Synthesis, Structure Characterization, and Evaluation in Microglia Cultures of Neuromelanin Analogues Suitable for Modeling Parkinson's Disease

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Synthetic neuromelanins activate microglia

Keywords

Neuromelanin; iron; amyloid fibrils; synthetic melanin; microglia; Parkinson's disease.

Abbreviations

βLG: native bovine β-lactoglobulin; CD: circular dichroism; COX-2: cyclooxygenase-2; DA: dopamine; EPR: electron paramagnetic resonance; Eu: eumelanin; fLG: fibrillar β-lactoglobulin; ICP-OES: inductively coupled plasma - optical emission spectrometry; IL-1β: interleukin-1 beta; iNOS: inducible nitric oxide synthase; LC-MS: liquid chromatography-mass spectrometry; NM: neuromelanin; Pheo: pheomelanin; PD: Parkinson's disease.

Melanic conjugates of fibrillar β-lactoglobulin mimic human neuromelanin structure and induce activation of microglia cells

Abstract

In the *substantia nigra* of human brain, neuromelanin (NM) released by degenerating neurons can activate microglia with consequent neurodegeneration, typical of Parkinson's disease (PD). Synthetic analogues of NM were prepared to develop a PD model reproducing the neuropathological conditions of the disease. Soluble melanin-protein conjugates were obtained by melanization of fibrillated β-lactoglobulin (fLG). The melanic portion of the conjugates contains either eumelanic (EufLG) or mixed eumelanic/pheomelanic composition (PheofLG), the latter better simulating natural NMs. In addition, the conjugates can be loaded with controlled amounts of iron. Upon melanization, PheofLG-Fe conjugates maintain the amyloid cross-β protein core as the only structurally organized element, similarly to human NMs. The similarity in composition and structural organization with the natural pigment is reflected by the ability of synthetic NMs to activate microglia, showing potential of the novel conjugates to model NM induced neuroinflammation. Thus, synthetic NM/microglia constitute a new model to develop anti-Parkinson drugs.

Neuromelanins (NMs) are dark pigments that accumulate during aging in various human brain areas and particularly in dopaminergic neurons of *substantia nigra* and in noradrenergic neurons of *locus coeruleus* (Zecca *et al.* 2004, 2008a). NMs are contained into cytoplasmatic organelles surrounded by a double membrane, suggesting that they are generated through an autophagic mechanism (Sulzer *et al.* 2000; Zucca *et al.* 2014). The formation of NM represents a protective process, preventing the accumulation of reactive quinones originated by oxidation of catecholamine neurotransmitters in the cytosol (Sulzer *et al.* 2000), and in addition NM pigment exhibits strong metal ion binding capacity, thus blocking another possible source of toxicity (Zecca *et al.* 2008b). Among metal ions, NM has peculiar affinity for iron, which can reach the remarkable amount up to about 10 μg/mg of pigment (Shima *et al.* 1997; Zecca *et al.* 1996, 2004, 2008a). The metal is bound in the iron(III) form and mostly organized in multinuclear clusters of high spin, pseudo-octahedral centers containing oxo and hydroxo bridges (Zecca *et al.* 1996; Aime *et al.* 1997), with a structure bearing resemblance to that present in the ferritin iron core (Zecca *et al.* 2001). Iron(III) is also present in mononuclear centers, bound to oxygen atoms of catechol moieties and probably hydroxo groups (Zucca *et al.* 2015). Iron accumulation into NM may play a role in the involvement of NM in neurodegenerative diseases, as the *substantia nigra* and to lesser extent *locus coeruleus* are the two brain areas mostly affected by Parkinson's disease (PD) (Hirsch *et al.* 1988; Pakkenberg *et al.* 1991; German *et al.* 1992; Gesi *et al.* 2000). All NMs are characterized by three organic moieties covalently linked to each other (melanin, protein, and lipid), and inorganic iron (Zucca *et al.* 2015). The biosynthesis of NM occurs in the cytosol, upon accumulation of catecholamines, particularly dopamine (DA), when their inclusion into synaptic vesicles is insufficient (Sulzer *et al.* 2000). Catecholamines are easily oxidized to the corresponding quinones, likely by iron-mediated or enzymatic catalysis, and by oxidizing species produced by oxidative stress. The quinones initiate the pathway of NM formation by reacting with nucleophilic amino acid residues, particularly Cys, of peptides (e.g. glutathione) and proteins (Engelen *et al.* 2012). The melanic portion of NM thus contains polymerized catecholamine residues (eumelanin, Eu) and polymerized Cys-DA residues (pheomelanin, Pheo), characterized by dihydroxyindole and benzothiazine units, respectively, in an approximate 3:1 ratio (Wakamatsu *et al.* 2003; Bush *et al.* 2006; Ito and Wakamatsu 2008). X-ray powder analysis of NMs isolated from all brain areas consistently shows that the protein content is organized in fibrillar aggregates with typical cross-β structure (Zecca *et al.* 2008a), which suggests that the initial step of NM formation depends on the presence of fibrillar protein seeds, with which catecholamine quinones apparently react more readily than

with other protein material (Zucca *et al.* 2015). The protein sites of quinone linkage then act as centers of growth of the melanic component of NM, by successive attachment of other quinones, as shown in experiments of protein modification by DA-quinones (Nicolis *et al.* 2008; Ferrari *et al.* 2013). This explains why the melanic component of NM is not structurally organized, unlike human peripheral melanins or other naturally occurring melanins, which are typically characterized by stacked, sheet-like oligomers (d'Ischia *et al.* 2014). Iron plays a role in promoting the oxidation of catechol residues that occur as intermediates in the various steps of addition of quinones to the growing melanic oligomers, and remains trapped by the chelating groups of these oligomers, which contain negatively charged O-donor atoms for which iron(III) has high affinity.

Studying human NMs is difficult, due to the small amount that can be isolated from human brain and their essential insolubility. Thus, synthetic NM analogues provide a useful tool to elucidate their structure, properties and reactivity. Additionally, synthetic NM analogues can have an important role in the study of neurodegenerative and neuroinflammatory processes mediated by microglia activation, in cell cultures and in *in vivo* animal models of PD, as previously reported by using human NM (Wilms *et al.* 2003; Zecca *et al.* 2008c; Zhang *et al.* 2011). During the disease, pigmented neurons of the *substantia nigra* selectively loose functionality and die (Hirsch *et al.* 1988), releasing their NM into the extracellular environment. This extracellular NM induces microglia activation (McGeer *et al.* 1988; Langston *et al.* 1999) and produces damage to other neurons by self-perpetuating cycles of neuroinflammation and neurodegeneration (Zecca *et al.* 2003, 2006; Zucca *et al.* 2014). These noxious cycles have been shown to be triggered by NM both in *in vitro* and *in vivo* models of PD (Wilms *et al.* 2003; Zecca *et al.* 2008c; Zhang *et al.* 2011).

Currently, PD is reproduced in animal models by the acute insult produced on dopaminergic neurons by administration of exogenous toxins such as rotenone, paraquat, 6-hydroxydopamine or MPTP (Dauer and Przedborski 2003; Hattori and Sato 2007; Boukhzar *et al.* 2016). However, the sudden and extensive damage produced on neurons by these treatments is far from the condition of natural progression of the disease. The availability of synthetic NMs capable of inducing microglia activation in a manner similar to the activation/neuroinflammation caused by the natural pigment in parkinsonian *substantia nigra* could have important applications in biological and pharmacological assays aimed at gaining an understanding of the mechanism of PD progression and testing new drugs.

In this paper, we describe new NM analogues based on the conjugation of DA melanin with a fibrillar protein. As in our previous study of melanin-albumin conjugates, we kept the size of

the DA-melanin component reduced, in order to confer sufficient solubility to the resulting conjugates, enabling their characterization by various techniques, and easier handling in biological experiments. We show here that the fibrillar nature of the protein component is preserved after melanization and this particular structure of the protein core has an impact on the properties of the synthetic NM analogue. The protein used in this study is β-lactoglobulin (βLG), a relatively small (18.4 kDa) protein present in milk and belonging to the lipocalin family, with binding properties towards a variety of lipophilic molecules such as cholesterol and vitamin D (Kontopidis *et al.* 2004). The structure of βLG contains a central β-barrel motif (the so-called calyx), which provides the ligand binding site, surrounded by three α-helices (Kontopidis *et al.* 2004). The main reasons for focusing on βLG as source of the protein component of the new NM analogues were that it contains an accessible and reactive Cys (Cys121), and it can be converted into amyloid fibrillar form (fLG) (Dave *et al.* 2013). The melanic portion of the melanin-protein conjugates was prepared with both Eu and Pheo composition to better model these components of NM (Ito and Wakamatsu 2008).

Materials and methods

Reagents

All reagents were purchased from Sigma–Aldrich (St. Louis, MO, USA). βLG was in polymorphic forms A and B that differ for the substitutions of Asp for Gly at position 64 and Val for Ala at position 118. All buffers and solutions were prepared with Milli-Q water. To remove contaminating metal ions, all glassware was washed with 6 % vol/vol nitric acid in Milli-Q water, and then rinsed extensively with Milli-Q water before use.

Synthesis of DA melanins

A synthetic pheomelanin (PheoDA) was prepared by autoxidation of DA in presence of L-Cysteine as previously described (Bridelli *et al.* 1999). A synthetic pheomelanin with iron(III) (PheoDA-Fe) was also prepared with an initial iron/DA ratio of 1/20. DA (225 mg), L-Cys (29.7 mg), and $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ in the case of PheoDA-Fe, were dissolved in 100 mL of 50 mM phosphate buffer (pH 7.4). The mixture was kept protected from light, in air at 37 °C, for 4 days. The obtained black-brown pigment was centrifuged at 7000 g for 20 min and washed firstly with an acetic acid solution (1 % vol/vol) and then with a sodium chloride solution (9 % wt/vol). The product was incubated with 25 mL of a 0.15 M Na_2-EDTA solution for 8 h, centrifuged and suspended again with 25 mL of 0.15 M Na_2-EDTA solution for 14 h. Several washes with MilliQ water were performed and synthetic melanins were

freeze-dried and stored at -20 °C. The amount of total iron incorporated in PheoDA-Fe was measured with inductively coupled plasma - optical emission spectrometry (ICP-OES) as reported below. Iron concentration was 11.4 ± 0.7 μg/mg of melanin.

Preparation of fLG

fLG was obtained as reported by Gosal and colleagues (Gosal *et al.* 2002). A 30 mg/mL solution of βLG in Milli-Q water was prepared and adjusted at pH 2 by using 6 M hydrochloric acid. The sample was kept at 80 °C for 20 h under stirring. The presence of fibrils was confirmed by Congo Red (Klunk *et al.* 1989) and thioflavin T spectroscopic assays.

Thioflavin T spectroscopic assay

This assay was performed as reported by LeVine (LeVine 1995). Thioflavin T stock solution was prepared by solving 8 mg of compound in 10 mL of 10 mM phosphate buffer (pH 7.4); this solution was diluted 1:50 with phosphate buffer on the day of analysis to generate the working solution. The fluorescence intensity of the working solution was measured by a Perkin Elmer spectrofluorimeter with an excitation wavelength of 440 nm (slit width 5 nm) and recording the emission at 482 nm (slit width 10 nm). An aliquot of untreated protein solution (5-10 μl) was added, stirred for 1 min, and the resulting mixture was measured. The same protocol was used for fLG solution. A measured intensity above the control sample indicated the presence of fibrils. This assay was also used for conjugates, which were dissolved in 10 mM phosphate buffer (pH 7.4) at the concentration of 1 mg/mL.

Preparation of melanin-βLG and melanin-fLG conjugates

Protein-melanin conjugates were prepared by autoxidation of DA in presence of βLG or fLG, following the procedure previously reported for melanin-albumin conjugates (Ferrari *et al.* 2013). For melanin-βLG conjugates, 450 mg of DA and βLG were dissolved in 200 mL of 50 mM phosphate buffer (pH 7.4) and allowed to react in air at 37 °C for 4 days, protected from light. The samples were prepared using the two different DA/βLG (w/w) ratios of 1:2 and 2:1. After 4 days of incubation, a brown solution was obtained with no appreciable precipitation of melanin. The same protocol was used for the synthesis of melanin-fLG conjugates, using a dilute solution of fLG.

The solutions of conjugates thus obtained were dialyzed using tubing with a 10 kDa cutoff against milliQ water for 3 days, with a buffer-to-sample volume ratio of 50:1, replacing the

water six times to eliminate unreacted DA and small DA-quinone oligomers not bound to protein. Conjugates were then freeze-dried to obtain a brown-black solid.
The same method was used to prepare Pheo conjugates of βLG and fLG by adding L-Cys to initial reaction mixture, with a 5:1 molar ratio DA:Cys. Conjugates containing iron(III) ions were also prepared, by adding $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ corresponding to an initial molar ratio of 0.05 of Fe with respect to DA.
A summary of synthesized melanin-βLG and melanin-fLG conjugates is reported in Table 1; they are labeled according to the initial ratios between the reagents employed.

NMR spectroscopy quantification of protein content

The protein contents of conjugates were determined after complete acid hydrolysis and NMR quantification of amino acids, according to the method previously described (Ferrari *et al.* 2013). The analysis of each sample was carried out in duplicate. The same analysis was performed also on βLG and fLG in order to validate the method.

NMR spectra of melanin-βLG and melanin-fLG conjugates

Suitable solutions for NMR investigation of the conjugates were prepared by treating the lyophilized samples (about 1 mg/mL) with deuterium oxide under stirring for two days, protected from light. The ^{1}H-NMR spectra of the solutions were recorded on a Bruker AVANCE 400 spectrometer. Due to the limited concentration of the solutions, the spectra were acquired accumulating a large (>2000) number of scans. For comparative purposes, βLG and fLG spectra were recorded with the same parameters.

Analysis of the protein modification sites in the melanic conjugates by controlled proteolysis and liquid chromatography-mass spectrometry (LC-MS) analysis

Proteolysis of βLG, fLG, melanin-βLG and melanin-fLG conjugates was carried out after a denaturation process in the presence of a reducing agent. To a 10^{-5} M solution of protein in 20 mM ammonium bicarbonate buffer (pH 8.0), 50 mM dithiothreitol and 6 M guanidine hydrochloride were added and the mixture heated to 60 °C for 1 h. After cooling, iodoacetamide, which reacts with the free Cys preventing the formation of disulfide bonds, was added to a final concentration of 0.2 M. The mixture was then allowed to react for 1 h at 37 °C protected from light. The solution was dialyzed overnight against ammonium bicarbonate buffer, remove denaturating agents. The proteolysis was carried out using two different enzymes: first with trypsin for 24 h and then with pepsin for 24 h at 37 °C after

adjusting the pH to 1.3 with hydrochloric acid. The amount of proteolytic enzyme was 2 % (wt/wt) with respect to protein or fibrils. Samples of 1 mL containing approximately 1.5 mg of the conjugate were digested using the same procedure. After the proteolytic treatment, the samples were centrifuged to eliminate the melanic precipitate, and the solutions of the resulting peptide fragments were analyzed by LC-MS. The cleaved peptides were separated and analyzed in automated LC-MS/MS mode using a LCQ ADV MAX ion trap mass spectrometer coupled with an automatic injector system Surveyor HPLC (Thermo Finnigan, San José, CA, USA).

The elution was performed using 0.1 % vol/vol formic acid in distilled water (solvent A) and 0.1 % vol/vol formic acid in acetonitrile (solvent B), with a flow rate of 0.2 mL/min; elution started with 98 % solvent A for 5 min, followed by a linear gradient from 98 % to 55 % A in 60 min.

The MS/MS spectra obtained by collision-induced dissociation were recorded with an isolation width of 2 Th (*m/z*), the activation amplitude was around 35 % of ejection RF amplitude of the instrument. For the analysis of protein fragments, mass spectrometer was set such that one full MS scan was followed by zoomscan and MS/MS scan on the most intense ion from the MS spectrum. To identify the modified residues, the acquired MS/MS spectra were automatically searched against protein database for βLG using the SEQUEST® algorithm incorporated into Bioworks 3.1 software (Thermo Finnigan, San Jose, CA). All Cys in peptide fragments were found to be modified by iodoacetamide (+57 amu).

Circular dichroism (CD) spectroscopy

CD spectra were recorded between 190 and 260 nm with a Jasco J-715 spectropolarimeter. Melanin-βLG and melanin-fLG conjugates were dissolved in 0.05 M phosphate buffer (pH 7.4) at the concentration of 1 mg/mL and diluted 1/10 with the same buffer before recording the spectra. Six scans were recorded for each sample using an optical cell with 0.1 cm path length.

Iron analysis with ICP-OES

A Perkin Elmer ICP-OES Optima 3300 DV was used for all the measurements, following operating conditions suggested by the manufacturer. The linearity range of intensity *vs.* iron concentration (0.1 - 10 mg/L) was obtained using standard solutions prepared from a 1 mg/mL stock solution; the limit of detection was 5 µg/L Fe, the limit of quantification was 15 µg/L Fe. Weighted amounts of each sample were treated with 2 mL of Ultrapure 65 % vol/vol

nitric acid, Merck, refluxed for 15 min, evaporated to a small volume and diluted to 10 mL with ultrapure water. The clear solutions thereby obtained were analyzed for the determination of the iron content. Reagent blanks were prepared following the same procedure applied to samples. All experiments were performed in triplicate.

Electron paramagnetic resonance (EPR) spectroscopy measurements

EPR spectra were recorded at 10 K with an X band Bruker Elexsys E580 spectrometer. The samples were prepared by dissolving each conjugate in MilliQ water upon stirring for 24 h at 28 °C. The dissolved samples were then loaded in iron-free quartz EPR tubes. Multiple scans were collected for each spectrum using the following spectrometer settings: high power (6.33 mW), high modulation amplitude (10 G) and wide scan range (4000 G) in order to simultaneously observe the iron(III) signal (g = 4.3) and the melanic radical signal (g = 2.0). Spectra were also collected in a narrower scan range in order to obtain a better resolved spectrum for the two signals.

The quantification of EPR-active iron(III) bound to conjugates, based on the g = 4.3 signal, was performed according to the protocol published by Chasteen and coworkers (Bou-Abdallah and Chasteen 2008). The calibration curve was constructed using Fe^{3+}-EDTA standard solutions prepared from a stock solution of known concentration of Fe^{3+} in hydrochloric acid (Iron Standard Solution in 0.1 M hydrochloric acid Prolabo).

X-ray diffraction

Lyophilized melanins and protein/melanin samples were sealed in thin glass capillaries of 0.7 mm diameter. In order to avoid degradation, samples were stored at -18 °C and kept at room temperature only for the time required for the measurement. Powder X-ray diffraction patterns were collected with a Bruker D8 Discover diffractometer operating in Debye Scherrer geometry, equipped with a copper anode (CuKα) and an area detector (GADDS). The peculiar geometry and the presence of an area detector allowed us to collect reasonable good statistics data even with small amount of samples (few mg). Diffraction patterns in the angular range 2θ=5-44° were obtained by merging three different frames with acquisition time of 1-4 h each.

The data were represented as plots of counts *vs.* Q (A^{-1}), Q being the magnitude of the scattering vector, the momentum transfer in the scattering process of the X-ray photon elastically scattered through an angle 2θ. The relationship between Q and the scattering angle

2θ is Q = 4π sin θ/λ, where λ= 1.54148 Å is the X-ray photon wavelength. The Q peak maximum is related to the lattice spacing *d*, by the relation $d = 2\pi/Q$.
Particular care was spent in order to subtract the background contribution from the pattern, mainly arising from the capillary, whose signal sometimes revealed to be of the same order of magnitude of the sample. To this purpose, reference scans were collected on a portion of empty capillary with the same acquisition time and then subtracted to the raw data. Especially in the case of samples containing iron, the unavoidable fluorescence caused by working with Cu anode led to a significant increase of the background signal and a further reduction of the signal/noise ratio. In order to improve the data contrast in such cases, some diffractograms were treated by means of last square digital polynomial smoothing filter Savitzky-Golay (90 points) to smooth fluctuation of data.

Human NM isolation

NM was isolated from human *substantia nigra* post-mortem tissues as already described in our previous works (Zecca *et al.* 2008a, 2008b; Engelen *et al.* 2012). For tissue treatments and ethics policies refer to previous papers (Zecca *et al.* 2008a; Engelen *et al.* 2012).

Primary microglia stimulation

The samples used to investigate the effects of different kinds of synthetic melanins, as well as βLG and fLG, were prepared by weighing about 1 mg in a sterile glass tube which was then sterilized with methanol. After evaporation under nitrogen flux, the samples were suspended in sterile 50 mM phosphate buffer (pH 7.4), with a concentration of 1 mg/mL. These were then allowed to rehydrate for 4 days under mild agitation and protected from light. The same procedure was followed to prepare human NM suspension with a concentration of 0.5 mg/mL. Then, suspensions of synthetic melanins, βLG, fLG and human NM were diluted to 1, 2, 10, or 30 μg/mL for cell treatments.

Primary microglia

Mixed glial cell cultures, containing both astrocytes and microglial cells, were established from rat Sprague–Dawley P2 pups. Briefly, after dissection, hippocampi and cortices were dissociated by treatment with trypsin (0.25 %) and DNAse-I (Sigma-Aldrich, St. Louis, MO) for 15 min at 37 °C, followed by fragmentation with a fire-polished Pasteur pipette. Dissociated cells were plated on poly-lysine coated T75 flasks in minimal essential medium (E-MEM, Invitrogen, Life Technologies, Carlsbad, CA) supplemented with 20 % fetal bovine

serum (Gibco, Life Technologies, Carlsbad, CA) and glucose (5.5 g/L). Microglial cells were harvested from 10-14-days-old cultures by orbital shaking for 30 min at 1300 rpm. Shaken microglia were re-plated on poly-L-ornithine-coated tissue culture dishes at a density of 1×10^6 per dish. To minimize the activation of microglia, half of the medium in which microglia were kept after shaking from mixed glial cultures was replaced with fresh low (1 %) serum medium.

Microglia were treated with synthetic melanins or human NM ranging from 1 to 30 μg/mL for 4 h. Control microglia were exposed to vehicle alone (0.05 M phosphate buffer, pH 7.4). At the end of stimulation, microglia were washed and harvested with TRIZOL for q-PCR analysis or fixed for electron microscopy (see below). Transmission electron microscopy was performed on microglia exposed for 4 h to vehicle (control), to PheofLG-1/2-Fe (10 μg/mL) as representative among synthetic melanins, and to human NM (10 μg/mL). All the experimental procedures followed the guidelines established by the European legislation (Directive 2010/63/EU), and the Italian Legislation (L.D. no 26/2014).

Reverse transcriptase-coupled PCR

The expression levels of the pro-inflammatory markers inducible nitric oxide synthase (iNOS), interleukin-1 beta (IL-1β), cyclooxygenase-2 (COX-2) and the pro-regenerative markers arginase-1, mannose receptor C type 1 also known as CD206 were quantified using real-time PCR.

Total RNA was isolated from rat primary microglia using Direct-zol RNA isolation kit (ZymoResearch) following the manufacturer's protocol. To remove any contaminating genomic DNA, total RNA was digested with DNase-I. cDNA synthesis was performed using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) and Random Hexamers as primer. The resulting cDNAs were amplified using TaqMan® Gene Expression Assay (Applied Biosystems). The mRNA expression was normalized to the label of glyceraldehyde-3-phosphate dehydrogenase mRNA. All of the PCR experiments were performed in duplicate to verify the results.

Statistical analysis

All data (mRNA fold changes in microglia cultures after treatment with synthetic NMs, βLG, fLG, and human NM) were collected from three or more independent experiments and values were expressed as means ± standard errors. Statistical analysis was performed using paired t-

test (before and after) on mRNA fold changes of each treatment *vs.* control. Values of P less than 0.05 were considered statistically significant.

Electron microscopy of microglia cells

Cells were fixed as a monolayer in a mixture of 4 % paraformaldehyde and 2 % glutaraldehyde in cacodylate buffer (0.12 M, pH 7.4) for 4 h at 4 °C. Then, cells were extensively washed with cacodylate buffer and subsequently post-fixed for 1 h on ice in a mixture of 1 % osmium tetroxide and 1.5 % potassium ferrocyanide in cacodylate buffer. After several washes with ultrapure water, samples were "en bloc" stained with 0.5 % uranyl acetate in water overnight at 4 °C. Finally samples were dehydrated in a graded ethanol series, then infiltrated for 2 h in a mixture 1:1 (vol/vol) of ethanol and Epon and subsequently in 100 % Epon, twice for 1 h. Then polymerization was performed for 24 h in an oven at 60 °C. Ultrathin sections (80 nm) were prepared using an ultramicrotome (Leica Ultracut; Leica Microsystems GmBH, Wien, Austria), collected on nickel grids and stained with saturated uranyl acetate for 5 min, washed and then stained with 3 mM lead citrate for 5 min. Finally the sections were photographed using a transmission electron microscope LEO 912AB (Advanced Light and Electron Microscopy BioImaging Center - San Raffaele Scientific Institute).

Results

Synthesis of conjugates

We obtained soluble melanin-protein conjugates by carrying out the oxidation of DA under controlled conditions and in the presence of adequate amounts of protein. The DA *vs.* fLG, or βLG, ratios employed in the preparation of the samples studied here were therefore optimized to obtain products differing for their melanic *vs.* protein ratio but with sufficient solubility to enable their spectroscopic characterization. Increasing the DA/protein ratio in the synthesis above 2/1 leads to more heterogeneous mixtures of products, with formation of insoluble fractions. Melanin-protein conjugates with mixed Eu and Pheo components were obtained, to better mimic human NMs (Ito and Wakamatsu 2008). These were prepared using DA and L-Cys, to generate the Cys-DA derivative necessary to obtain the benzothiazine units of Pheo. In addition, iron was incorporated into the conjugates because this element is an essential component of the natural pigment. The various conjugates studied here are described in Table 1 and labeled according to the initial ratios between the reagents employed in their preparation. In the conjugates prepared with fLG, the presence of fibrils was confirmed by the

thioflavin T assay and CD spectroscopy (Figures S1 and S2). Although it was not possible to quantify the amount of fibrils in the conjugates with these techniques, it is clear that the melanization process does not disrupt their overall structure.

Protein and fibril content

Since the presence of a significant melanic portion interferes with the use of usual methods for protein quantification in the melanin-protein conjugates (i.e., Bradford or Lowry methods), we followed a method described previously (Ferrari *et al.* 2013), involving protein hydrolysis and NMR analysis of the amino acids, to determine the amount of protein in the conjugates (Table 1). In general, the protein content was similar in the conjugates derived from either fLG or βLG prepared with the same DA/protein ratio, hence the protein structure does not appear to have influence on the melanization process. As expected, the protein content was lower in the conjugates synthesized with an excess of DA. Nonetheless, the protein content is not proportional to the initial DA/protein ratio since DA is not completely converted into melanin. However, the addition of iron salt promotes the formation of melanin when DA is in excess in the reaction mixture. Initially iron(II) is distributed between DA and protein, but with the progress of the reaction it is completely oxidized and the protein-bound fraction released and transferred to the strongly chelating groups of the melanic polymer.

Identification of melanization sites

To identify the protein residues constituting the melanization sites of the protein, the conjugates were denaturated and digested with trypsin and pepsin. The resulting fragments were analyzed with LC-MS and compared with those obtained from the digestion of βLG and fLG alone. The fragments identified upon digestion of the latter proteins but absent in the analysis of the melanin-protein conjugates must be bound to the melanic portion and contain the residues reacting with DA-quinones. In fact, proteolytic enzymes are unable to reach the portion of the protein covalently bound to melanin, so that proteolysis does not occur. The digestion of βLG and fLG allowed the coverage of over 90 % of the amino acid sequence (Tables S1 and S2). The peptides containing the DA-quinone reaction sites, and missing from the analysis of the melanin-protein conjugates are reported in Table S3. Some difference in the identified fragments between the conjugates derived from βLG and fLG can be noted. For the fibrillary conjugates the sites of preferred DA-quinone reaction appear to be Cys121 and His146, because the fragments containing these residues were missing in all digested samples. Upon protein hydrolysis, the disulfide bridges are preserved (Dave *et al.* 2013) and therefore

all the other cysteines are unable to react with DA-quinones. Other residues likely react with DA-quinones, since other fragments were absent in some of the samples. Interestingly, fragments containing Cys121 were identified in the conjugates derived from native βLG, indicating that Cys121 is much less accessible to DA-quinones with respect to fLG. On the other hand, His146 is accessible to DA-quinones both in βLG and fLG types of conjugates.

CD spectra

The intense absorption of the melanic portion makes it difficult to obtain reliable CD spectra of synthetic NMs, but even with this limitation, some useful information can be obtained from the analysis of the CD profiles. The spectra are collected in Figure S2, while Table S4 contains numerical data of secondary structure content from fitting of the spectra. As expected, fibrillation of βLG produces a marked reduction in α-helix and an increase in β-sheet and random. The melanin-fLG conjugates have secondary structure content similar to fLG, indicating that β-sheet remains dominant after melanization. The reaction of fLG with DA-quinones induces some modification in the fibril secondary structure, but the intense absorption of the melanic component could have an influence on the spectra.

NMR spectra of melanin-protein conjugates

Comparing the ^{1}H-NMR spectra of βLG and fLG it can be noted that the amide proton signals around 6.5 ppm for native βLG are absent in the spectrum of fLG (Figure S3). This is due to the fast exchange of the amide protons of fibrils with D_2O. Furthermore, the pattern of aromatic signals is clearly different for βLG and fLG.

The spectra of melanin-fLG conjugates reveal important differences in the aromatic region with respect to fLG, particularly for conjugates prepared with an excess of protein (Figures 1 and 2). In general the signals are broader, since these derivatives have larger molecular weight and probably less conformational mobility in solution. However, some sharp signals attributable to Phe, Tyr, and Trp residues can be seen in the spectra of EufLG-1/2 and PheofLG-1/2. This indicates the presence of conformationally free portions in melanized fibrils, which probably undergo partial loss of structure upon formation of the DA-melanin oligomers. A similar sharpening effect of several aromatic amino acid signals was observed for the melanin-albumin conjugates (Ferrari *et al.* 2013). The fibrillar structure is only partly disrupted, since the sharp signals overlap with other broad peaks. This conclusion is consistent with the thioflavin T assay and CD spectra.

The partial disruption of the fibrillar structure in conjugates seems further enhanced by the presence of iron(III). Indeed, in the NMR spectra of EufLG-1/2-Fe and PheofLG-1/2-Fe the sharp signals are more evident than for their iron-free counterparts. Iron(III) increases the rate of DA oxidation and hence the amount of DA-quinones that reacts with the protein. This probably spreads DA oligomerization to different sites of the fibrils, leading to local denaturation and increased mobility. Furthermore, the presence of iron could directly affect fibril formation and stability (Guzzi *et al.* 2015). On the other hand, in EufLG-2/1 and PheofLG-2/1 spectra sharp peaks are absent, indicating that fibrils retains their structure and that the melanic portion is more extended and forms larger oligomers, with a slow rotational motion in solution, resulting in broader NMR peaks (Figures 1 and 2).
The spectra of conjugates prepared with native βLG (Figure S4) are very similar regardless of the presence of iron or Cys. The spectra do not show sharp peaks, indicating that DA modification does not induce loss of structure in significant portions of βLG. Here, only a slight broadening of the peaks can be appreciated, due to the increased molecular weight of the conjugates with respect to free βLG.

Iron binding

The binding capacity for iron by βLG is not known. Our data suggest that in fLG and βLG conjugates iron is mostly bound to the melanic portion of the conjugates, as the amount of iron is larger when this portion is more extended (Table 2). This is true for both Eu and Pheo conjugates, but the latter show slightly lower ability to coordinate iron(III). This was noted also previously with melanin-albumin conjugates (Ferrari *et al.* 2013).
The amount of iron tightly bound in the melanin-protein-Fe conjugates is slightly lower than that found in NM isolated from *substantia nigra*. This is due to the less extended melanic portion in the conjugates studied here with respect to human NM (Zecca *et al.* 2004, 2008a; Engelen *et al.* 2012), for the necessity to obtain them in a soluble form. However, NM *in vivo* is not completely saturated with iron and the concentration of this metal is lower when NM is in tissues with respect to that measured in isolated pigments. In fact, during isolation from brain tissues human NM binds additional iron deriving from other cellular compartments (Shima *et al.* 1997; Zecca *et al.* 2001, 2004, 2008a).

EPR spectra

As for human NMs (Zecca *et al.* 1996, 2004, 2008a), the low-temperature EPR spectra of the melanin-protein-Fe conjugates show two types of signals, attributable to high spin iron(III) in

mononuclear sites at g = 4.3 (Figure S5) and to the organic radical of the melanin component (at g = 2.0). Quantification of the iron EPR signals shows that only a very small fraction of total iron is EPR-detectable (Table 2). The amount of iron in these mononuclear sites is independent of the protein structure or the type of Eu or Pheo polymer. Thus, in all melanin-protein derivatives almost all the iron must be associated in oligonuclear clusters similar to those present in human NM.

X-ray diffraction

The X-ray diffraction spectra of βLG and fLG bear remarkable resemblance, with typical signatures of β-sheet motifs motifs (Gosal *et al.* 2004; Fowler *et al.* 2006). The spectrum of βLG displays two main peaks at 2θ = 8.5° and 19.7° (Q = 0.60 and 1.39 $Å^{-1}$) which can be related to lattice distances of 10.4 Å and 4.5 Å, respectively (Figure S6). The broad peaks are typical for powder protein samples. For fLG, more intense and narrower maxima are observed at 2θ = 8.9° and 19.4° (Q = 0.62 and 1.37 $Å^{-1}$) corresponding to distances of 9.9 Å and 4.6 Å (Figure S6). These match the distinctive features of cross β-sheet structures of amyloid aggregates, with antiparallel strand spacing of 4.7 Å and intersheet separation of 8-10 Å (Makin and Serpell 2005). However, fLG displays sharper and more intense peaks, reflecting the increased conformational homogeneity due to the long ranging repeating cross β-sheet structure. The additional reflection at d = 3.7 Å, present in all the spectra, may be correlated to hydrogen-bonded water molecules to the protein and ice-like structured (Lepault *et al.* 1997). The melanic component does not alter the main X-ray diffraction patterns, as shown for PheofLG-1/2 (Figure S7). However, the spectrum shows a further weak peak at 2θ = 21.3° (Q = 1.51 $Å^{-1}$), corresponding to a spacing of 4.16 Å, which is in accordance with those of some natural melanins (Casadevall *et al.* 2012), and can be related to minor fractions of DA melanin bound in the conjugate.

The presence of iron introduces some modifications in the X-ray diffraction profile both for PheoβLG-Fe and EufLG-Fe samples (Figure S8 and S9). The amplitude of the peak corresponding to β interstrand spacing of 4.5 Å for βLG and 4.6 Å for fLG is noticeably reduced with respect to water reflection. An additional weak broad peak at 2θ ~ 15.5° (Q ~ 1.1 $Å^{-1}$), corresponding to d ~5.7 Å, can be noted but in general the large and diffuse peaks are suggestive of enhanced amorphous character that may be due to lyophilization of samples. The x-ray diffraction spectra of all synthetic NMs are shown in Figure S10.

Microglia activation

Primary rat microglia were exposed to βLG, fLG and seven synthetic NMs, at a concentration of 10 μg/mL for 4 h. Human NM from s*ubstantia nigra* was used as reference stimulus, for its known role as microglia activator, in *in vitro* and *in vivo* models of PD (Zecca *et al.* 2008c; Zhang *et al.* 2011). The effect of synthetic NMs and proteins on microglial activation was assessed by q-PCR analysis of typical pro-inflammatory (IL-1β, COX-2 and iNOS) and anti-inflammatory (arginase-1 and CD206) genes. Synthetic NMs mimic human NM in inducing a pro-inflammatory response, as indicated by significant increase in iNOS, IL-1β and COX-2 transcripts (Figure 3a, b and c). βLG shows a stronger pro-inflammatory effect than fLG. Furthermore, iron seems to play a role in the pro-inflammatory activity. To strengthen this hypothesis we performed a concentration-response study of microglia activation upon treatment with PheofLG-1/2-Fe with respect to human NM. Microglia were treated with 1, 2, 10 and 30 μg/mL of both compounds for 4 h. At a concentration $\geq$ 10 μg/mL the pro-inflammatory action of PheofLG-1/2-Fe was even stronger than that elicited by human NM (Figure 3d, e, f, g, h).

The ability of cultured microglia to rapidly phagocytose and completely degrade NM particles during activation upon exposure to NM itself was shown previously (Zhang *et al.* 2011). Here, we evaluated by transmission electron microscopy microglia exposed to PheofLG-1/2-Fe (Figure 4a, b, c) and human NM (Figure 4d, e, f), as well as microglia exposed to vehicle alone (Figure 11S). After 4 h of incubation, both PheofLG-1/2-Fe (Figure 4a) and human NM (Figure 4d) were efficiently phagocytosed by microglia. Both synthetic and natural melanins highly accumulated inside specialized phagosomes for subsequent degradation.

Discussion

We synthesized and characterized soluble analogues of human NM reproducing its main structural features and capable of activating microglia as NM does in pathological processes of PD. The synthetic NMs have an approximate ratio of protein/melanin of 1:1, lower than in human NMs, where it ranges from 1:1 to 1:5 (Engelen *et al.* 2012). The reduced melanin fraction limits the iron binding capability so that also the amount of metal is lower than in human NMs (Zecca *et al.* 2008a). Comparing the properties of synthetic NMs derived from βLG and fLG reveals important differences and shows that melanin-fLG conjugates are better mimics of human NM, both in terms of structure and properties.

The primary DA-quinone binding site is Cys121 as expected (Tse *et al.* 1976; Whitehead *et al.* 2001; Nicolis *et al.* 2008), but it is extensively modified only in melanin-fLG conjugates, because the compact structure of native βLG hinders the access to Cys121. The sheet-like

arrangement of peptide chains in fLG increases the exposure of amino acid side chains, facilitating the reaction with DA-quinones. This is an important result, which clarifies why human NM is probably formed starting form a fibrillar protein core.

The only structural element recognized in human NMs is the fibrillar protein core (Zecca *et al.* 2008a). Unlike peripheral melanins, with the typical stacked arrangement of Eu "protomolecules" at about 3.4 Å (Meredith and Sarna 2006), the melanic component of NM is not structurally organized, as it grows like branches of a tree at the protein reactive sites (Ferrari *et al.* 2013). In fact, X-ray powder analysis of melanin-fLG conjugates display a structural motif (~4.6 Å) similar to that of human NMs from several brain areas characterized by ~4.7 Å separation (Zecca *et al.* 2008a). This structural parameter corresponds to the separation between hydrogen-bonded backbone of peptide chains in β-sheets present in the cross-β structure of amyloid fibrils (Makin and Serpell 2005). The same pathway occurs in the synthesis of melanin-fLG conjugates. The NMR spectra of conjugates show that upon melanization some of the peptides on the surface of the fibrils undergo a loss of β-sheet structure and gain conformational mobility, resulting in sharp signals. The presence of iron(III) further contributes to sharpening of the signals. However, when the melanic portion is more extended and forms larger oligomers, as in EufLG-2/1 and PheofLG-2/1 conjugates, the spectra show only broad peaks, due to the slow rotational motion in solution.

As previously noted for melanin-albumin conjugates (Ferrari *et al.* 2013), iron(III) is bound essentially to the catecholato residues of the Eu component. The presence of soft sulfur donors of Pheo benzothiazine and benzothiazole moieties reduces the coordination ability of hard iron(III) centers. The strong tendency of these centers to associate in clusters with oxo/hydroxo bridges makes most of the iron contained in the conjugates EPR non-detectable, leaving EPR signal at g=4.3 as the signature of the minor fraction of iron(III) bound in mononuclear sites. The ratio of EPR-detectable *vs.* EPR-inactive iron(III) in human NM has not been determined yet. However, from the apparent intensity ratio of iron(III)/free radical signals, which is similar to those of synthetic NMs, it is likely that also in human NM only a small fraction of total iron is bound in EPR-active form (Gerlach *et al.* 1995; Aime *et al.* 1997). This shows that the melanic oligomers prevent precipitation of iron(III) hydroxide, suggesting that NM acts as an efficient iron deposit inside pigmented neurons (Zecca *et al.* 2001, 2004) preventing its toxicity (Zecca *et al.*, 2008c).

The better structural analogy between melanin-fLG-Fe conjugates and human NMs suggests their potential application in biological and pharmacological assays as NM substitutes. Analysis of the microglial phenotype shows that globular βLG induces higher pro-

inflammatory activity than fLG, in line with the current view that small, soluble pre-fibrillar species, rather than fibrils, are the most toxic forms of misfolded proteins (Walsh and Selkoe, 2007; Winner *et al.* 2011; Brouillette *et al.* 2012; Simón *et al.* 2012). The differential action of βLG and fLG is lost upon conjugation with the melanin component which surrounds the protein core. In fact both melanin-βLG-Fe and melanin-fLG-Fe conjugates mimic the pro-inflammatory action of human NM, being even more active at higher concentrations. It is noteworthy that PheofLG-1/2-Fe, which better mimics human NM composition and structure, displays a pro-inflammatory action very similar to that of human NM.

A recent study showed that synthetic melanins induce microglia activation upon injection in mice and up-regulate pro-inflammatory mediators in microglial cells *in vitro* (Viceconte *et al.* 2015). These compounds contain only a melanic portion and are essentially insoluble. The conjugates described here are more realistic models of human NMs, and better mimic their composition (DA-derived melanin, fibrillar protein core, and iron content). They are soluble, can be accurately quantified, and are well characterized, so that they can be proposed to replace human NM as useful tool to induce chronic neuroinflammation and neuronal degeneration in cell cultures and animal models of PD. In particular, they will better reproduce the events occurring in PD brain during disease progression than the currently used toxins, and can be therefore used to test the efficacy of new drugs aiming to block neuroinflammation and neuronal death.

Synthetic NMs containing iron can also be used to investigate the role of NM and iron in relaxation and saturation exchange processes in magnetic resonance imaging. Imaging of NM in human brain were extensively reported but without quantification of NM content (Sasaki *et al.* 2006; Kashihara *et al.* 2011). With the present NM analogues we are developing calibration standards for quantification of NM content in human brain by magnetic resonance imaging. Indeed we are generating adequate sequences and procedures for accurate monitoring of PD progression and possibly establishing an early diagnosis of PD.

Acknowledgments and conflict of interest disclosure

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The authors declare no competing financial interests.

Supporting information

Additional Supporting information may be found online in the supporting information tab for this article.

Figure S1. Thioflavin T fluorescence spectra of βLG, fLG, and conjugates.

Figure S2. Circular dichroism spectra.

Figure S3. ^{1}H-NMR spectra of βLG and fLG.

Figure S4. ^{1}H-NMR spectra of melanin-βLG conjugates.

Figure S5. EPR spectrum in the g=4.3 region of PheoβLG-1/2-Fe.

Figure S6. X-ray diffraction patterns of βLG and fLG.

Figure S7. X-ray diffraction patterns of fLG and PheofLG-1/2.

Figure S8. X-ray diffraction patterns of βLG and PheoβLG-1/2-Fe.

Figure S9. X-ray diffraction patterns of fLG and EufLG-2/1-Fe

Figure S10. X-ray diffraction spectra for whole melanins conjugates investigated.

Figure S11. Transmission electron microscopy image of microglia exposed to vehicle.

Table S1. Peptide fragments identified in βLG digestion.

Table S2. Peptide fragments identified in fLG digestion.

Table S3. Peptide fragments not identified in melanin-fLG and melanin-βLG conjugates.

Table S4. Secondary structure content of the protein portion of melanin-conjugates.

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Figure legends

Figure 1. ^{1}H-NMR spectra of fLG conjugates. ^{1}H-NMR spectra recorded in D_2O of Eu-type conjugates: (I) EufLG-2/1-Fe; (II) EufLG-1/2-Fe; (III) EufLG-2/1 and (IV) EufLG-1/2.

Figure 2. ^{1}H-NMR spectra of fLG conjugates. ^{1}H-NMR spectra recorded in D_2O of Pheo-type conjugates: (I) PheofLG-2/1-Fe; (II) PheofLG-1/2-Fe; (III) PheofLG-2/1 and (IV) PheofLG-1/2.

Figure 3. Relative changes in qRT-PCR analyses in microglia cells treated with human and synthetic NMs. Relative changes in qRT-PCR analyses in microglia cells treated with 10 μg/mL solutions of seven different compounds for 4 h are reported for iNOS (**a**), IL-1β (**b**) and COX-2 (**c**). PheoDA and PheoDA-Fe indicate DA-melanins without protein as explained in Supplementary information. Data are expressed as mean fold increases ± SEM (n = 3) of treated microglia compared with untreated microglia, with values set to 0. Dose-response relative changes of iNOS (**d**), IL-1β (**e**), COX-2 (**f**), arginase-1 (**g**) and CD206 (**h**) are reported for microglia treated for 4 h with 1, 2, 10 or 30 μg/mL solutions of PheofLG-1/2-Fe (as representative sample for synthetic NMs) or human NM. Data are expressed as fold increases compared with untreated microglia with values set to 0. The p values have been obtained with paired t-test (before and after); * $p< 0.05$; ** $p < 0.01$;*** $p < 0.001$.

Figure 4. Electron microscopy images of microglia exposed to synthetic and human NM. Microglia exposed for 4 h to 10 μg/mL of PheofLG-1/2-Fe (**a-c**) and to 10 μg/mL of human NM (**d-f**). Both compounds appears as dark and electron-dense pigments that were actively phagocytosed by microglia cells (arrows in **a** and **d**). The synthetic and human NM are engulfed inside specialized phagosomes for future breakdown and degradation of the pigments (higher magnification in panels **b** and **e**, respectively). Sometimes we have observed a double membrane limiting the vacuoles containing the phagocytosed pigment (both PheofLG-1/2-Fe and human NM), probably depending on the state of phagocytosis pathway, and/or their sequestration by autophagosomes, and/or fusion with lysosomes or other vacuoles (arrowhead in **c** and **f** indicating the double membrane) as normally occurs (Klionsky *et al.* 2014). Scale bar = 2 μm in A and D; scale bar = 1 μm in B and E. Scale bar = 500 nm in C and F.

Table 1. Summary of the conditions for the preparation of water soluble melanin-fLG and melanin-βLG conjugates. Protein content (as percentage by weight) of melanin-fLG and melanin-βLG conjugates is reported in the last column.

Sample name	**DA/protein (wt/wt ratio)**	**DA/Cys (mol ratio)**	**Fe/DA (mol ratio)**	**Protein content (%)**
EufLG-2/1	2/1	-	-	45.7
EufLG-1/2	1/2	-	-	56.3
EufLG-2/1-Fe	2/1	-	1/20	36.7
EufLG-1/2-Fe	1/2	-	1/20	55.0
PheofLG-2/1	2/1	5/1	-	39.1
PheofLG-1/2	1/2	5/1	-	56.9
PheofLG-2/1-Fe	2/1	5/1	1/20	38.5
PheofLG-1/2-Fe	1/2	5/1	1/20	59.2
EuβLG-1/2	1/2	-	-	56.4
EuβLG-1/2-Fe	1/2	-	1/20	59.1
PheoβLG-1/2	1/2	5/1	-	54.0
PheoβLG-1/2-Fe	1/2	5/1	1/20	58.6

Table 2. Iron content, measured by inductively coupled plasma - optical emission spectrometry, EPR-active iron(III), and its percentage with respect to total iron content in melanin-fLG-Fe and melanin-βLG-Fe conjugates.

Sample	**Total Fe (µg/mg)**	**EPR- detectable Fe (µg/mg)**	**% EPR-detectable Fe**
EufLG-2/1-Fe	14.25 ± 0.05	0.02	0.15
EufLG-1/2-Fe	4.66 ± 0.03	0.02	0.40
EuβLG-1/2-Fe	4.8 ± 1.1	0.03	0.76
PheofLG-2/1-Fe	10.2 ± 1.7	0.01	0.10
PheofLG-1/2-Fe	2.9 ± 0.6	0.04	1.35
PheoβLG-1/2-Fe	5.3 ± 1.1	0.02	0.40