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Low molar excess of 4-oxo-2-nonenal and 4-hydroxy-2-nonenal promote oligomerization of alpha-synuclein through different pathways

Leire Almandoz-Gil[a], Hedvig Welander[a], Elisabet Ihse[a], Payam Emami Khoonsari[b], Sravani Musunuri[b], Christofer Lendel[c], Jessica Sigvardson[d], Mikael Karlsson[e], Martin Ingelsson[a], Kim Kultima[b,1], Joakim Bergström[a,*,1]

[a] Department of Public Health and Caring Sciences, Molecular Geriatrics, Uppsala University, SE-751 85 Uppsala, Sweden
[b] Department of Medical Sciences, Uppsala University, SE-751 85 Uppsala, Sweden
[c] Department of Chemistry, KTH Royal Institute of Technology, SE-100 44 Stockholm, Sweden
[d] BioArctic AB, Warfvinges väg 35, SE-112 51 Stockholm, Sweden
[e] Department of Engineering Sciences, Uppsala University, SE-751 21 Uppsala, Sweden

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ABSTRACT

Aggregated alpha-synuclein is the main component of Lewy bodies, intraneuronal inclusions found in brains with Parkinson's disease and dementia with Lewy bodies. A body of evidence implicates oxidative stress in the pathogenesis of these diseases. For example, a large excess (30:1, aldehyde:protein) of the lipid peroxidation end products 4-oxo-2-nonenal (ONE) or 4-hydroxy-2-nonenal (HNE) can induce alpha-synuclein oligomer formation. The objective of the study was to investigate the effect of these reactive aldehydes on alpha-synuclein at a lower molar excess (3:1) at both physiological (7.4) and acidic (5.4) pH. As observed by size-exclusion chromatography, ONE rapidly induced the formation of alpha-synuclein oligomers at both pH values, but the effect was less pronounced under the acidic condition. In contrast, only a small proportion of alpha-synuclein oligomers were formed with low excess HNE-treatment at physiological pH and no oligomers at all under the acidic condition. With prolonged incubation times (up to 96 h), more alpha-synuclein was oligomerized at physiological pH for both ONE and HNE. As determined by Western blot, ONE-oligomers were more SDS-stable and to a higher-degree cross-linked as compared to the HNE-induced oligomers. However, as shown by their greater sensitivity to proteinase K treatment, ONE-oligomers, exhibited a less compact structure than HNE-oligomers. As indicated by mass spectrometry, ONE modified most Lys residues, whereas HNE primarily modified the His50 residue and fewer Lys residues, albeit to a higher degree than ONE. Taken together, our data show that the aldehydes ONE and HNE can modify alpha-synuclein and induce oligomerization, even at low molar excess, but to a higher degree at physiological pH and seemingly through different pathways.

1. Introduction

Intraneuronal inclusions of aggregated alpha-synuclein, known as Lewy bodies, are the neuropathological hallmark of Parkinson's disease (PD) and dementia with Lewy bodies (DLB) [1]. The physiological function of alpha-synuclein has not been completely elucidated, but the native protein has been suggested to be involved in neurotransmitter release [2], modulation of synaptic plasticity [3] and vesicle recycling [4]. Furthermore, recent studies indicate that alpha-synuclein might have an important role in the assembly process of soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) complexes, by binding to the SNARE protein VAMP2 [5,6].

Alpha-synuclein consists of 140 amino acid residues and can be divided into three structurally distinct domains: a lipid-binding amphipathic N-terminal region (1–60), a central hydrophobic region (residues 61–95) and a negatively charged C-terminal region (residues 96–140). During the aggregation process, natively unstructured monomeric alpha-synuclein undergo conformational changes into more folded intermediate molecular species with increasing molecular weight, such as oligomers and protofibrils [7]. Continued

Abbreviations: PD, Parkinson's disease; DLB, dementia with Lewy bodies; ONE, 4-oxo-2-nonenal; HNE, 4-hydroxy-2-nonenal; SEC, size-exclusion chromatography; AFM, atomic force microscopy; PK, proteinase K; CD, circular dichroism; ELISA, enzyme-linked immunosorbent assay

* Corresponding author.
E-mail address: joakim.bergstrom@pubcare.uu.se (J. Bergström).
[1] These authors contributed equally to this work.

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polymerization ultimately gives rise to insoluble β-sheet rich fibrils [8,9]. The formed oligomers can either be on-pathway, i.e. on their way of forming fibrils, or off-pathway, i.e. adopting a kinetically stable conformational state that precludes them from polymerizing into fibrils [10]. The underlying mechanisms by which aggregated forms of alpha-synuclein cause neurodegeneration are not completely understood, but growing evidence suggests that oligomers display particularly neurotoxic properties [11–14].

Six point mutations (A30P, E46K, H50Q, G51D, A53E and A53T) have been found to be associated to early onset familial forms of PD and DLB [15–20]. Furthermore, patients carrying duplications and triplications of the alpha-synuclein gene develop familial forms of the diseases [21,22], indicating a crucial role of alpha-synuclein aggregation in the pathogenesis of PD and DLB.

Oxidative stress appears to play a central role in the pathogenesis of neurodegenerative disorders. The brain has a high consumption of oxygen in combination with a low level of antioxidant scavenging enzymes [23]. Moreover, a high amount of polyunsaturated fatty acids (PUFAs), and high levels of redox transition metals lead to an excess production of reactive oxygen species (ROS) [24–26]. As ROS are highly unstable, they can initiate lipid peroxidation of PUFAs, which is a central event of free radical mediated injury [26]. In this process, a range of secondary products are generated, such as the reactive aldehydes 4-oxo-2-nonenal (ONE) and 4-hydroxy-2-nonenal (HNE) [27–29]. Both ONE and HNE can form covalent adducts with proteins, thereby potentially altering their structure and thus impairing protein function [30]. The chemical structures of ONE and HNE are nearly identical and they only differ at their C4 position, where ONE has a carbonyl group and HNE has a hydroxyl group (Fig. 1A) [30–32].

It has been shown that HNE-modified proteins can be observed to a higher extent in nigral neurons in both PD and DLB [33,34]. Moreover, it has been demonstrated that both ONE and HNE readily react with alpha-synuclein and induce aggregation in vitro [35–40]. Alpha-synuclein can be modified by ONE and HNE via Michael addition, which generates adducts through side chains of Cys, His or Lys residues [41,42]. Moreover, ONE and HNE also modify proteins through Schiff base formation with Lys residues [41,42]. However, the chemistry of ONE is much more diverse, e.g., the Schiff base over time can give rise to a 4-ketoamide which is isomeric to a Lys-ONE Michael adduct [43]. Alpha-synuclein contains no Cys residues, one His (at position 50) and fifteen Lys residues (located between amino acids 6 and 102) (Fig. 1B). Our group has recently demonstrated that ONE and HNE-induced alpha-synuclein oligomers differ in morphological and biochemical characteristics [40]. However, in these previous studies a high molar excess (20:1 – 30:1) of ONE and HNE were used [35–40]. Here, we wanted to investigate the effect of the aldehydes on alpha-synuclein oligomerization at a lower, and probably more physiological, molar excess (3:1). Moreover, as acidic pH has been shown to increase the aggregation rate for unmodified alpha-synuclein [7,44], we also wanted to investigate the effect of the aldehydes on oligomerization at an acidic pH.

2. Material and methods

2.1. Chemicals

ONE (5 mg/ml) in 99% methyl acetate and HNE (10 mg/ml) was supplied in 99% ethanol, (Cayman Chemical Ann Arbor, MI). Recombinant alpha-synuclein was expressed and purified as previously described [36]. Acetonitrile (ACN), acetic acid (HAc), trifluoroacetic acid (TFA) and ammonium bicarbonate (AmBi) were obtained from Merck (Darmstadt, Germany). Trypsin and Glu-C (sequencing grade from bovine pancreas and Staphylococcus aureus, respectively; Roche diagnostic, Basel, Switzerland) was used for digestion of each alpha-synuclein sample.

2.2. Protein determination

The alpha-synuclein concentration was determined using Pierce BCA kit (Thermo Scientific, Rockford, IL) according to manufacturer's instructions.

2.3. Modification of alpha-synuclein with ONE or HNE

Recombinant alpha-synuclein (140 μM) was buffer exchanged to a 50 mM sodium phosphate buffer, pH 5.4 or pH 7.4, using Zeba™ desalt spin columns (Thermo Scientific). To generate aldehyde modified alpha-synuclein species, ONE or HNE was added to alpha-synuclein (140 μM), with a final concentration equivalent of a molar ratio of 3:1 (ONE/HNE: alpha-synuclein). Then, samples were quiescently incubated at 37 °C for six different time points: 15 min, 30 min, 45 min, 1 h, 8 h and 24 h. In a separate experiment, samples were incubated for 96 h.

2.4. Production of unmodified alpha-synuclein oligomers through lyophilization

The unmodified oligomers were prepared according to previous protocols [45,46]. First, 600 μL of recombinant alpha-synuclein at a concentration of in MilliQ water was lyophilized using a VirTis

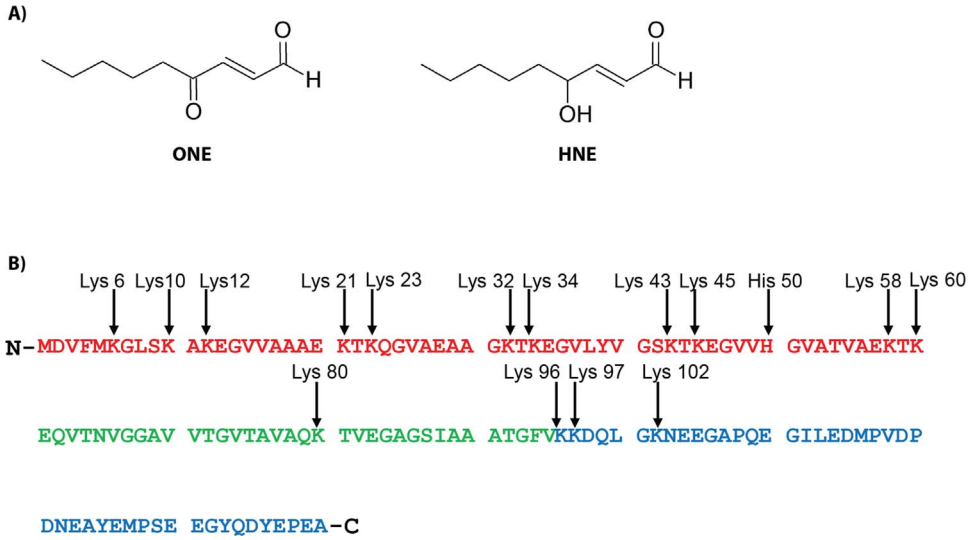

Fig. 1. The chemical structure of 4-oxo-2-nonenal (ONE) and 4-hydroxy-2-nonenal (HNE) (A). The alpha-synuclein amino acid sequence indicating His and Lys residues (B). Alpha-synuclein's three different structural regions: N-terminus (1–60, red), the hydrophobic core region (61–95, green) and the C-terminus (96–140, blue) (B).

benchtop K freeze dryer (SP Scientific, Warminster, PA). The samples was reconstituted in PBS to a final concentration of and filtered through a Corning Costar Spin X Centrifuge Tube Filter 0.45 μm (Sigma-Aldrich, St. Louis, MO), immediately before incubation at 37 °C for 24 h quiescently.

2.5. Size exclusion chromatography

The ONE- or HNE-modified alpha-synuclein samples were diluted to 20 μM using 50 mM Tris/0.15 M NaCl, pH 7.4 and were centrifuged at 16.000 × *g* for 5 min before injecting 10 μL onto a Superose 6 PC 3.2/30 column (GE Healthcare, Uppsala, Sweden). A 50 mM Tris/0.15 M NaCl, pH 7.4 buffer was used as mobile phase with a flow rate of 50 μL/min. The UV absorbance was monitored at 214 nm. Unmodified oligomeric samples at 640 μM were centrifuged at 16.000 × *g* for 5 min and 50 μL injected onto a Superose 6 Increase 5/150 GL column (GE Healthcare) using 50 mM Tris/0.15 M NaCl, pH 7.4 as mobile phase with a flow rate of 0.2 ml/min.

In a separate experiment, 25 μL of 20 μM 96 h HNE-modified alpha-synuclein samples incubated at pH 7.4 were injected onto a Superose 6 Increase 5/150 GL column (GE Healthcare) using 50 mM Tris/0.15 M NaCl, pH 7.4 as mobile phase with a flow rate of 0.2 ml/min. The oligomeric and monomeric fractions were collected and stored at −20 °C for subsequent enzyme-linked immunosorbent assay (ELISA) measurements.

High molecular weight and low molecular weight gel filtration calibrations kits (GE Healthcare) were used to estimate the molecular weight of the alpha-synuclein species. The following globular standards were included: blue dextran (2000 kDa), thyroglobulin (669 kDa), ferritin (440 kDa), aldolase (158 kDa), conalbumin (75 kDa), ovalbumin (43 kDa), carbonic anhydrase (29 kDa), and ribonuclease (14 kDa) (Sigma-Aldrich).

2.6. Atomic force microscopy

For atomic force microscopy analysis (AFM) analysis, an XE-150 large-sample AFM system (Park systems, Santa Clara, CA) equipped with a 150 × 150 mm xy scanner was used. All measurements were performed with silicon-based AFM probes (ACTA; Applied NanoStructures, Santa Clara, CA). ONE- or HNE- 96 h treated, pH 5.4 or pH 7.4, alpha-synuclein samples (10 μL at 35 μM diluted in distilled water) were adsorbed to a freshly cleaved mica surface (Veeco, Cambridge, UK) overnight at RT. Before analysis, the mica was washed with distilled water and dried with nitrogen gas. All samples were analyzed at ambient temperature in true non-contact mode.

2.7. SDS-PAGE Western blotting

One μL of the 140 μM aliquots of ONE- or HNE-treated alpha-synuclein from each time point (15 min, 30 min, 45 min, 1 h, 8 h, 24 h and 96 h) was mixed with 19 μL of ultrapure water. Next, 7 μL from each sample was mixed in 2 x Laemmli sample buffer (Sigma-Aldrich) and subsequently separated by SDS-PAGE on 10–20% Tricine gels (Life Technologies, Carlsbad, CA). After electrophoresis, the proteins were transferred to an immobilon FL polyvinyldifluoride membrane (Millipore, Bedford, MA). A monoclonal alpha-synuclein antibody (211, Santa Cruz Biotechnology, Santa Cruz, CA) at 0.5 μg/ml and a fluorescent goat anti-mouse DyLight 800 (Thermo Scientific) at 0.1 μg/ml were used as primary and secondary antibodies. The membrane was scanned at 800 nm, using an Odyssey fluorescence scanner (LI-COR Biosciences, Lincoln, NE). A protein molecular weight marker (10–250 kDa) was used (LI-COR Biosciences).

2.8. Proteinase K digestion and dot blot

ONE- or HNE-modified alpha-synuclein samples at a concentration of 0.2 mg/ml (incubated with the aldehydes for 96 h at pH 5.4 or 7.4) were treated with 0, 10, 40 and 80 μg/ml proteinase K (PK) (Sigma-Aldrich) in 50 mM sodium phosphate buffer, pH 7.4, for 30 min at 37 °C and the reaction was stopped by incubating the samples at 95 °C for 5 min. One μL (200 ng of protein) of sample was coated onto a 0.2 μm nitrocellulose membrane (Bio-Rad, Hercules, CA) and the membrane was blocked in 5% milk powder diluted in 50 mM Tris/0.15 M NaCl, pH 7.4. The membrane was incubated with a polyclonal alpha-synuclein antibody (Fl-140, Santa Cruz Biotechnology) at 0.5 μg/ml for 1 h. A fluorescent goat anti-rabbit-800 (Thermo Scientific) at 0.1 μg/ml was used as secondary antibody and the blot was scanned at 700 nm and 800 nm, using an Odyssey fluorescence scanner (LI-COR Biosciences).

2.9. Circular dichroism

Unmodified and ONE- or HNE-treated alpha-synuclein (140 μM) was incubated for 96 h and diluted in 50 mM sodium phosphate buffer, pH 5.4 or 7.4, to a final concentration of 14 μM. The circular dichroism (CD) spectra were recorded at 25 °C in 50 mM sodium phosphate buffer, pH 5.4 or pH 7.4 using a Jasco J-810 spectropolarimeter (Jasco, Easton, MD). The measurements were carried out using a 1 mm cuvette and the displayed data represent the average of ten scans with the buffer background subtracted. Spectra for pure oligomers were estimated by treating the recorded data as weighted sum of the spectra of monomer and oligomers:

$$CD_{observed} = x \cdot CD_{monomer} + (1 - x) \cdot CD_{oligomers}$$

with x equal to the population of monomer observed in the SEC experiments. Monomer populations for the displayed data were 0.83 and 0.40 for ONE treated samples at pH 5.4 and pH 7.4, respectively and 0.67 for HNE-treated sample at pH 7.4.

2.10. On-filter digestion of alpha-synuclein

An on-filter digestion protocol was used for digestion of the samples using 3 kDa filters (Merck Millipore). Centrifugation was performed at a centrifugal force of 14.000 × *g* throughout the protocol. Aliquots corresponding to 100 μg of protein sample were diluted in 50 mM AmBi. The samples were transferred to spin filters which had been pre-washed with 250 μL of 20% ACN for 15 min and then 500 μL of MQ water for 20 min. The samples were then centrifuged for 10 min to remove interfering substances. An additional volume of 200 μL of 20% ACN in 100 mM AmBi was added and the filters were spun for 15 min followed by 150 μL of 20% ACN in 100 mM AmBi and 150 μL of 100 mM AmBi, and centrifugation for another 15 min. Finally, a volume of 100 μL of 100 mM AmBi was added together with trypsin or Glu-C to yield a final protease enzyme/protein concentration of 2.5% (w/w). The digestion was performed at 37 °C overnight. The digests were spun through the filter for 20 min to collect the peptides. An additional volume of 100 μL of 20% ACN, 1% HAc was added and the filters were spun for 10 min and pooled with the first tryptic or Glu-C peptide filtrate. The collected filtrates were vacuum centrifuged to dryness using a Speedvac system ISS110 (Thermo Scientific). The peptide mixtures were reconstituted in 100 μL of 0.1% formic acid.

2.11. Liquid chromatography-tandem mass spectrometry

The peptide extracts were analyzed using a Q Exactive quadrupole orbitrap mass spectrometer (Thermo Scientific) coupled to a Ultimate 3000 (Dionex Thermo Scientific, Sunnyvale, CA) HPLC through a heated electrospray ionization probe (HESI II, Thermo Scientific). Ten μL of peptide extract were analyzed in duplicate and the peptide separations were performed on an Acclaim RSCL column (50-μm inner diameter, 150 mm long). The separations were performed at a flow rate of 400 μL/min with mobile phases A (water with 0.1% formic acid) and B (98% acetonitrile and 0.1% formic acid). A 25 min gradient from 0% B

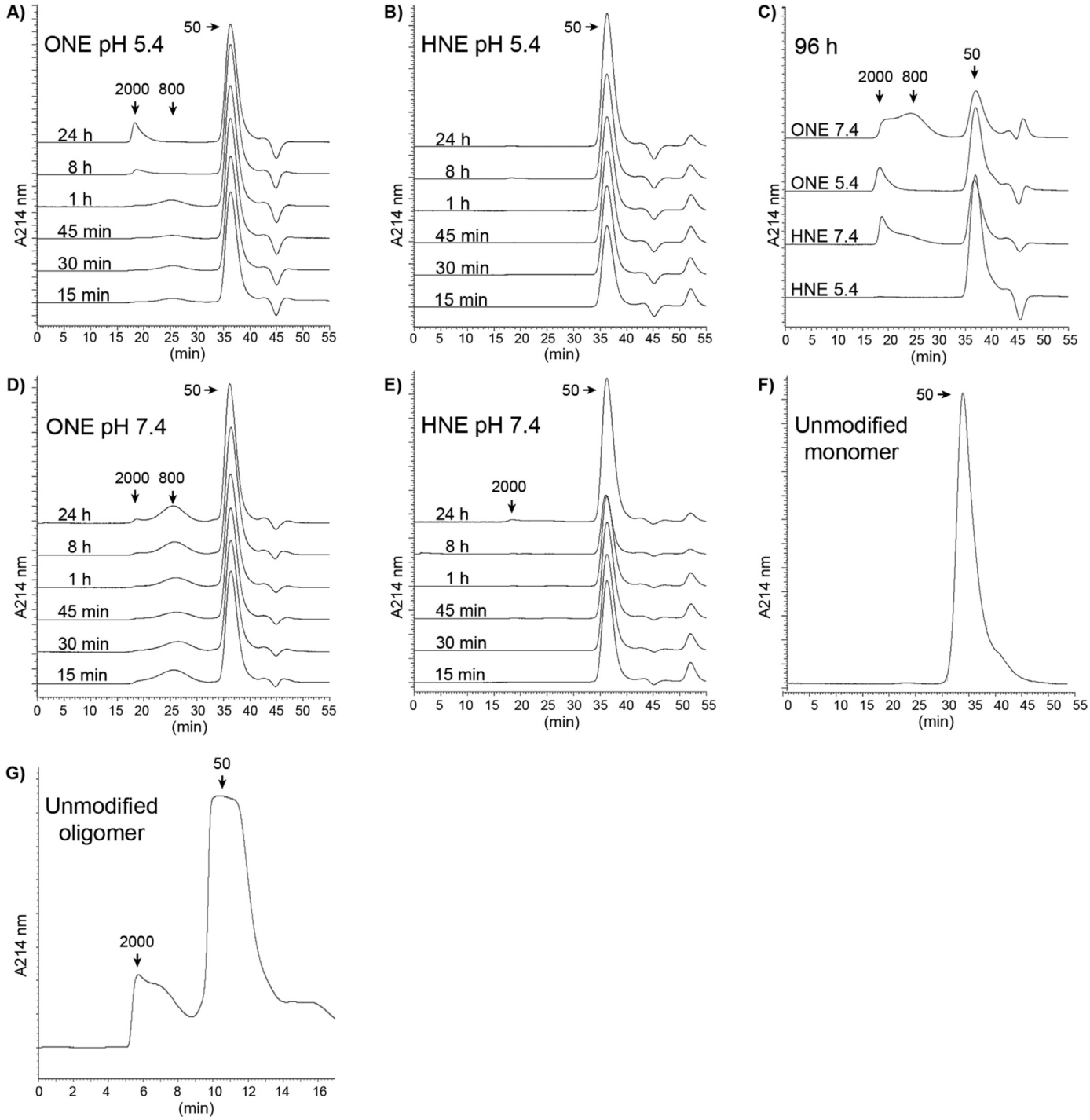

Fig. 2. Size exclusion chromatography analysis of alpha-synuclein after incubation with ONE or HNE at pH 5.4 and 7.4 between 15 min – 96 h at 37 °C. ONE induced alpha-synuclein oligomers at pH 5.4 after 15 min (~ 800 kDa) and they increased in size (up to ~ 2000 kDa) with prolonged incubation (A). ONE-induced alpha-synuclein oligomers (~ 800 kDa) formed at pH 7.4 already after 15 min and with increasing incubation time a small peak around ~ 2000 kDa could also be observed (D). No alpha-synuclein oligomers were formed at pH 5.4 with HNE treatment (B). At pH 7.4, HNE induced a small population of alpha-synuclein oligomers observed only after 24 h (E). After 96 h of incubation, increased amount of oligomers with a molecular weight about 2000 kDa could be detected at pH 5.4 for ONE and at pH 7.4 for HNE (C). After 96 h of incubation with ONE at pH 7.4, two oligomeric peaks at ~ 800 and ~ 2000 kDa could be seen to a much higher degree. No oligomers could be observed with HNE at pH 5.4 after 96 h (C). Unmodified monomeric alpha-synuclein eluted with a molecular weight of ~ 50 kDa (F). Oligomers produced through lyophilization of alpha-synuclein eluted as a broad peak with a maximum at 2000 kDa (G).

to 55% B followed by a washing step with 100% B for 2 min and the column was re-equilibrated for 5 min at 600yL/min. The Q Exactive instrument was operated in full profile MS mode (70,000 resolution, scan range 350–1600 *m/z*, maximum injection time 240 ms, a maximum AGC of 1×10^6), followed by eight consecutive MS/MS (17,500 resolution stored in centroid mode, maximum injection time 60 ms, relative collision energy of 28, isolation window of 3.0 *m/z*, no offset). The acquired data were converted to the .mgf format and subjected to peptide identification using X! Tandem (version Piledriver, 2015.04.01.1) (PMID: 14976030) against the human Ensembl GRCh37 database. The search parameters were set to Taxonomy: Homo sapiens, Enzyme: trypsin or glu-C, variable modifications: Oxidation (M), Deamidated (NQ), HNE (CHK), 4-ONE (H) and 4-ONE (K). For refinement was used HNE-Delta:H_2O(H), HNE-Delta:H_2O(K), 4-ONE

+Delta:$H_2O(-1)$ (H) and 4-ONE+Delta:$H_2O(-1)$(K). Peptide tolerance: 5 ppm, MS/MS tolerance: 50 ppm and maximum 2 missed cleavage sites. Valid identification log score < −0.4 corresponded to false discovery rate of < 0.01. In addition, all modifications found were manually curated.

The quantification of peptides was performed in OpenMS [pmid: 18366760]. The data was centroided using PeakPickerHiRes and features were quantified using FeatureFinderMetabo [PMC3879626] with default parameters (except for the following: noise_threshold_int: 50000; max_trace_length: 400; local_rt_range: 40; local_mz_range: 40; charge_lower_bound: 1; charge_upper_bound: 5; isotope _model: Peptide; use_smoothed_intensities: FALSE). The features across the runs were linked using FeatureLinkerUnlabeledQT with the following parameters: distance_RT: 25 s; distance_MZ: 10 ppm. To calculate the relative fraction of modified peptide, the ratio between the modified and corresponding unmodified peptide was calculated. This ratio was then used to calculate the relative amount between pH 7.4 and pH 5.4. For Lys 154 Da & 156 Da modification only peptides cleaved with Glu-C and no missed cleavages were used and for His 156 Da modification only peptides cleaved with trypsin and no missed cleavages were used.

2.12. Enzyme-linked immunosorbent assay (ELISA)

For the ELISA, half-area high-binding 96-well polystyrene plates (Corning Inc, Corning, NY) were coated with 50 ng of HNE-treated alpha-synuclein samples (15 min, 30 min, 45 min, 1 h, 8 h, 24 h and 96 h) diluted in 50 mM sodium phosphate/0.15 M NaCl, overnight at 4 °C. The plate was blocked with 50 mM sodium phosphate/0.15 M NaCl, pH 7.4 supplemented with 1% bovine serum albumin for 2 h. Next, the plate was incubated for 1 h at RT with 0.33 μg/ml of a mouse monoclonal antibody (mAb3249; R & D Systems, Minneapolis, MN), specifically detecting HNE adducts of His residues, diluted in 50 mM sodium phosphate/0.15 M NaCl, pH 7.4 supplemented with 1% BSA and 0.05% Tween-20. The plate was incubated with goat anti-mouse-HRP antibody at 0.08 μg/ml (Thermo Scientific) in 50 mM sodium phosphate/0.15 M NaCl, pH 7.4 supplemented with 1% BSA and 0.05% Tween-20 for 1 h. The K-blue aqueous solution (Neogen, Lansing, MI) was used as a substrate for HRP. Before measurement, the reaction was stopped using 1 M H_2SO_4. Each sample was measured in duplicate and the ELISA analysis was performed in three separate experiments. The absorbance was measured at 450 nm using an Infinite M200 Pro microplate reader (Tecan, Männedorf, Switzerland). The signal of the blank (no coated antigen) was subtracted from the sample signal.

For the analysis of HNE His modifications in the oligomeric and monomeric fractions of HNE-treated, pH 7.4 alpha-synuclein samples at the 96 h time point, the above protocol was used with the following changes: a serial dilution of oligomeric and monomeric fractions of HNE-treated alpha-synuclein samples were coated on the plate. In addition to an ELISA with HNE modified histidine antibody, the samples were incubated with 1 μg/ml of Fl-140 (Santa Cruz Biotechnology) alpha-synuclein polyclonal primary antibody and a goat-anti-rabbit secondary antibody at 0.04 μg/ml (Thermo Scientific) to determine the concentration of alpha-synuclein.

3. Results

3.1. Size exclusion chromatography analysis

In order to study the kinetics of alpha-synuclein oligomer formation with the incubation of ONE and HNE at a molar ratio of 3:1 (aldehyde: protein) at pH 5.4 and pH 7.4, samples were collected and analyzed by size exclusion chromatography (SEC) after 15 min, 30 min, 45 min, 1 h, 8 h and 24 h (Fig. 2). The analyses showed that ONE was able to induce oligomers at pH 5.4 and 7.4, although fewer oligomers were formed at pH 5.4 (Fig. 2 A and D). After 15 min at pH 7.4, an 800 kDa oligomeric peak could be observed and it reached its maximum after 24 h (~ 14% of the monomeric peak area). A small shoulder peak at ~ 2000 kDa, representing larger oligomeric species, could also be seen after 24 h. In contrast, at pH 5.4 a smaller ~ 800 kDa peak initially formed (between 15 min – 1 h), which after 8 h of incubation had shifted to a size of about ~ 2000 kDa, and the peak area continued to increase up to 24 h (~ 7% of the monomeric peak area). At both pH values, monomeric alpha-synuclein, eluting with a molecular weight of about ~ 50 kDa, was the dominating molecular species. For HNE at pH 7.4, a small amount of alpha-synuclein oligomers (~ 0.5% of the monomeric peak) with a molecular weight of ~ 2000 kDa could be observed after 24 h (Fig. 2E). No oligomers were formed at pH 5.4 with HNE treatment and only a monomeric peak could be observed (Fig. 2B).

An earlier study using higher excess of HNE (30:1) showed an increased formation of alpha-synuclein oligomers with time [35] and therefore we also investigated if alpha-synuclein oligomerization increased with longer incubation. After 96 h of incubation with ONE or HNE, an increased amount of alpha-synuclein oligomers could be seen in all samples at both pH, except for HNE at pH 5.4 where still no oligomers could be observed (Fig. 2C). For ONE at pH 5.4, the oligomers eluted at ~ 2000 kDa and had increased in amount (~ 15% of the monomeric peak). At pH 7.4, the oligomeric species eluted in a broad peak with two distinct maxima at ~ 800 kDa and ~ 2000 kDa, which constituted the dominating molecular species (~ 144% of the monomeric peak). For HNE at pH 7.4, the oligomeric peak area was much greater (~ 33% of the monomeric peak) compared to 24 h of incubation. Although the majority of the oligomers eluted at ~ 2000 kDa, the peak had a broad appearance covering molecular species ranging in size between ~ 800 and 2000 kDa.

In order to compare the size distribution of ONE- and HNE-induced oligomers to unmodified oligomers, we generated alpha-synuclein oligomers by lyophilization of native protein followed by a quiescent incubation at 37 °C for 24 h [45,46] (Fig. 2G). The elution profile of the unmodified oligomers showed a broad peak of oligomeric species, similar to that found in ONE 7.4 samples, but with a main peak at 2000 kDa. A predominating monomeric peak could also be observed at 50 kDa.

3.2. Atomic force microscopy analysis

Non-contact AFM analysis was performed on the ONE- and HNE-treated alpha-synuclein samples to characterize the morphology of the oligomers (Fig. 3). In the ONE pH 5.4, ONE pH 7.4, HNE pH 7.4 and unmodified samples, the largest oligomeric species measured 6–8 nm of height and approximately 100 nm of width and had a rounded appearance. Occasionally these rounded structures exhibited a more bent shape, particularly in the ONE 5.4 and HNE 7.4 samples. These larger structures presumably correspond to the oligomers of around 2000 kDa that were observed in the SEC analysis. Additionally, smaller spherical oligomeric structures with a height of approximately 2–4 nm and a width of 30–100 nm could also be observed; most likely corresponding to the smaller sized oligomers observed by SEC analysis.

3.3. Western blot analysis

Western blot analysis was performed on the aldehyde-treated alpha-synuclein samples to assess the presence of SDS-stable oligomeric species (i.e. the degree of cross-linking). For ONE-treated samples, low-molecular weight oligomers ranging in size between 35 and 100 kDa, corresponding to dimers to hexamers, were observed at both pH 5.4 and pH 7.4 between 15 and 60 min of incubation (Fig. 4).

Furthermore, a high-molecular weight smear band (~ 100–260 kDa) could also be observed at this time, but it was much more pronounced in samples incubated at pH 7.4. At both pH values, the 35–100 kDa oligomeric species decreased with increasing incubation times (1–96 h), whereas the higher molecular weight smear (~ 100–300 kDa) increased in intensity. At the latest time points (24 and

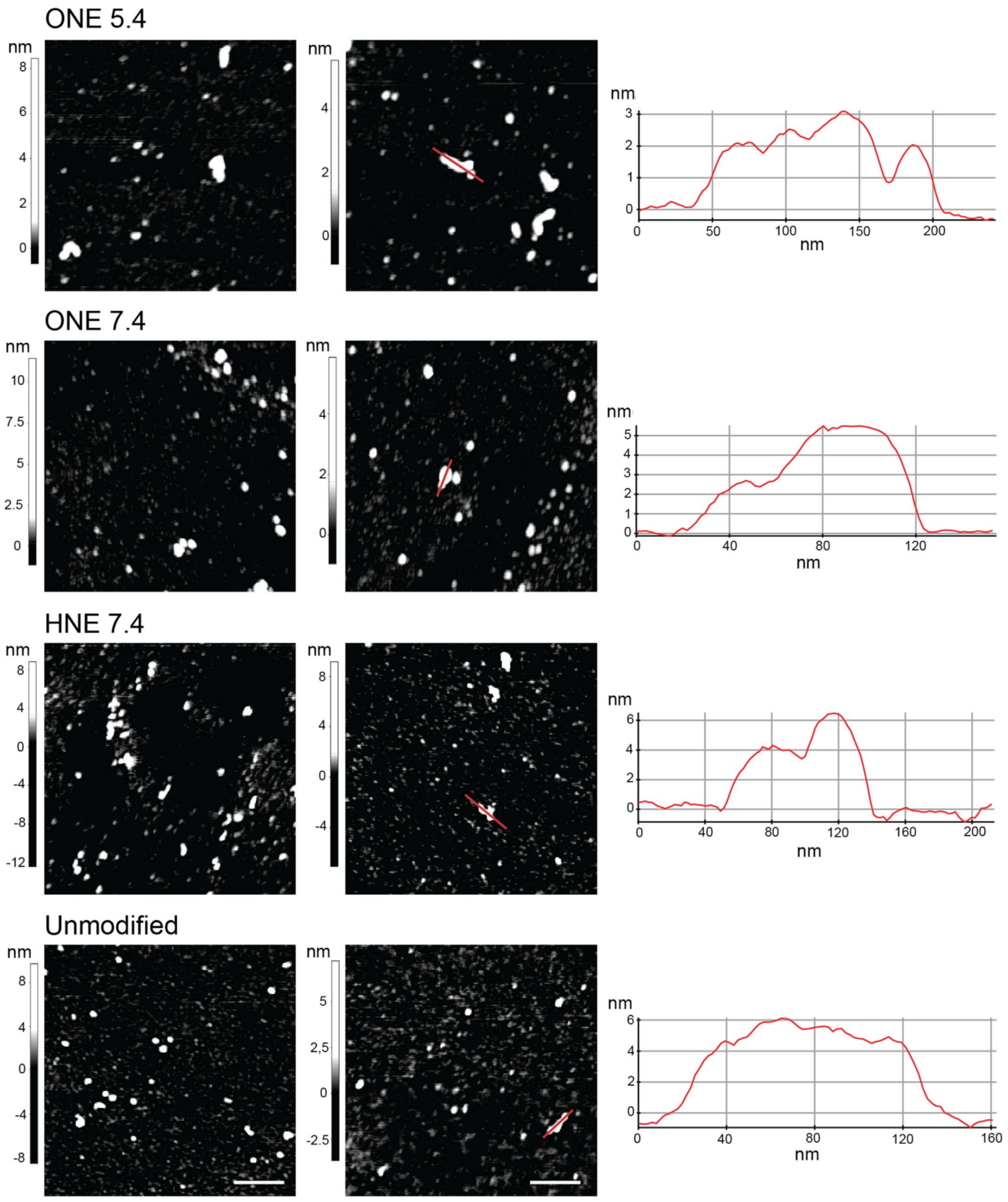

Fig. 3. Representative atomic force microscopy images of alpha-synuclein samples treated with 96 h ONE at pH 5.4 or 7.4, HNE at 7.4 and unmodified oligomers produced from lyophilized alpha-synuclein. Scan size 1 μm, scale bar 200 nm.

96 h), oligomeric species not entering the gel could be observed to a higher degree at pH 5.4. In addition, a strongly labeled monomeric band was observed at all time points for both pH, but decreased in intensity over time. For HNE, in addition to a strongly labeled monomeric band at 15 kDa, a faint dimeric band was visible at pH 7.4 already after 15 min. The intensity of the dimeric band increased with

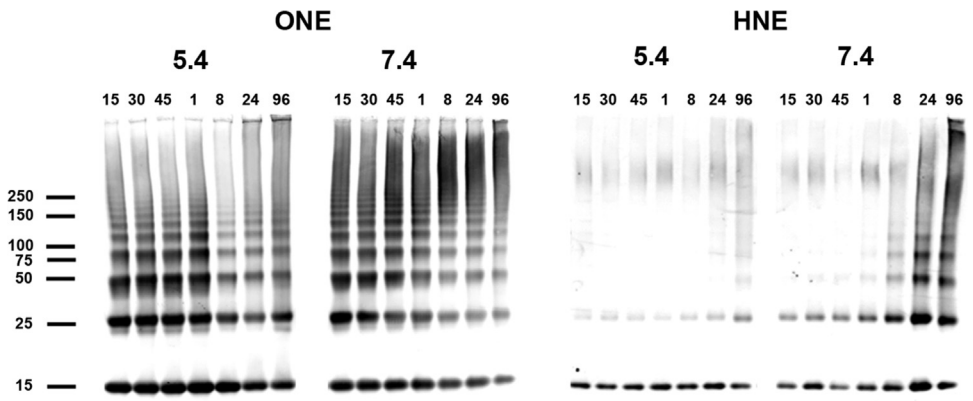

Fig. 4. Western blot analysis of the aldehyde-treated alpha-synuclein samples at 15 min – 96 h. Low-molecular weight oligomers, as well as high-molecular weight smear bands, were detected for ONE-treated samples at both pH 5.4 and pH 7.4 already after 15 min of incubation. With increasing incubation times, the amount of high-molecular weight oligomers increased at both pH values, whereas monomeric and low-molecular oligomeric alpha-synuclein bands decreased in intensity. For HNE, a monomeric band was seen for all time points at both pH and was the dominating species. In addition, a faint high-molecular weight oligomeric band was seen at 24 and 96 h for HNE-treated samples at pH 5.4. The same pattern was observed at pH 7.4, but the intensity of the band was stronger. The different time points are indicated at the top of the wells.

prolonged incubation time, and at 24 and 96 h of incubation low molecular weight oligomers (up to pentamers) could be detected. In addition, at these time points a high molecular weight smear (100–300 kDa) was also observed. For pH 5.4, a monomeric 15 kDa alpha-synuclein band was the predominating molecular species. With increasing incubation time, a faint dimer band and a faint high-molecular smear (100–300 kDa) could also be observed.

3.4. Proteinase K digestion

To study the compactness of the ONE- or HNE-treated alpha-synuclein, samples were taken after 96 h and treated with PK followed by immunoblot analysis (Fig. 5).

The highest PK resistance (80 μg/ml) was seen for ONE- and HNE-treated samples at pH 7.4, whereas ONE-treated samples at pH 5.4 were less PK-resistant (40 μg/ml). In contrast, unmodified monomeric alpha-synuclein, as well as HNE-treated alpha-synuclein incubated at pH 5.4, were sensitive to PK treatment as no immune-reactive material could be observed even at the lowest PK concentration (10 μg/ml).

3.5. Circular dichroism analysis

CD was performed to investigate the secondary structure of ONE- and HNE-treated alpha-synuclein. Unmodified monomeric alpha-synuclein displayed a CD spectrum with its spectral minimum around 200 nm, which is typical for random coil structure (Fig. 6A and B).

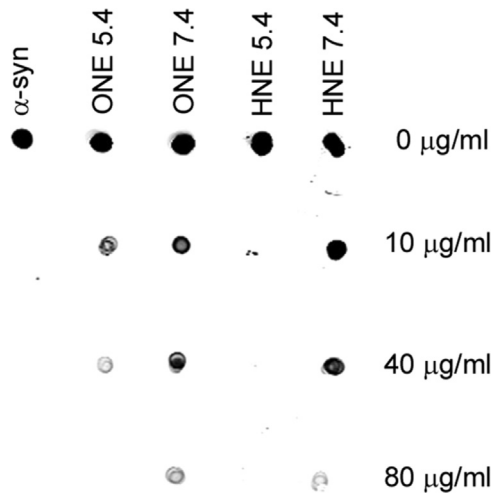

Fig. 5. Investigation of the compactness of ONE or HNE-treated samples by proteinase K (PK) digestion. The highest PK resistance was noticed for both ONE- and HNE-treated samples at pH 7.4. Unmodified monomeric alpha-synuclein and HNE-treated samples at pH 5.4 showed no PK resistance at all.

Addition of ONE or HNE at pH 7.4 resulted in a distinct change in the CD spectrum with decreasing amplitude of the negative peak around 200 nm and a small increase of the negative amplitude in the 210–230 nm spectral region (Fig. 6B). The alpha-synuclein sample treated with HNE at pH 5.4 did not change the shape of the spectrum (the amplitude difference might be attributed to small concentration differences) (Fig. 6A). As the measured CD spectra resulted from contributions from all protein conformations present in the sample, we also estimated the spectra of pure oligomers by treating the data as a weighted average of monomer and oligomers (Fig. 6C and D). The resulting oligomer spectra clearly differed from that of the monomeric protein with broad minimum around 220 nm and maximum between 190 and 200 nm. Such appearance is in agreement with formation of β-sheet secondary structure, as previously reported for ONE- and HNE-treated alpha-synuclein [35,40].

3.6. Mass spectrometry analysis

To specifically determine the residues modified by ONE and HNE at the different pH, mass spectrometry analysis was performed on samples incubated for 24 h (Fig. 7 and tandem mass spectrometry raw data in Supplementary data). We chose to analyze the samples after 24 h because it is a time point just prior to the observed increase in oligomer formation for both ONE- and HNE-treated alpha-synuclein. In addition to trypsin, which selectively cleaves peptide bonds after arginine and lysine, an amino acid that can be modified by ONE and HNE, the samples were digested with endoproteinase Glu-C, which cleaves after glutamic acid.

ONE and HNE can modify Cys, Lys and His residues [41,42] (alpha-synuclein has 15 Lys residues and one His residue). We detected additions of 136 or 154 Da for ONE and 138 or 156 Da for HNE. For ONE-treated alpha-synuclein, a modification of 154 Da, was found at positions K6, K10, K12, K21, K23, K32, K43, K45, K58, K60, K80 and K96 at both pH values. This modification could either represent a Michael addition or a 4-ketoamide [43]. However, as the 4-ketoamides are reported to be more stable and long-lived than Michael additions of ONE on Lys residues, most likely the 154 Da addition represents a 4-ketoamide [47]. The 136 Da additions represent either a Schiff base reaction on a Lys or a dehydrated Michael addition [48]. The 136 Da additions were found in all positions were 154 Da additions were found. No peptides were found that contained a 136 or 154 Da modification of the His50 residue. For HNE-treated alpha-synuclein, a 156 Da Michael addition reaction was detected at residues K6, K12, K34, K43, K45, H50, K60, K80 and K96. The lone difference between pH 5.4 and pH 7.4 was the modification of K43, which was modified at pH 7.4 but was not detected at pH 5.4. An addition of 138 Da, representing either a Schiff base reaction or a dehydrated Michael's addition, was found at positions

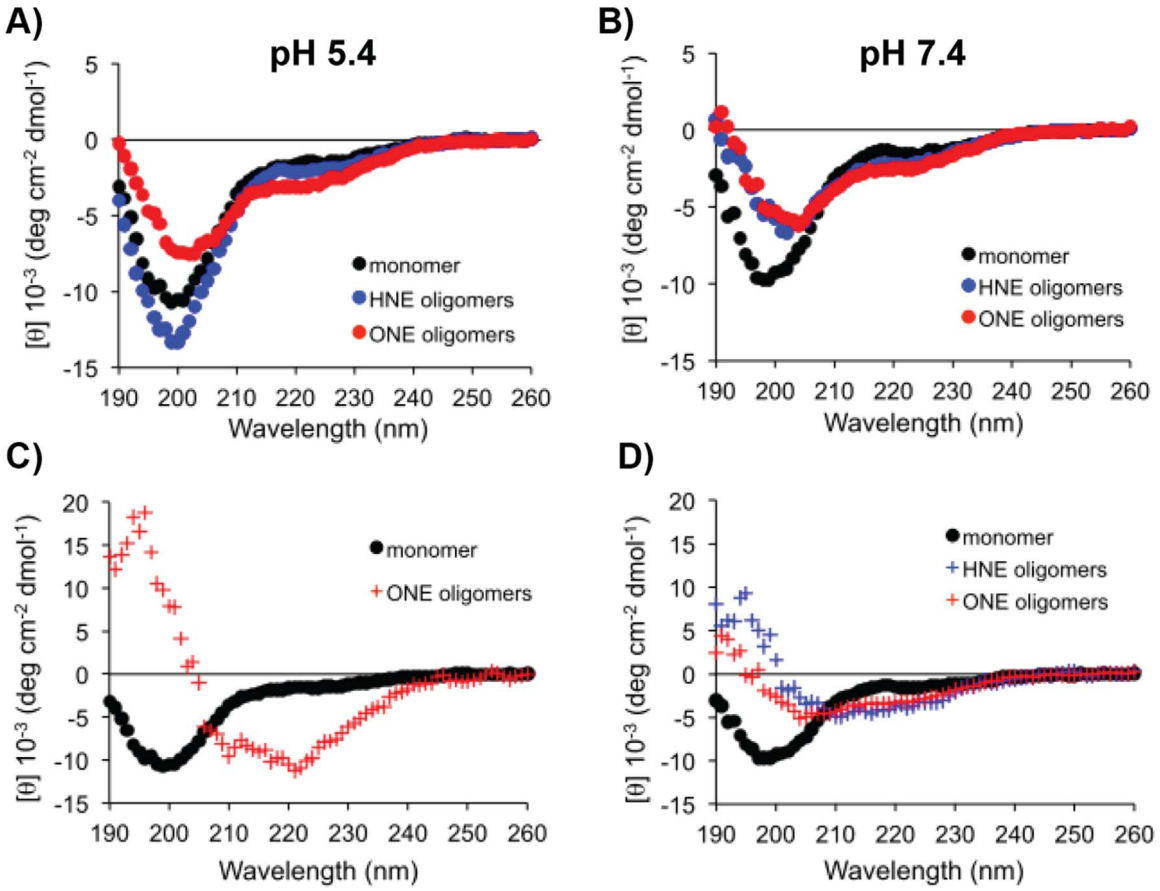

Fig. 6. Circular dichroism (CD) spectra of unmodified monomeric, ONE- or HNE-treated alpha-synuclein at pH 5.4 (A, C) and at pH 7.4 (B, D). The recorded CD data for the samples containing a mixture of monomer and oligomers is shown in panel A and B. In panel C and D, the spectrum of monomeric alpha-synuclein is compared to the estimated spectra of pure oligomers (see Section 2).

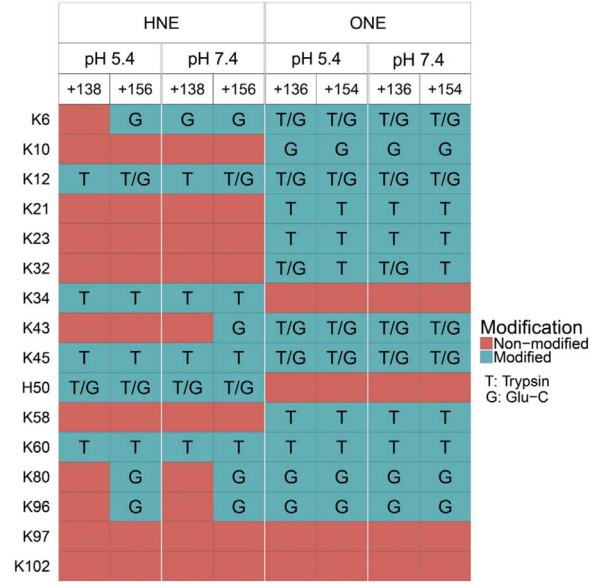

Fig. 7. His and Lys residues modified by HNE and ONE at 24 h, at pH 5.4 or 7.4, detected by mass spectrometry. Additions of 138/156 Da were detected for HNE and 136/154 Da for ONE. The modifications were found in peptides digested by trypsin (T) or Glu-C (G) digestion and for some sites by both digestion buffers.

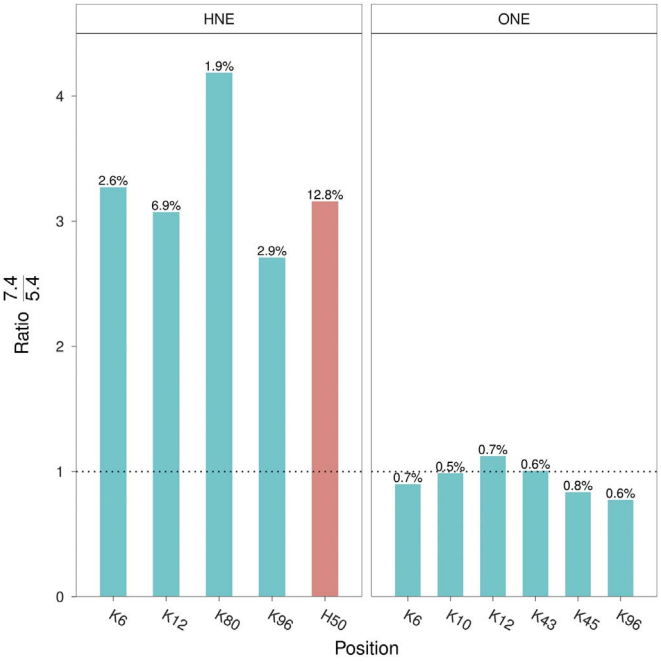

Fig. 8. Ratio between percentages of modified peptides (+154 or +156) at pH 7.4 and percentages of modified peptides at pH 5.4 for His (red) and Lys (blue) residues in HNE (+154) and ONE (+156) modified alpha-synuclein at 24 h, measured by semi-quantitative mass spectrometry. Percentages on top of bars indicate the percentage of modified peptides at pH 7.4.

K12, K34, K45, H50, K60 for HNE at both pH values, with an additional modification being found at K6 at pH 7.4. Neither ONE, nor HNE modified residues K97 or K102.

In order to investigate the amount of ONE or HNE modified

molecules, we also performed a semi-quantitative mass spectrometry analysis by quantifying the relative amount of modified peptides by ONE or HNE (Fig. 8). In this experiment, only Glu-C cleaved peptides were analyzed, except for His50 where only trypsin cleaved peptides were analyzed, and only additions of 154 or 156 Da were quantified. More HNE modified peptides were found at pH 7.4 than at pH 5.4, but there were no differences in the percentage of modifications between pH 7.4 and pH 5.4 for ONE modified peptides (Fig. 8). More HNE modified peptides were found compared to ONE modified peptides. The highest fraction of modified peptide was for the HNE His50 position, with approximately 13% of modified peptides vs. corresponding unmodified peptide.

3.7. Indirect ELISA analysis of HNE His modifications over time

As the His50 residue has been reported to be important for HNE-induced oligomer formation [35,38,39,44,49], we were interested in investigating HNE additions to His50 at different time points. We therefore analyzed HNE modifications of alpha-synuclein over time in an indirect ELISA using a monoclonal antibody specifically recognizing HNE adducts of His residues. The results indicated that the His residue was HNE-modified already after 15 min and, at most time points, alpha-synuclein was significantly less HNE-modified at the His residue at pH 5.4 compared to 7.4 (Fig. 9).

3.8. Indirect ELISA analysis of HNE-modified histidine in the oligomeric and monomeric fraction of HNE pH 7.4 samples

As the mass spectrometry analysis detected aldehyde modifications in all alpha-synuclein species contained in the sample (i.e. both monomers and oligomers), we wanted to compare the levels of HNE His modification at pH 7.4 between monomers and oligomers at the 96 h time point. An oligomeric and a monomeric fraction were isolated through size exclusion chromatography and probed with an antibody against HNE-modified histidine residues in an indirect ELISA. Since the concentration of alpha-synuclein in the two fractions varied, we performed an alpha-synuclein ELISA to measure the relative ratio between HNE modified histidine and the total amount of alpha-synuclein (Fig. 10). The results showed significantly higher levels of HNE modified histidine in the oligomeric fraction at most sample dilutions.

4. Discussion

We and others have shown that ONE and HNE can promote the formation of alpha-synuclein oligomers with defined structural properties [35,37,38,40]. In these earlier studies high molar excess has been used for ONE and HNE (20–30:1), but here we could show that even a low molar excess of aldehyde can promote the formation of alpha-synuclein oligomers.

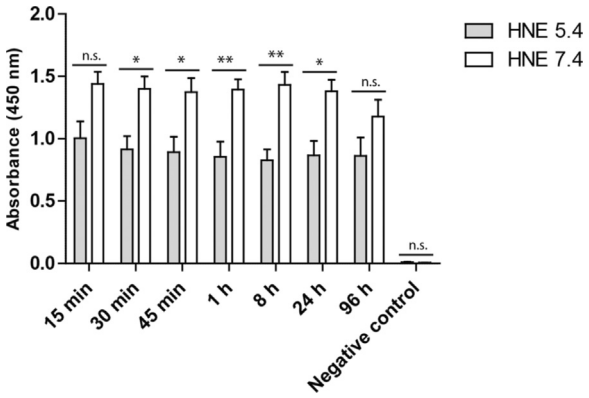

Fig. 9. Indirect ELISA analysis of HNE His modifications over time. Error bars represent the standard error of the mean (SEM) and each experiment was performed three times with sample duplicates. *P < 0,05; **P < 0,01; ***P < 0001, two-way ANOVA followed by Bonferroni post-hoc test, error bars represent the standard error of the mean (SEM).

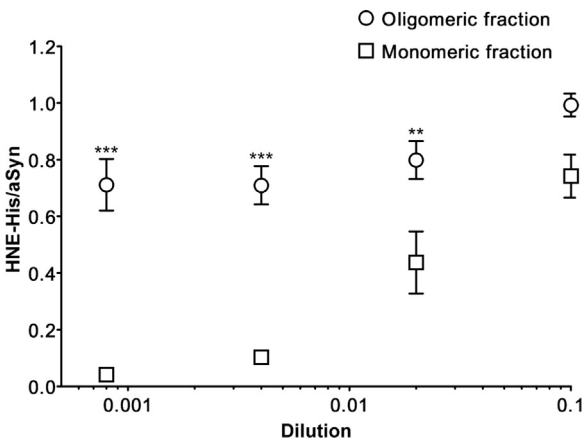

Fig. 10. HNE-Histidine modifications analyzed by indirect ELISA. Alpha-synuclein was incubated with HNE at pH 7.4 for 96 h and the resulting sample was separated into an oligomeric and a monomeric fraction using size exclusion chromatography. The fractions were diluted and probed with an antibody recognizing HNE-histidine adducts and an antibody against full-length alpha-synuclein on an indirect ELISA. The ratio of the signal from both ELISAs indicates the amount of HNE-modified histidines in relation to the alpha-synuclein concentration. Three different samples were used; each was analyzed by ELISA two or three times with duplicates. *P < 0,05; **P < 0,01; ***P < 0001, two-way ANOVA followed by Bonferroni post-hoc test, error bars represent the standard error of the mean (SEM).

By SEC and western blot we could demonstrate that ONE induced oligomer formation already after 15 min at both pH values. On the other hand, HNE-modified alpha-synuclein only formed detectable oligomers after 24 h and only at pH 7.4. For both aldehydes, the amount and size of the oligomers increased with prolonged incubation (i.e. up to 96 h). The oligomers had a rounded appearance, as observed by AFM, and resembled oligomers formed by unmodified alpha-synuclein. The ONE- and HNE-induced oligomers were SDS-stable to a certain extent as evidenced in the western blot analysis, which indicates that the alpha-synuclein molecules were intermolecularly cross-linked. The finding that ONE induced SDS-stable oligomers to a higher degree agrees with previous studies showing that ONE is a more potent protein cross-linker than HNE [42,50]. This can be explained by a carbonyl group at position C4 of ONE instead of a hydroxyl group at the same position of HNE [30,31,42]. Additionally, the oligomers formed by the presence of ONE and HNE were PK resistant and had β-sheet structure, thus indicating that low molecular aldehyde excess also leads to the formation of compact oligomers with a defined secondary structure.

For the first time we compared the modification pattern of ONE- and HNE-treated alpha-synuclein by mass spectrometry. Although more modified Lys residues could be identified for ONE than for HNE, our semi-quantitative analysis showed that the total amount of modifications actually was lower. Furthermore, only HNE modifications of the His50 residue could be observed. As ONE induced cross-linking to a higher degree, a potential explanation for these findings could be that the extensive cross-linking involving His-Lys or Lys-Lys residues between alpha-synuclein molecules hinder the identification of modified peptides by MS/MS analysis [30,47].

A previous mass spectrometry study of alpha-synuclein treated with high molar excess of HNE only found modifications at Lys60 and Lys96 at physiological pH [37]. This is in contrast with the current study where we found eight HNE-modified Lys residues both in N-terminal and mid region of alpha-synuclein.

Earlier studies have indicated that alpha-synuclein is primarily modified by HNE at His50 [38,39,49,51]. The mass spectrometry results showed that His50 was the residue most often modified by HNE, at both pH values. An explanation could be that HNE-His Michael additions are more stable than the HNE-Lys counterpart [48]. Additionally,

the ELISA analysis of HNE-modified His residues showed that His50 was indeed HNE-modified at both acidic and physiological pH, and this occurred within 15 min of incubation and only a subtle increase could be observed over time. Our ELISA analysis also showed a significant increase in modification of the His50 residue in oligomers compared to monomeric protein. Nonetheless, based on our observations of HNE-treated samples at acidic pH, the modification of His50 alone does not seem to trigger aggregation.

It is somewhat surprising that the oligomerization propensity was abolished at a lower pH for HNE, as the opposite has been shown for unmodified alpha-synuclein [7]. As both mass spectrometry and ELISA data showed that alpha-synuclein was indeed HNE modified at an acidic pH, albeit to a lower degree, the lack of oligomerization most likely cannot be explained by a lack of modification.

Taken together, although both ONE and HNE can modify alpha-synuclein, oligomer formation seems to occur through different pathways presumably due to their different chemistry. ONE causes rapid cross-linking of alpha-synuclein leading to an oligomerization at both physiological and acidic pH. However, oligomers generated at lower pH are less compact, take longer time to form, and are larger in size. This indicates that alpha-synuclein under acidic conditions is less prone to adopt a compact oligomeric structure. In contrast, although HNE modifies alpha-synuclein immediately, primarily the His50 residue, oligomer formation only occurs with prolonged incubation times (> 24 h) and involving fewer cross-linking events. Although HNE can bind to alpha-synuclein at an acidic pH, these modifications cannot promote oligomerization even with increased incubation times. This study highlights the unique ability of alpha-synuclein to form diverse oligomeric structures under different conditions.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.freeradbiomed.2017.07.004.

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