis.

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