

Binding Modes of Phthalocyanines to Amyloid β Peptide and Their Effects on Amyloid Fibril Formation

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ABSTRACT The inherent tendency of proteins to convert from their native states into amyloid aggregates is associated with a range of human disorders, including Alzheimer's and Parkinson's diseases. In that sense, the use of small molecules as probes for the structural and toxic mechanism related to amyloid aggregation has become an active area of research. Compared with other compounds, the structural and molecular basis behind the inhibitory interaction of phthalocyanine tetrasulfonate (PcTS) with proteins such as α S and tau has been well established, contributing to a better understanding of the amyloid aggregation process in these proteins. We present here the structural characterization of the binding of PcTS and its Cu(II) and Zn(II)-loaded forms to the amyloid β -peptide ($A\beta$) and the impact of these interactions on the peptide amyloid fibril assembly. Elucidation of the PcTS binding modes to $A\beta_{40}$ revealed the involvement of specific aromatic and hydrophobic interactions in the formation of the $A\beta_{40}$ -PcTS complex, ascribed to a binding mode in which the planarity and hydrophobicity of the aromatic ring system in the phthalocyanine act as main structural determinants for the interaction. Our results demonstrated that formation of the $A\beta_{40}$ -PcTS complex does not interfere with the progression of the peptide toward the formation of amyloid fibrils. On the other hand, conjugation of Zn(II) but not Cu(II) at the center of the PcTS macrocyclic ring modified substantially the binding profile of this phthalocyanine to $A\beta_{40}$ and became crucial to reverse the effects of metal-free PcTS on the fibril assembly of the peptide. Overall, our results provide a firm basis to understand the structural rules directing phthalocyanine-protein interactions and their implications on the amyloid fibril assembly of the target proteins; in particular, our results contradict the hypothesis that PcTS might have similar mechanisms of action in slowing the formation of a variety of pathological aggregates.

INTRODUCTION

Neurodegeneration is characterized by the progressive loss of neuronal cells and the decline of motor and cognitive functions (1). Different neuropathological, genetic, and biochemical studies support the role of protein amyloidogenesis in the development of neurodegenerative disorders (2,3). Although their amino acid sequences and the biological environments in which they function have coevolved to maintain peptides and proteins in their soluble states, in some circumstances, they can convert into nonfunctional and potentially

damaging protein aggregates (4). A detailed understanding of the mechanism by which proteins of wide structural diversity are transformed into potentially damaging aggregates is therefore of high clinical importance.

Alzheimer's disease (AD) is the most common cause of dementia among neurodegenerative disorders in the elderly population, affecting ~25 million people worldwide (5). Neurodegeneration in AD is characterized by the progressive accumulation of extracellular senile plaques consisting of amyloid β -peptide ($A\beta$) and intracellular fibrillary tangles consisting of Tau protein (6). The $A\beta$ is a normally secreted, small peptide (39–43 residues) that results from processing of the larger amyloid precursor protein (APP) (7,8). Extensive genetic and cell viability studies

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support a key role for the A β peptide in AD neurodegeneration and oxidative stress (9–11). In its monomeric state, A β is best described as an ensemble of structurally heterogeneous conformations, with propensities for β -strand structure at two hydrophobic regions (Leu17–Ala21 and Ile31–Val36), and turn or bend-like structures at the Asp7–Glu11 and Phe20–Ser26 segments (12–14). By contrast, due to the complexity of the structural conversions occurring during the amyloid aggregation process, the mechanism(s) underlying the structural transition from the monomeric A β to its aggregated form(s) remains poorly described (4).

Currently, no preventive therapy is available for AD and other protein misfolded diseases (15–17). The ongoing clinical trial strategies for AD include the use of small modified peptides, antibodies, and chemical drugs targeting either A β aggregation, clearance, or production (15,16). The continued failures of biomolecules in late clinical trials, including the recent failure of the promising antibody Solanezumab, highlights the urgent need for a better understanding of the aggregation phenomena, its role in the disease progression, and the structural requirements and mechanistic basis of drug candidates. The discovery of small molecules targeting disease-associated protein aggregation is considered one of the most active therapeutic approaches toward neurodegenerative disorders (18). Not only have they been shown to modulate the aggregation of amyloid proteins both *in vitro* and *in vivo*, but they also have been used as molecular and structural probes to gain insight into the amyloid aggregation process (19–21). From the screening of large libraries of small compounds, a range of potential candidates with a wide range of chemical structures were found to modulate the aggregation of distinct amyloid proteins (21–34). Notably, poly-aromatic scaffolds belonging to the chemical classes of flavonoids, polyphenols, porphyrins, and phthalocyanines were predominantly identified by these screenings (21–31).

Recently, distinct variants of phthalocyanines and porphyrins have been tested for their ability to impair the amyloid assembly process of proteins linked to neurodegeneration. These molecules are cyclic tetrapyrroles, a class of compounds whose distinguished characteristic is the planarity and hydrophobicity of its aromatic ring system. The phthalocyanine tetrasulfonate (PcTS) compounds are among the most widely investigated tetrapyrroles. The structure of PcTS contains four sulfonic acid groups at the borders of the aromatic rings, whereas the center of the molecule can remain ligand free or coordinated to metal ions of various valences (Fig. S1). Besides the demonstrated prophylactic and therapeutic effects of PcTS in scrapie disease (25,27,35–37), this compound suppressed the filament assembly of α S, a key protein involved in the pathology of Parkinson's disease, leading to the formation of a variety of non-toxic, amorphous α S aggregates (21,38). Interestingly, PcTS was also effective in inhibiting the misfolding and aggregation of vesicle-bound α S (39). The potential of PcTS as an amyloid modulator was

also explored on the protein Tau and the A β peptide. PcTS was able to interfere with Tau filament assembly by inducing the conversion of the protein into non-toxic soluble oligomers. From a neuroblastoma-cell-based assay, it was demonstrated that PcTS was also an effective modulator of Tau-induced filament formation (40). In the case of the A β peptide, it was shown that PcTS was able to induce the clearance of metal-induced toxic A β oligomers and their conversion into an amyloid fibrillar meshwork, which coincidentally reduced the toxic activity of A β in cellular assays (41). More recently, it was reported that a novel carboxylated Zn(II)-loaded derivative delayed the formation of A β oligomers and inhibited A β toxicity in neuronal cell lines (42).

Compared with other compounds that show activity as amyloid inhibitors, the structural and molecular basis behind the inhibitory interaction of PcTS with proteins such as α S and Tau has been well established, contributing to a better understanding of the aggregation process in these proteins. In the case of the protein α S, structural characterization of the α S-PcTS complexes indicated that the inhibition of α S amyloid fibril formation in the presence of this compound was a direct consequence of its binding to the N-terminus of the protein (21). The fact that the interaction was shown to be dependent on the presence of the aromatic residues contained in that region demonstrated unequivocally the role of the aromatic moieties as anchoring groups for PcTS binding to α S. These studies revealed also that specific aromatic interaction with the Tyr39 residue provides a central mechanistic basis for the inhibitory process of PcTS on α S fibril formation, whereas the residue-specific structural characterization of the α S-PcTS complex provided the basis for the rational design of non-amyloidogenic species of α S, highlighting the role of aromatic interactions in driving α S amyloid assembly. Added to that, these studies allowed the identification of low-order stacked aggregates of PcTS as the active amyloid inhibitory species (28), whose selective effects on the aromatic moieties in the protein sequence were attributed to their large aromatic surface area.

In the same direction, it was demonstrated that PcTS inhibits the formation of Tau filaments *in vitro* and in cells by selectively interacting with the aromatic residues Tyr197, Tyr310, Phe346, Phe378, and Tyr394 in the central domain of Tau and converting the protein into soluble oligomers (40). That study revealed also detailed insights into the mechanism of Tau-aggregation inhibition and the structure and dynamics of soluble Tau oligomers, demonstrating that the structure of off-pathway oligomers of Tau is distinct from the structure of toxic Tau oligomers.

The fact that PcTS molecules were shown to block different types of disease-associated protein aggregation, such as that of α -synuclein and Tau proteins, raised the possibility that this compound might have similar structural and molecular mechanisms of action in slowing the formation of a variety of pathological aggregates. In that direction, in this work, we sought to delineate the structural basis of the

interaction of PcTS and metal-loaded variants of this phthalocyanine with $A\beta_{40}$ and to analyze its implications for amyloid fibril formation of the peptide. Elucidation of the PcTS binding modes to $A\beta_{40}$ revealed that both aromatic and hydrophobic interactions play a key role in the process of molecular recognition, ascribed to an interaction mode in which the planarity and hydrophobicity of the aromatic ring system in the phthalocyanine molecule act as main structural determinants for the interaction with $A\beta_{40}$. In contrast with previous studies on other amyloid proteins, the formation of $A\beta_{40}$ -PcTS complexes did not interfere with the progression of the peptide toward the formation of mature amyloid fibrils. The binding profile of PcTS(Cu(II)) to $A\beta_{40}$ and its effects on the peptide amyloid fibril formation paralleled that of metal-free PcTS. On the other hand, our results demonstrated that conjugation of Zn(II) at the center of the PcTS macrocyclic ring modified substantially the binding profile of this phthalocyanine to $A\beta_{40}$ and became crucial to reverse the effects of metal-free PcTS on the fibril assembly of the peptide. In this case, the coordination geometry preferences of the Zn(II) ion conjugated at the central core of PcTS constituted the key structural factor behind the inhibitory interaction of this phthalocyanine with $A\beta_{40}$. Overall, the results reported in this work provide a firm basis to understand the structural rules directing phthalocyanine-protein interactions and their implications on the amyloid fibril assembly of the target proteins; in particular, our results contradict the hypothesis that PcTS might have similar mechanisms of action in slowing the formation of a variety of pathological aggregates.

MATERIALS AND METHODS

Sample preparation and reagents

Non-labeled and ^{15}N isotopically enriched $A\beta_{40}$ samples were purchased from EZBiolab (Carmel, IN) and Alexotech (Oslo, Norway), respectively. Peptide samples were prepared according to the alkaline dissolution protocol (12). Lyophilized peptide (1 mg) was dissolved in 400 μL of 10 mM NaOH, incubated for 30 min in ice, aliquoted, and stored at -80°C . Immediately before recording the NMR experiments, one or more $A\beta_{40}$ aliquots were diluted in 20 mM TRIS buffer. The pH was then adjusted to 7.5 and samples were centrifuged at $20,000 \times g$ to eliminate potentially pre-formed aggregates (43). The peptide concentration was measured by ultraviolet spectroscopy using a molar extinction coefficient, $\epsilon_{280\text{ nm}} = 1490\text{ cm}^{-1}\text{ M}^{-1}$. Purity of the $A\beta_{40}$ solutions was assessed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). PcTS was purchased from MP Biomedicals (Solon, OH); Zn(II) phthalocyanine tetrasulfonic acid (PcTS(Zn(II))) was purchased from Frontier Scientific (Logan, UT); Cu(II) phthalocyanine tetrasulfonic acid (PcTS(Cu(II))) was purchased from Sigma-Aldrich (St. Louis, MO).

Aggregation assay

Aggregation kinetics measurements were performed with 50 μM peptide samples dissolved in 20 mM TRIS buffer at pH 7.5. Samples were incubated at 37°C under constant stirring in the absence and presence of phthalocyanines. Although the Thioflavin T (ThioT) fluorescence assay is

usually used to monitor amyloid fibril formation, in the case of phthalocyanine-treated samples, the reliability of ThioT as an indicator of fibril inhibition may be questionable, since these small molecules interfere significantly and alter ThioT fluorescence in the absence of proteins due to their intrinsic absorbance and fluorescent properties (44). Accordingly, in this work, we used an NMR-based approach that measures the consumption of the monomeric state of the amyloid protein/peptide during the progression of the aggregation process (21,45,46). The amount of soluble $A\beta$ monomers at different time points of the aggregation assay was determined by integration of the protein NMR resonances in the aliphatic region of the one-dimensional (1D) ^1H NMR spectrum (0.7–1.0 ppm). Aggregation yields were normalized to the initial values of the free $A\beta$ peptide sample as previously described (45). Endpoint aliquots of aggregation assays were loaded in 8–23% gradient SDS-PAGE (47) and were visualized using a silver staining protocol as previously described (48).

NMR spectroscopy

NMR spectra were acquired on a 600 MHz Avance III (Bruker, Billerica, MA) equipped with a cryogenically cooled triple-resonance $^1\text{H}(^{13}\text{C}/^{15}\text{N})$ TCI probe. Heteronuclear NMR experiments were performed with pulsed-field-gradient-enhanced pulse sequences on 100 μM ^{15}N -labeled peptide samples in 20 mM TRIS buffer (pH 7.5) at 15°C . 1D ^1H -NMR experiments were acquired at 15°C on 50 μM unlabeled $A\beta_{40}$ dissolved in deuterated 20 mM TRIS buffer (pH 7.5). Aggregation did not occur under these low temperature conditions and absence of stirring.

For the mapping experiments, ^1H - ^{15}N heteronuclear single quantum correlation (HSQC) amide cross-peaks affected during PcTS titration were identified by comparing their intensities (I) with those of the same cross-peaks in the data set of samples lacking the tetrapyrrolic compound (I_0) (49). Mean weighted chemical-shift displacements (^1H - ^{15}N MWCS) were calculated as $[(\Delta\delta^1\text{H})^2 + (\Delta\delta^{15}\text{N})^2/25]^{1/2}$ (49).

Pulsed-field-gradient-NMR experiments were acquired at 15°C on 100 μM unlabeled $A\beta_{40}$ peptide samples dissolved in D_2O and containing dioxane as an internal radius standard and viscosity probe. NMR spectra were recorded in the absence and presence of phthalocyanines. A series of 20 1D spectra were collected as a function of gradient amplitude. The gradient strength was shifted from 0.68 to 32 G cm^{-1} in a linear manner.

Acquisition, processing, and visualization of the spectra were performed by using TOPSPIN 2.0 (Bruker) and Sparky (Goddard and Kelner, University of California San Francisco).

Electron microscopy

Ten-microliter aliquots withdrawn from aggregation reactions were adsorbed onto Formvar/carbon-coated copper grids (Pella, Redding, CA) and negative stained with 2% (w/v) uranyl acetate. Images were obtained at various magnifications (1000–90,000 \times) using a Philips CM120 transmission electron microscope.

RESULTS

PcTS is not able to impair $A\beta_{40}$ amyloid fibril assembly

The ability of PcTS to interfere with $A\beta_{40}$ amyloid assembly was analyzed by using NMR, SDS-PAGE, and transmission electron microscopy (TEM). As shown in Fig. 1, A and B, the time course of aggregation of free $A\beta_{40}$ monitored as monomer consumption by 1D ^1H -NMR spectroscopy was comparable to that measured in the presence of PcTS. Indeed, we found that PcTS did not reduce the amount of

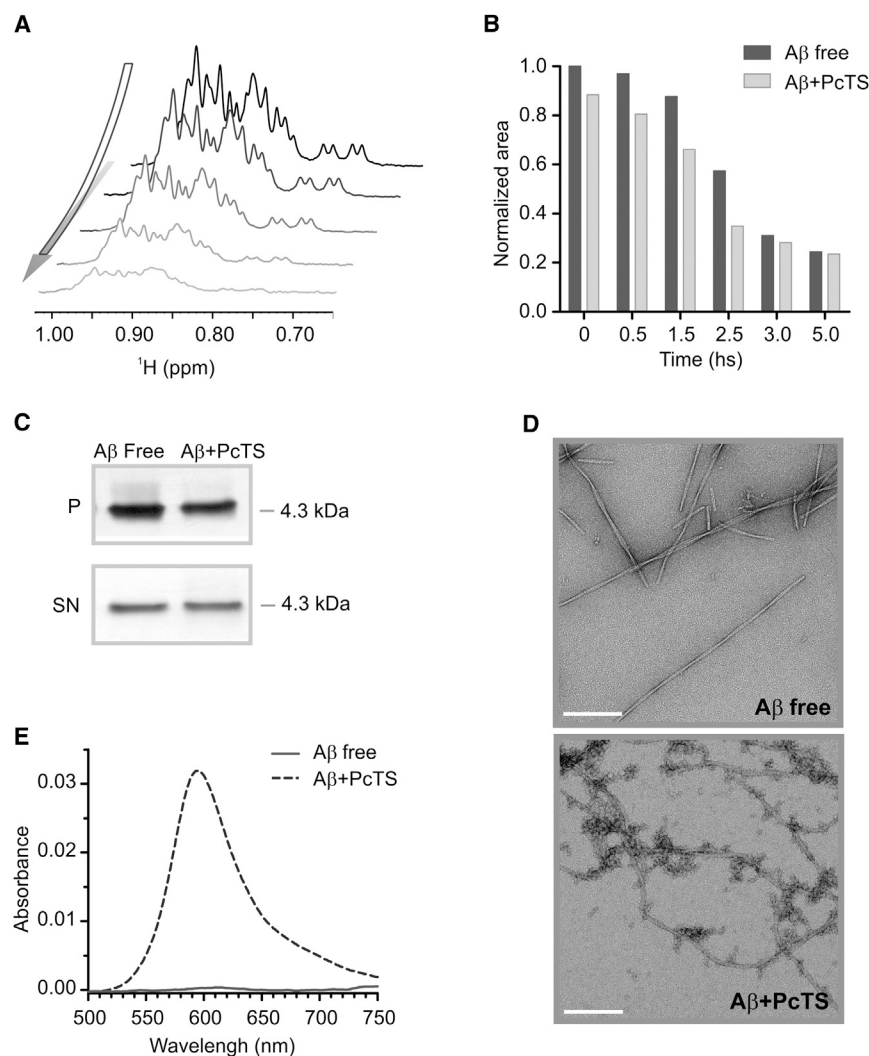


FIGURE 1 Analysis of PcTS effects on $A\beta_{40}$ amyloid fibril formation. (A) 1D ^1H -NMR spectra (aliphatic region) of $50\ \mu\text{M}$ $A\beta_{40}$ as a function of aggregation time. Monomer consumption was quantified by integration of the NMR signals in the 0.7–1.0 ppm spectral region. (B) Level of remaining soluble $A\beta_{40}$ monomers in the absence (black bars) and presence (gray bars) of $150\ \mu\text{M}$ PcTS, determined by 1D ^1H -NMR spectroscopy. (C) SDS-PAGE of $A\beta_{40}$ peptide soluble (SN) and insoluble (P) fractions of the end points of the aggregation assays ($50\ \mu\text{M}$ $A\beta_{40}$) in the absence (control) and presence of $150\ \mu\text{M}$ PcTS. (D) Representative negative-stain electron microscopy images of $A\beta_{40}$ aggregates ($50\ \mu\text{M}$ $A\beta_{40}$ samples) generated in the absence and presence of $150\ \mu\text{M}$ PcTS. Scale bars, 100 nm. (E) Electronic absorption spectra corresponding to the pellet fraction of $50\ \mu\text{M}$ $A\beta_{40}$ aggregations obtained in the absence (solid line) or presence (dashed line) of $150\ \mu\text{M}$ PcTS.

aggregated $A\beta_{40}$ (Fig. 1 C). When the final product of aggregation of $A\beta_{40}$ in PcTS-treated samples was analyzed by TEM, the images revealed the presence of amyloid fibrils decorated with a variety of small, apparently amorphous species (Fig. 1 D). Consistent with this, absorption spectroscopy on extensively washed $A\beta_{40}$ aggregates from PcTS-treated samples indicated that PcTS molecules were incorporated into the $A\beta_{40}$ amyloid fibrils (Fig. 1 E). Altogether, the results demonstrate that even though PcTS appears to be efficiently incorporated into the final product of aggregation of $A\beta_{40}$, the cyclic tetrapyrrole does not inhibit the amyloid fibril formation of the peptide.

Formation of the $A\beta_{40}$ -PcTS complex at the N- and C-termini is mediated by aromatic and hydrophobic interactions

To assess whether the absence of an inhibitory effect of PcTS on $A\beta_{40}$ amyloid assembly might be linked to the lack of interaction of the cyclic tetrapyrrole with monomeric

$A\beta_{40}$, we used ^1H - ^{15}N HSQC spectra. The central region of the ^1H - ^{15}N HSQC spectrum of a $100\ \mu\text{M}$ sample of uniformly ^{15}N -labeled $A\beta_{40}$ recorded in 20 mM TRIS buffer (pH 7.5) at 15°C is shown in Fig. 2 A.

The resonances were well resolved and sharp, with a limited dispersion of chemical shifts, reflecting the disordered nature and the high degree of mobility of the backbone that characterize the monomeric state of $A\beta_{40}$ peptide. Upon titration of ^{15}N -enriched $A\beta_{40}$ with increasing concentrations of PcTS, the ^1H - ^{15}N HSQC spectra demonstrated measurable broadening and chemical-shift changes in a discrete number of residues distributed throughout the peptide sequence (Fig. 2, A and B). This behavior indicates that a certain number of signals from free and bound states of $A\beta_{40}$ cannot be resolved and are averaged in a manner that leads to resonance line broadening, indicative of a system undergoing intermediate exchange on the NMR timescale. In other words, the broadening is caused by an exchange of $A\beta_{40}$ molecules between the free and PcTS-bound states, as previously reported for the αS and

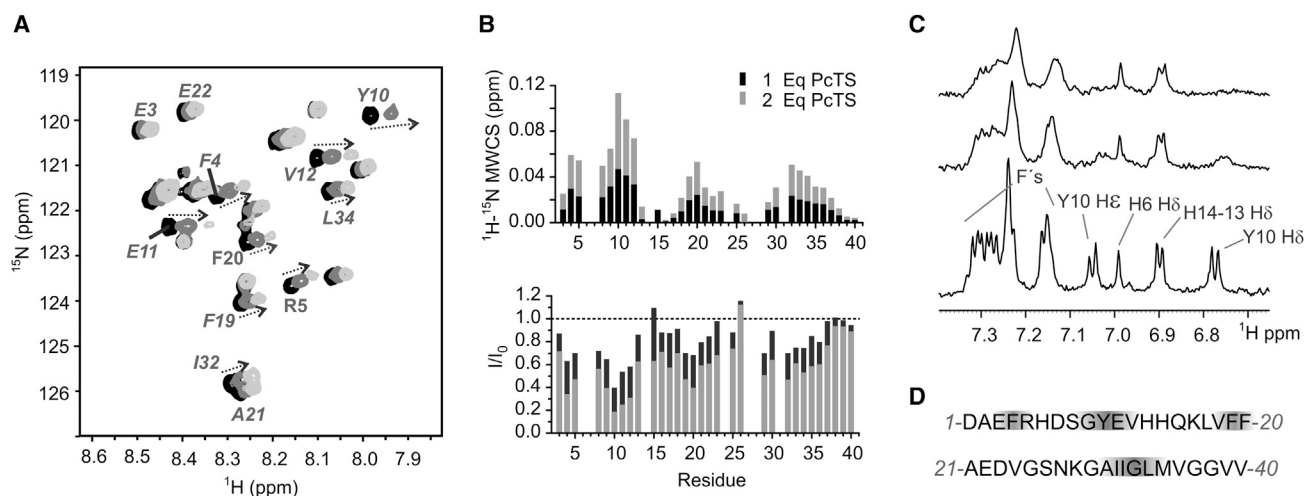


FIGURE 2 Analysis of PcTS binding to Aβ₄₀ by NMR. (A) Overlaid ¹H-¹⁵N HSQC spectra of 100 μM Aβ₄₀ in the absence (black) and presence (dark gray) of 100 and 200 μM PcTS (gray). Amino acid residues broadened significantly or beyond detection are identified. (B) I/I₀ profiles and differences in the mean-weighted chemical-shift displacements (¹H-¹⁵N MWCS) of the backbone amide groups of 100 μM Aβ₄₀ in the presence of 100 μM (black) and 200 μM PcTS (gray). (C) 1D ¹H-NMR of aromatic side chains of 50 μM Aβ₄₀ in the absence (lower trace) and presence of 12.5 μM (middle trace) and 25 μM PcTS (upper trace). The slight effects observed on His13 and His14 upon addition of PcTS are likely due to its proximity to Tyr10. (D) Primary sequence of Aβ₄₀ indicating in gray the identified PcTS binding regions.

Tau proteins (21,28,40). The effects became evident at 100 μM PcTS and the broadening of resonances was further pronounced at 200 μM PcTS. A detailed analysis of ¹H-¹⁵N backbone amide signals allowed us to identify four major binding interfaces for PcTS in Aβ₄₀: aa3–5, 8–12, 18–23, and 32–37 (Fig. 2 B), as reflected by the intensities and chemical-shift interaction profiles. A close analysis of the cross-peaks exhibiting severe broadening effects and chemical-shift displacements upon titration with PcTS revealed that they correspond to regions located in the proximity of aromatic residues (Phe in position 4, Tyr in position 10, and two Phe in positions 19 and 20) and to a hydrophobic segment at the C-terminus (Fig. 2, B and D).

Considering that the degree of perturbation observed on the backbone amide groups of Aβ₄₀ in the presence of the PcTS compound might be influenced also by the occurrence of other structural events, such as solvent-exchange phenomena triggered by the ligand-peptide interaction, to probe further the role of aromatic residues in the Aβ₄₀-PcTS interaction, we conducted NMR experiments aimed to directly detect the resonances of the aromatic side chains of Aβ₄₀. The 1D ¹H-NMR spectrum of Aβ₄₀ shows well-resolved clusters of resonances in the 6.5–7.5 ppm range, comprising the side chains of different aromatic residues: Phe4, His6, Tyr10, His13, His14, Phe19, and Phe20 (Fig. 2 C). The distribution of these residues throughout the Aβ₄₀ sequence provides excellent probes for exploring the binding features of the PcTS ligand to the peptide. Clearly, the binding features observed for PcTS confirm the direct role played by the aromatic side chains of Tyr10 of Aβ₄₀ in the interaction process (Fig. 2 C). Although complicated by severe signal overlapping, the broadening effects induced by PcTS on

the cluster of resonances assigned to Phe residues at positions 4, 19, and 20 became also evident. Interestingly, the titration experiments showed only small perturbations caused by PcTS on the resonances assigned to the imidazole rings of histidines at positions 6, 13, and 14, likely due to the proximity of these residues to Phe4 and Tyr10, suggesting a lack of interaction of the phthalocyanine with the imidazole rings at these sites.

To assess further the assembly state of the Aβ₄₀ peptide in the absence and presence of PcTS, we performed pulsed-field gradient NMR experiments. Pulsed-field gradient NMR on the free and PcTS-complexed states of the Aβ₄₀ peptide showed identical decay curves of NMR signal intensity (Fig. S2). The results obtained from the curves showed that the free Aβ₄₀ and its PcTS-complexed form have the same apparent diffusion coefficient of $1.08 \times 10^{-8} \text{ cm}^2 \text{ s}^{-1}$ at 15°C and thus the same assembly state. A calculated hydrodynamic radius of ~1.5 nm demonstrates that the NMR-visible states of the peptide are mainly monomeric, in good agreement with previous reports of the hydrodynamic properties of monomeric Aβ₄₀ peptide (50). Added to that, the absence of noticeable changes in the hydrodynamic properties of the Aβ₄₀ peptide upon addition of PcTS (Fig. S2) indicates that the binding of the phthalocyanine to monomeric Aβ₄₀ would proceed via low-order stacked species of the compound, as previously reported for the interactions of PcTS with the protein α-synuclein (28). These results allow us to conclude that 1) PcTS binds to the monomeric form of Aβ₄₀; 2) both N- and C-terminal regions of Aβ₄₀ represent binding interfaces for PcTS, ascribed to an interaction mode in which the planarity and hydrophobicity of the aromatic ring system in the

phthalocyanine molecule act as main structural determinants for the interaction with $A\beta_{40}$; 3) Phe4, Tyr10, Phe at positions 19 and 20, and residues 32–37 constitute the main anchoring groups for PcTS binding to $A\beta_{40}$, whereas no direct interaction was observed with the cluster of histidines in positions 6, 13, and 14; and 4) formation of $A\beta_{40}$ -PcTS complexes does not interfere with the progression of the peptide toward the formation of mature amyloid fibrils.

PcTS(Zn(II)) but not PcTS(Cu(II)) inhibits amyloid fibril formation of $A\beta_{40}$

It is well documented that the incorporation of different metal cations into the central core of phthalocyanines strongly influences the biological activity of this type of molecule (27,28,36,37,51–54). Consistent with evidence that relates Cu(II) and Zn(II) to the etiology of AD (55,56), we decided to evaluate the effects of the Cu(II)- and Zn(II)-bound forms of PcTS on $A\beta_{40}$ amyloid fibril assembly (Fig. 3, A–D). Our TEM studies showed that the PcTS(Zn(II)) derivative completely abolished $A\beta_{40}$ amyloid fibril formation, inducing a variety of small, amorphous, non-fibrillar $A\beta_{40}$ aggregates (Fig. 3 B), whereas PcTS(Cu(II))-treated samples showed an amyloid fibril morphology similar to those of free $A\beta_{40}$ samples (Fig. S3). Interestingly, Fig. 3 C shows that $A\beta_{40}$ monomers are rapidly assembled into non-detectable NMR species upon addition of PcTS(Zn(II)), suggesting that PcTS(Zn(II))-treated samples became predominantly populated by an aggregated $A\beta_{40}$ state(s). As shown in Fig. 3 D, analysis of the final product of aggregated $A\beta_{40}$ -PcTS(Zn(II)) samples, performed by SDS-PAGE, confirm this finding. As observed for the PcTS-treated $A\beta_{40}$ samples, absorption spectroscopy on extensively washed $A\beta_{40}$ aggregates from PcTS(Zn(II))-treated samples indicates that this compound is incorporated into the $A\beta_{40}$ aggregates (Fig. S4). In addition, the observation of the characteristic PcTS(Zn(II)) electronic spectrum in the pellet fraction of the $A\beta_{40}$ aggregates demonstrates conclusively that the metal ion remains coordinated to the tetrapyrrolic macrocycle under these experimental conditions. On the other hand, incubation of $A\beta_{40}$ samples in the presence of PcTS(Cu(II)) resulted in SDS-PAGE profiles similar to that observed for metal-free PcTS (Fig. S3). Overall, these results demonstrate that coordination of Zn(II), but not Cu(II), into the PcTS scaffold renders phthalocyanine an efficient modulator of $A\beta_{40}$ amyloid fibril formation.

PcTS(Zn(II)) binds preferentially to the N-terminal histidine residues of $A\beta_{40}$

To evaluate the possibility that the distinct effects of PcTS(Zn(II)) and PcTS(Cu(II)) on $A\beta_{40}$ fibril formation correlate with the features of their binding to the peptide, we analyzed the interaction profiles of both ligands with

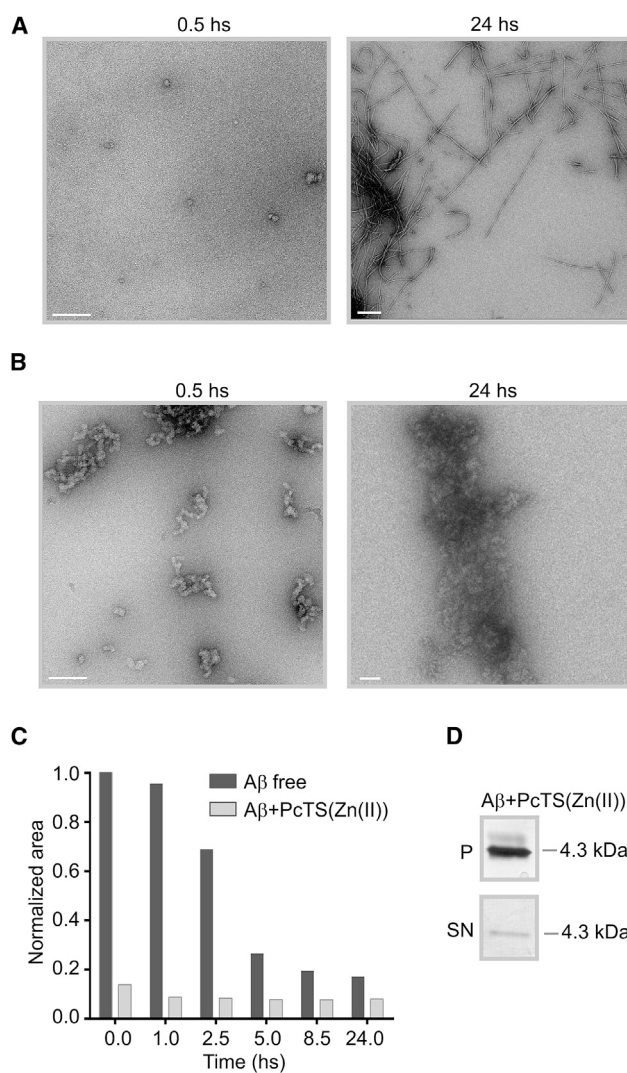


FIGURE 3 Analysis of PcTS(Zn(II)) effects on $A\beta_{40}$ amyloid assembly. (A and B) Representative negative-stain EM images of $A\beta_{40}$ aggregates (50 μ M $A\beta_{40}$ samples) generated in the absence (A) and presence of 150 μ M PcTS(Zn(II)) (B). The products of aggregation were analyzed at the time intervals of 0.5 and 24 h of the aggregation assay. Scale bars, 100 nm. (C) Level of remaining soluble $A\beta_{40}$ monomers in the absence (black bars) and presence (gray bars) of 150 μ M PcTS(Zn(II)) determined by 1D 1 H-NMR spectroscopy during the time course of the aggregation assay. (D) SDS-PAGE analysis of $A\beta_{40}$ peptide soluble (SN) and insoluble (P) fractions of the end point of the aggregation assays in the presence of three equivalents of PcTS(Zn(II)).

monomeric $A\beta_{40}$. To this purpose, we first used 1D 1 H-NMR spectroscopy by monitoring again the spectral region containing the resonances of aromatic side chains in $A\beta_{40}$. Whereas the binding features of PcTS(Cu(II)) to $A\beta_{40}$ resemble those described for the metal-free phthalocyanine (Fig. 4 A), the interaction profile of PcTS(Zn(II)) changed substantially, suggesting a role for the imidazole rings of histidine residues at positions 6, 13, and 14 of $A\beta_{40}$ as the main anchoring groups for PcTS(Zn(II)) binding. As reported for the interaction with metal-free PcTS,

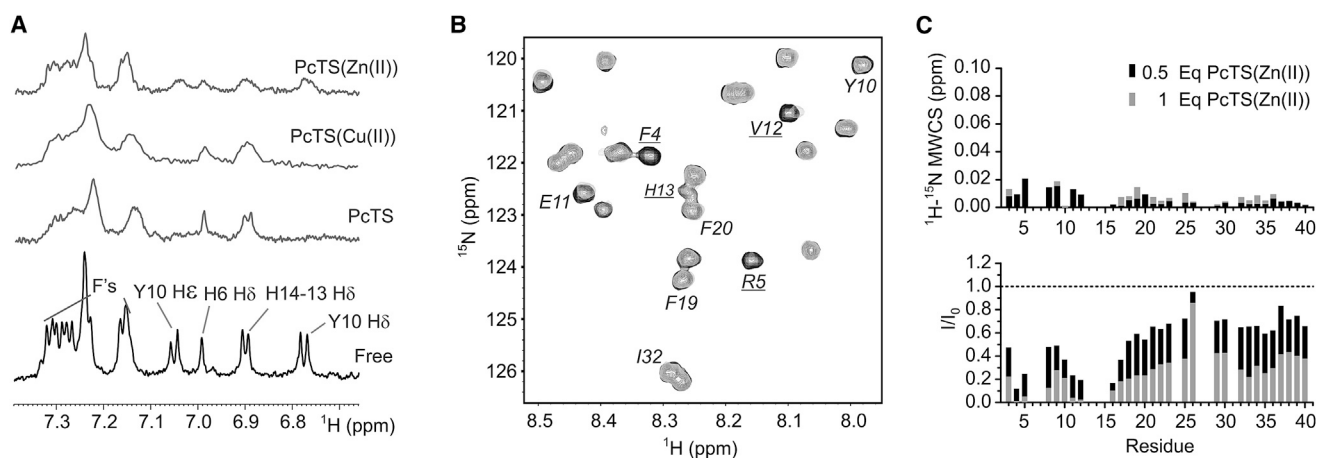


FIGURE 4 Analysis of PcTS(Zn(II)) binding to $A\beta_{40}$ by NMR. (A) 1D ^1H -NMR of aromatic side chains of $50\ \mu\text{M}$ $A\beta_{40}$ in the absence (*lower trace*) and presence of $25\ \mu\text{M}$ of PcTS and its Zn(II) and Cu(II) derivatives. The higher degree of signal broadening in the spectrum of the $A\beta_{40}$ -PcTS(Cu(II)) complex obeys the paramagnetic nature of Cu(II) ions. Spectra were registered at 15°C in deuterated $20\ \text{mM}$ TRIS_d buffer (pH 7.5). (B) Overlaid ^1H - ^{15}N HSQC spectra of $100\ \mu\text{M}$ $A\beta_{40}$ in the absence (*black*) and presence (*gray*) of $50\ \mu\text{M}$ PcTS(Zn(II)). Amino acid residues broadened significantly or beyond detection are underlined. (C) I/I_0 profiles and differences in the mean-weighted chemical-shift displacements of the backbone amide groups of $100\ \mu\text{M}$ $A\beta_{40}$ in the presence of $50\ \mu\text{M}$ (*black*) and $100\ \mu\text{M}$ PcTS(Zn(II)) (*gray*).

the hydrodynamic properties of $A\beta_{40}$ remained unchanged upon complexation with PcTS(Cu(II)) and PcTS(Zn(II)) (data not shown).

To gain further insight into the unique binding properties of PcTS(Zn(II)) to $A\beta_{40}$, we then used ^1H - ^{15}N HSQC spectroscopy (Fig. 4 B). Titration of $A\beta_{40}$ samples with 50 – $100\ \mu\text{M}$ PcTS(Zn(II)) caused a severe broadening, in some cases beyond detection, of a group of resonances centered at the His6 and His13–14 sites (Fig. 4 C). Thus, the combined analysis of 1D and 2D NMR titration experiments confirm that the imidazole rings of histidines at positions 6, 13, and 14 constitute the main anchoring groups for PcTS(Zn(II)) binding to $A\beta_{40}$. Added to the major effects centered on the resonances of His residues, the binding of PcTS(Zn(II)) to $A\beta_{40}$ causes a significant and general signal attenuation phenomenon. The general signal intensity reduction in the NMR spectra due to PcTS(Zn(II)) binding is consistent with the picture obtained by TEM and SDS-PAGE analysis, demonstrating that upon complexation with PcTS(Zn(II)), the $A\beta_{40}$ samples become mostly populated by an aggregated, non-fibrillar state(s).

DISCUSSION

The discovery and design of small molecules that efficiently target disease-associated protein aggregation became a promising tool for the development of therapeutic strategies. To understand fully, from a mechanistic perspective, the way these compounds might modulate protein aggregation, it is of paramount importance to decipher the structural and molecular basis of the implied protein-ligand interactions, but also to investigate the structural requirements of the small molecules that are critical for efficient and specific anti-amyloid activity.

In this work, we have delineated the structural basis for the interaction of PcTS with $A\beta_{40}$ and demonstrated that the nature of the metal ion incorporated into the heterocycle center modulates the binding features and the activity of PcTS on $A\beta_{40}$ amyloid fibril formation. The NMR analysis of $A\beta_{40}$ complexes with PcTS demonstrated unequivocally the role of the aromatic moieties as anchoring groups for PcTS binding to the monomeric state of $A\beta_{40}$, ascribed to a binding mode in which the planarity and hydrophobicity of the aromatic ring system in the phthalocyanine molecule act as main structural determinants for the interaction with $A\beta_{40}$.

Our studies revealed also that hydrophobic interactions between the macrocycle ring of PcTS and the central and C-terminal hydrophobic clusters of $A\beta_{40}$ contribute strongly to the binding process. Electrostatic interactions between negative sulfonates at the periphery of the aromatic ring in PcTS and suitably positioned positive centers, likely provided by the lysine and arginine residues located in the vicinity of the binding sites, might also have an auxiliary role as modulators of the interaction. Overall, the interaction scenario described here for $A\beta_{40}$ -PcTS seems to be a unique feature of this complex, since only aromatic interactions were proposed to mediate the inhibitory binding of PcTS to the proteins αS and Tau (21,40). Interestingly, these differences might provide a structural explanation for the lack of inhibitory activity of PcTS molecules against $A\beta_{40}$ amyloid fibril formation. The central and C-terminal hydrophobic segments that were strongly affected by PcTS binding to monomeric $A\beta_{40}$ constitute the two main β -sheet segments that characterize the architecture of $A\beta$ fibrils (57,58). Intramolecularly, the β -sheets are held together by hydrophobic interactions, and several works indicate that the proximity of the N- and C-terminal parts of the $A\beta$ sequence would be pre-ordered at the oligomeric and

monomeric levels as well (12–14,59), where the central and C-terminal hydrophobic segments might form a common binding interface, of a transient nature, capable of interacting with the hydrophobic PcTS species. Accommodation of the macrocycle ring of PcTS molecules into that transient hydrophobic interface would act then by enhancing or stabilizing rather than compromising early intra- and intermolecular interactions necessary for amyloid structural transitions. Added to the lack of inhibitory effects on $A\beta_{40}$ amyloid fibril assembly, the fact that PcTS molecules appear mostly incorporated into $A\beta_{40}$ aggregates gives further support to this hypothesis.

Since it was demonstrated that the conjugation of metal ions into the central cavity of phthalocyanines influences the self-association propensity and the biological activity of these compounds, we then extended our studies to Zn(II) and Cu(II)-loaded phthalocyanines. Our study revealed that metal incorporation into the architecture of PcTS influenced the activity of these compounds on $A\beta_{40}$ amyloid fibril assembly in different ways. Conjugation with Zn(II) resulted in a PcTS variant with inhibitory effects on $A\beta_{40}$ amyloid fibril formation. We found that PcTS(Zn(II)) produced a variety of small, apparently amorphous, non-fibrillar $A\beta_{40}$ aggregates. On the other hand, the $A\beta_{40}$ aggregates in the PcTS(Cu(II))-treated samples showed an amyloid morphology comparable with that of free $A\beta_{40}$ samples.

Considering that the phthalocyanines studied in our work exist in solution mostly as self-stacked aggregates (28,60), the differences observed in their activity against $A\beta_{40}$ fibril formation seems to be relatively insensitive to the tendencies of PcTS derivatives to self-associate. Interestingly, another metal-sensitive property of PcTS molecules that might influence their binding features and consequently their activity as amyloid inhibitors is related to the different properties of the metal ion coordinated into the core aromatic ring of the phthalocyanine. Indeed, the residual positive charge located at the metal ion, the preferred coordination stereochemistry of the metal ion, or its relative affinity for axial ligands might potentially act as critical structural determinants for the mode(s) of interaction of these compounds with protein target sites. In that sense, we noticed that coordination of Zn(II) to PcTS redirected the binding preferences of the molecule toward the N-terminal region, where the interaction was mostly centered on the histidine residues located at that region. It is well established that zinc binds to the same three His residues as copper, with K_{dapp} of $\sim 0.1 \mu\text{M}$ for Cu(II) and $\sim 2 \mu\text{M}$ for Zn(II) ions (55,56). However, when loaded into the central cavity of PcTS, only Zn(II) ions, not Cu(II), might be still able to accommodate histidine(s) into its coordination environment, likely reflecting a higher capability for the Zn(II)-metallated species to accommodate axial ligands out of the plane of the PcTS scaffold. Although more work is needed to confirm this hypothesis, this interpretation is in agreement with the more pronounced propensity of Zn(II)

ions to form flexible and open coordination geometries. Overall, these results indicate that the nature of the metal ion conjugated at the center of the phthalocyanine ring may modulate the binding features and anti-amyloid activity of the PcTS compound by targeting other binding sites in the $A\beta_{40}$ peptide.

Related to the molecular events behind the effects of PcTS(Zn(II)) on $A\beta_{40}$ amyloid fibril formation, it was shown that the N-terminal hydrophilic region of the free $A\beta$ adopts an extended conformation rich in PII helix that is proposed to be essential to keep the peptide soluble and protected from amorphous aggregation (61,62). Thus, we assume that the presence of the Zn(II)-loaded phthalocyanine might perturb the structural preferences at the N-terminal hydrophilic region by inducing the formation of a network of $A\beta$ peptides, in which the metal ion conjugated to the PcTS molecule would act to stabilize these structures by binding histidines belonging to adjacent peptides. Indeed, previous works demonstrated that Zn(II) ions can bridge intermolecular N-terminus interaction of the type $N\tau$ -Zn(II)- $N\tau$ between histidine imidazole rings of different $A\beta$ polypeptide chains, preventing the structural transitions of the peptide into amyloid-competent species (63,64). In that direction, recent studies focused on the interaction of heme centers with $A\beta$ amyloid confirmed the binding of the heme iron to the His residues in the N-terminal region of the peptide and indicated that this interaction was crucial to prevent the formation of $A\beta$ ordered fibrils through a mechanism that involved Fe(III)-mediated intermolecular histidine bridging (65,66).

Added to the fact that cyclic tetrapyrroles have been shown to block different types of disease-associated protein aggregation, the previous studies of the effects of PcTS on α -synuclein and Tau proteins raised the possibility that this compound might have similar mechanisms of action in slowing the formation of a variety of pathological aggregates. However, based on the fact that PcTS was not able to impair the amyloid fibril formation of $A\beta_{40}$, our work seems to contradict that hypothesis. Although more work is clearly needed, these results likely reflect the differences in the universe of structural conversions and driving forces that direct the amyloid aggregation of these proteins.

Overall, the results reported in this work provide a firm basis to understand the structural rules directing phthalocyanine-protein interactions and their implications for the amyloid fibril assembly of the target protein. Because the structural basis for the anti-amyloid effects of these molecules is starting to emerge, combined efforts from the fields of structural and cell biology are needed at this stage to elucidate the precise molecular mechanism(s) of action of these molecules.

SUPPORTING MATERIAL

Four figures are available at [http://www.biophysj.org/biophysj/supplemental/S0006-3495\(18\)30066-3](http://www.biophysj.org/biophysj/supplemental/S0006-3495(18)30066-3).

AUTHOR CONTRIBUTIONS

A.A.V.-G. and C.O.F. designed research. A.A.V.-G. and D.R. performed research. A.A.V.-G., T.F.O., M.A.M.-M., C.G., and C.O.F. analyzed data and wrote the article.

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