Inhibitors of Crystallin Aggregation

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Contents

Abstract

Cataract is one of the leadings causes of vision loss worldwide. The exact mechanism is unknown, but aggregation of crystallin proteins, which make up much of the protein content of the lens and are involved in maintaining the integrity of the lens, leads to the characteristic clouding of the lens. Given this, it is of great clinical importance to identify possible inhibitors of crystallin aggregation. Phthalocyanine compounds are known to inhibit aggregation of proteins such as tau and α-synuclein, which are related to neurodegeneration. Their effectiveness as inhibitors of crystallin aggregation has not been evaluated. To characterize the potential of phthalocyanines as inhibitors of crystallin aggregation, fluorescence proteostat aggregation assays and transmission electron microscopy (TEM) were used to detect the presence of aggregation via β-sheet character and determine specific aggregation morphology respectively. It was determined that porphyrazine 285 (PZ285) exhibited poor inhibition of crystallin aggregation due to its minimal solubility in aqueous solution. Phthalocyanine tetrasulfonate (PcTS) showed some reduction in crystallin aggregation in a concentration-dependent manner.

Introduction

The lens of the eye plays a critical role in vision; it focuses light onto the retina. The lens has a high protein concentration of which crystallins make up approximately 90% [1]. Short range interactions between crystallin proteins are important for maintaining lens transparency and refractive index [2]. α-Crystallin, a member of the small heat shock protein family, is known to have chaperone-like activity and thus is important for sequestering proteins whose native structure is compromised. The β- and γ-crystallins primarily play structural roles.

Cataract, which is associated with clouding of the eye lens (Fig. 1), occurs when light scattering high molecular weight aggregates form, which are largely made up of modified crystallin proteins [3].

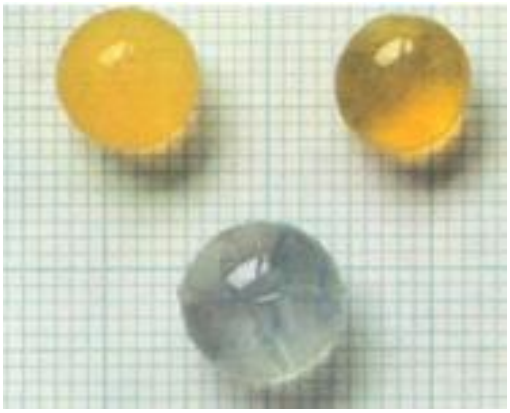


Figure 1: Example Lenses
On the top left, an aged lens with cataract is pictured. The top right is an aged lens without cataract. The bottommost lens is clear and young. (http://photobiology.info/Roberts.html)

The exact mechanism of cataractogenesis is not known. However, factors such as protein damage lead to malfunction of α-crystallin and minimize its effectiveness as a chaperone. In turn, uncontrollable aggregation of peptides and proteins takes place causing eye lens clouding and eye disease. The accumulation of post-translation modifications and low molecular weight

crystallin fragments are of particular interest as they may be contributing elements to protein aggregation in the lens [4]. As there is little protein turnover in the lens, post-translation modifications such as deamidation, truncation, phosphorylation, oxidation, glycation, cross-linking and aspartate isomerization build up over time [4, 5]. While the sites of such modifications have been well-documented, it is difficult to predict the exact effects as crystallin proteins in vivo each have their own set of post-translational modifications [4]. However, it has been demonstrated that these modifications can disrupt the native structure of crystallin proteins and subsequently affect their solubility and, in the case of α-crystallin, affect chaperone-like activity [5]. Numerous crystallin fragments, which accumulate as the lens ages, have been identified and are implicated in the development of cataract [6]. These fragments are found predominantly in the nuclear region of the lens which contains the oldest fiber cells and in regions of opacity [6]. Normally, the proteolytic system in the lens degrades peptides into their constituent amino acids. Possible explanations for the buildup of these crystallin peptides are the proteolytic systems become less effective or are overwhelmed or the tendency of these peptides to associate with crystallin proteins protects them from proteolytic enzymes [7]. One fragment of particular interest is the αA-66-80 peptide as it consists of part of the α-crystallin chaperone site, tends to form fibril-like aggregates, and impairs the chaperone-like activity of α-crystallin as well as inducing its aggregation [1, 7]. Crystallin fragments such as the αA-66-80 peptide are believed to be a result of enzymatic hydrolysis, but the protease(s) involved have not been identified [7]. A schematic depicting the suspected role of crystallin fragments is pictured below (Fig. 2).

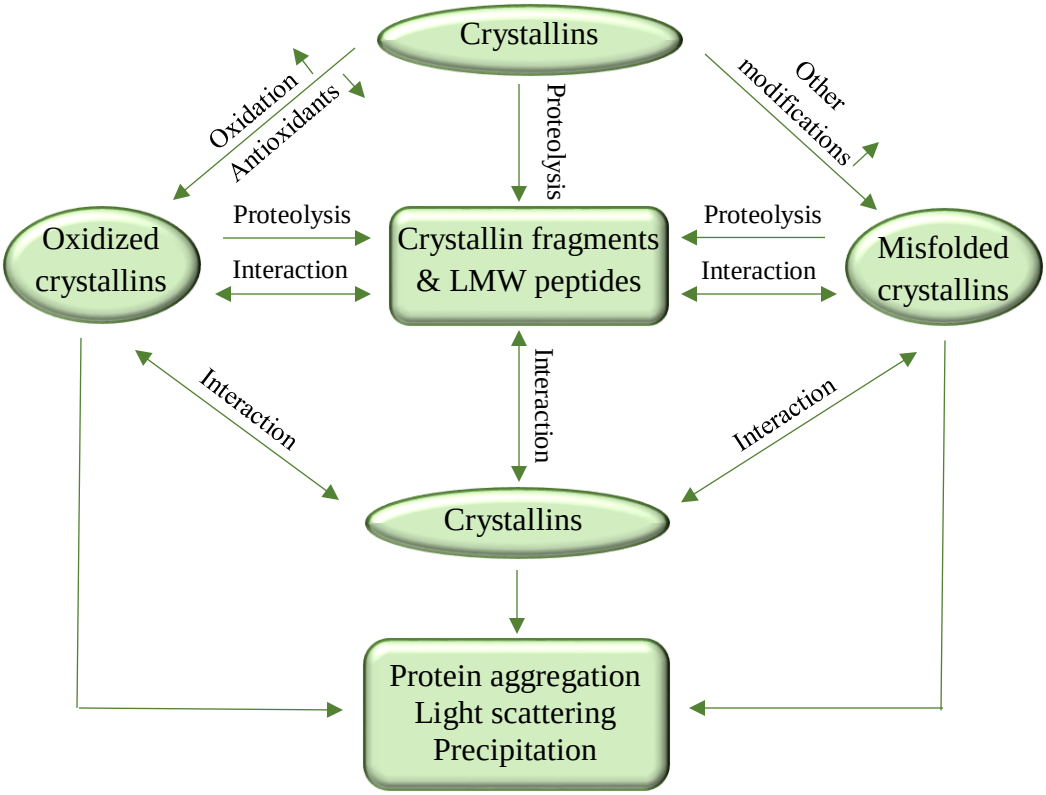


Figure 2: Schematic of the Role of Low Molecular Weight Peptides in Cataractogenesis [4]

Currently, there is no cure or preventive measures available. Surgery is performed when the resulting vision problems start to interfere with everyday life [8]. While the surgery is effective, it is not possible to address all the cases of cataract worldwide especially given that the incidence is increasing due to the ageing population [9]. Thus, the identification of potential therapeutic agents is of great interest. Toward the development of potential therapeutic agents for eye disease, several strategies have been employed. For instance, research has been conducted on small molecules and their ability to increase the chaperone activity of α-crystallin [10]. The other strategy involves use of inhibitors of peptide aggregation. One class of small molecule that has shown promise in preventing amyloid fibril formation, linked to neurodegeneration, is the phthalocyanine class of compounds (Fig 3) [11-14].

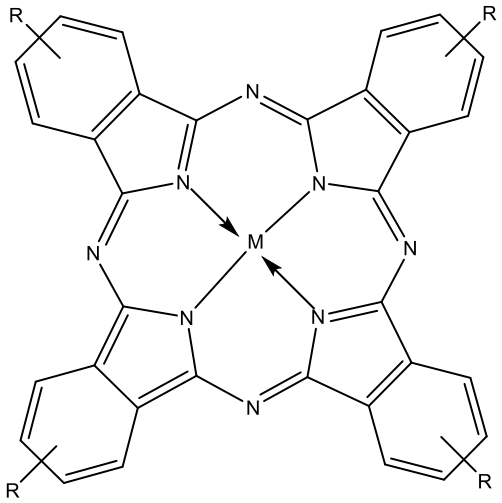


Figure 3: Generic Phthalocyanine Structure
Phthalocyanines can have a variety of peripheral functional groups (designated as R in the diagram) and are capable of coordinating a metal in their central cavity (designated as M in the structure). Arrows indicate metal coordination.

Phthalocyanines have been shown to have anti-scrapie properties and are able to prevent aggregation of a variety of neurodegeneration biomarkers, such as tau, amyloid-β protein, and α-synuclein [11-15]. Their effectiveness in preventing aggregation varied depending on the metal ions bound and its peripheral functional groups [11]. Phthalocyanines are planar compounds with extensive aromatic character and π-π interactions between phthalocyanines and the proteins of interest are believed to be the most significant [11-15]. Generally, the effectiveness of a phthalocyanine compound against amyloidogenesis and its anti-scrapie activity are correlated with the tendency of the phthalocyanine compound to self-associate, or "stack" [11-15].

Phthalocyanines have not been tested as inhibitors of crystallin aggregation. In this study, phthalocyanine tetrasulfonate (PcTS) and porphyrazine 285 (PZ285) (Fig. 4) are evaluated [16]. Porphyrazines are similar in structure to phthalocyanines; both are classified as tetrapyrroles [16].

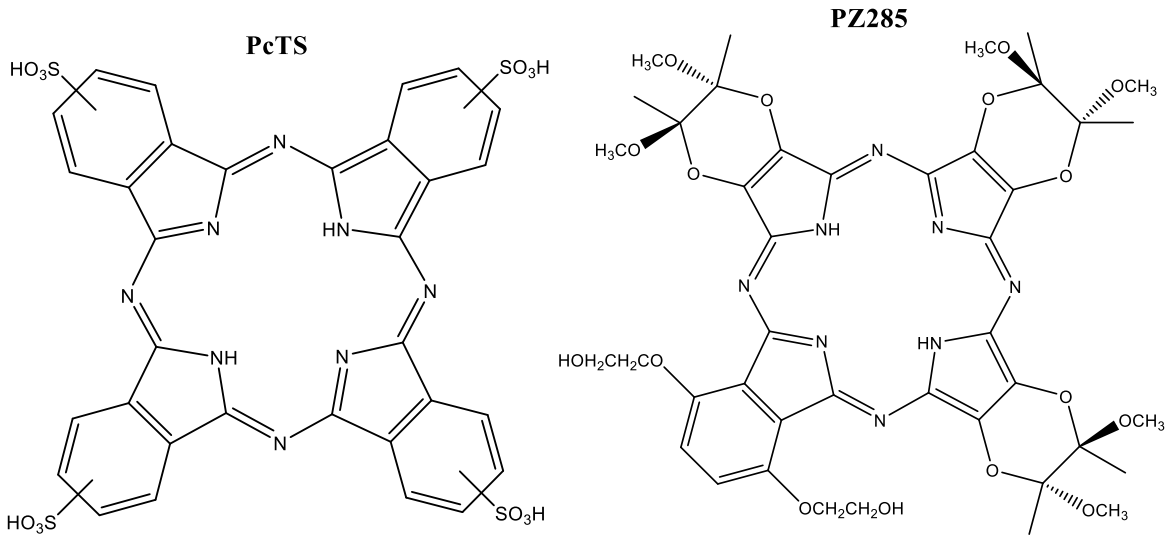


Figure 4: PcTS and PZ285 Structures

The ability of phthalocyanine tetrasulfonate to inhibit amyloid fibril formation in α-synuclein and amyloid-β protein and act as anti-scrapie agents has been studied [12-15]. α-Synuclein in the presence of PcTS was shown to form amorphous species as well as aggregates described as short, flat, and fibrillar that had negligible cytotoxicity [12, 13]. NMR spectroscopy has been used to characterize the binding interactions between α-synuclein and PcTS and it was found that the majority of binding occurs at the N-terminus with aromatic residues [12]. In the case of amyloid-β protein, PcTS induced the formation of an amyloid fibrillar meshwork when it was introduced to toxic amyloid-β oligomers that were formed by the addition of metals [11, 14]. The resulting meshwork mitigated the toxic effects of the oligomers [11, 14]. PcTS is believed to act as an anti-scrapie agent via π-π interactions with prion protein's aromatic side chains that likely serve to stabilize the protein's native structure and block interactions between native prion protein and infectious, misfolded prion protein [11, 15].

In this study, the abilities of phthalocyanine tetrasulfonate and porphyrazine 285 to inhibit the aggregation of αA-66-80 (SDRDKFVIFLDVKHF) crystallin peptide will be

evaluated. First, the morphologies of αA-66-80 peptide will be determined in order to have a basis for comparison when evaluating the inhibitors. Other α-crystallin peptides were investigated as well. This study will provide knowledge on the effectiveness of phthalocyanines as inhibitors of crystallin aggregation and will likely prompt further studies on optimizing the effectiveness of phthalocyanines as inhibitors through altering the identity of the metal coordinated and identities of the peripheral functional groups. The primary experimental techniques used were fluorescence proteostat aggregation assays to determine the extent of β-sheet structure, the basic motif of fibril aggregates, and transmission electron microscopy (TEM) to characterize the morphologies of the crystallin aggregates (oligomer, fibril, nanotubes, etc.).

Experimental

Reagents Used

Phthalocyanine tetrasulfonate (PcTS) was purchased from Sigma-Aldrich. Porphyrazine 285 (PZ285) was generously synthesized by Dr. Evan Trivedi of Oakland University's Department of Chemistry. For this study, 99.8% 2,2,2-trifluorethanol (TFE) was purchased from Acros Organics. TFE was used to attempt to dis-aggregate αA-66-80 crystallin peptide.

Peptides Used

Two peptides, αA-66-80 (SDRDKFVIFLDVKHF) crystallin peptide and αA-94-116 crystallin peptide (VEIHGKHNERQDDHGYISREFHR), were purchased from GenScript and both were of greater than 95% purity. The peptides were prepared in deionized water. 2.75 mM solutions of the peptides were used.

Peptide Sample Preparation

The solutions of the crystallin peptide (2.5 µL of 2.75 mM), without (2 µL water) and with the inhibitor (2 µL), were prepared in Tris-HCl buffer, pH 7.2 (22.5 µL). After 24 and 48 hours of incubation at room temperature in ambient light, the samples were measured by fluorescence or used for TEM analysis. Control experiments included the following solutions: 1) no peptide but with inhibitors (2 µL) and 2) only buffer. The concentration-dependent experiments were carried out by varying the concentration of the PcTS solution added.

Fluorescence Proteostat Aggregation Assays

The proteostat protein aggregation assay kit was purchased from Enzo. The proteostat detection reagent was prepared as directed, yielding a 0.15 mM solution. The kit also contains a positive control, aggregated lysozyme, and a negative control, native lysozyme. The controls were prepared as directed, yielding 40 µM solutions. The proteostat detection reagent, a

fluorescent probe, has much greater fluorescence intensity when bound to the β-sheet structure characteristic of amyloid fibrils than when it is bound to native protein structures. To peptide solutions, 1 μL of the proteostat detection reagent was added, and the mixture was incubated for 15 minutes in the dark. Next, fluorescence readings were taken using a take3 micro-volume plate and Biotek microplate reader, whose use was generously permitted by Dr. David Szlag. As per the Enzo instruction manual for the proteostat protein aggregation assay, readings were taken with an excitation wavelength of 550 nm and emission wavelength of 600 nm to evaluate levels of protein aggregation. Additional readings were taken with an excitation wavelength of 600 nm and emission wavelength of 625 nm as well as an excitation wavelength of 300 nm and emission wavelength of 625 nm to check for interference as phthalocyanine compounds have fluorescent properties. All assays were performed in triplicate. Error bars were created using the standard deviation.

Transmission Electron Microscopy (TEM)

The desired samples for each trial were prepared and allowed to incubate at room temperature in ambient light if necessary. 10 microliters of each sample were spotted on Formvar-carbon coated nickel grids. After 2 hours, the grids were rinsed with deionized water and blotted dry. A drop of 2% gluteraldehyde was spotted on each grid. After 5 minutes, the grids were rinsed with deionized water, blotted dry, and negatively stained with a drop of 1% aqueous uranyl acetate. After 5 minutes, the grids were rinsed with deionized water and blotted dry. The grids were subsequently imaged at 22kx and 56kx on a FEI Morgagni™ 268 transmission electron microscope by Dr. Victoria Kimler of Oakland University's Eye Research Institute.

Results

Characterization of αA-66-80 Crystallin Peptide Without Aging

Fluorescence Proteostat Aggregation Assay

The proteostat detection reagent was added to 0.25 mM αA-66-80 crystallin peptide solutions immediately upon preparation and fluorescence readings were taken in triplicate (Fig. 5). The high intensity of fluorescence indicates that the αA-66-80 crystallin peptide stock solution is aggregated despite being kept frozen as the peptide is extremely aggregation prone. Therefore, all experiments are done with pre-aggregated crystallin. Eventually, it would be beneficial to test the effects of phthalocyanines as aggregation inhibitors on crystallin that is not yet aggregated in order to fully evaluate its inhibition potential.

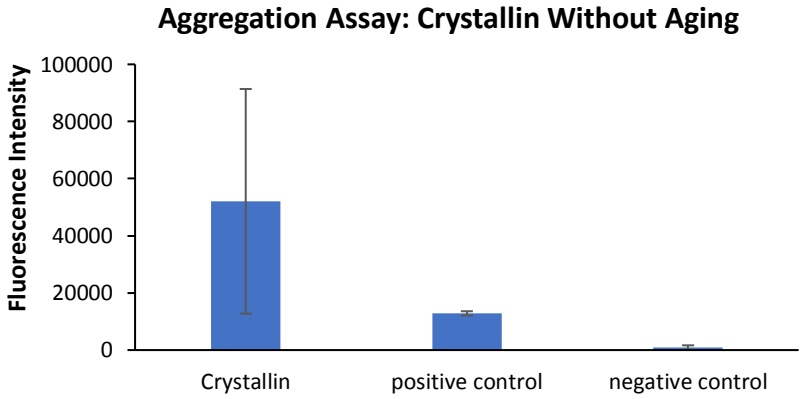


Figure 5: Aggregation Assay of α-66-80 Peptide Without Incubation Period
Excitation wavelength was 550 nm and the emission wavelength was 600 nm.

Transmission Electron Microscopy (TEM)

The αA-66-80 crystallin peptide solution was immediately applied to a grid. Representative images of the peptide are included below (Fig. 6). There are numerous long filaments (Fig. 6) as in the TEM images of the peptide which was pre-aggregated for 24-hours.

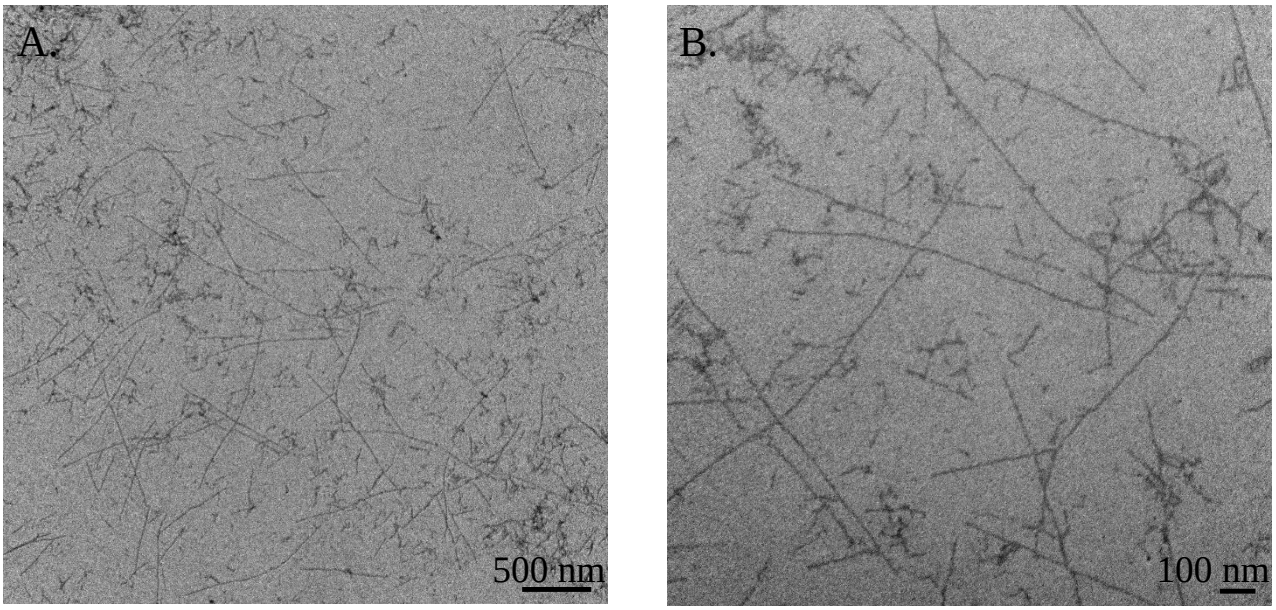


Figure 6: TEM Images of αA-66-80 Crystallin Peptide Without Aging
A. and B. αA-66-80 crystallin peptide at different magnification.

Addition of TFE to αA-66-80 Crystallin Peptide

Fluorescence Proteostat Aggregation Assay

To try to dis-aggregate αA-66-80 crystallin peptide, 30% (v/v) TFE was added. After a one-hour incubation period at room temperature in ambient light, the proteostat detection reagent was added. Fluorescence readings were taken in triplicate. Some experimental error resulted from the solutions containing TFE puddled on the take3 plate rather than forming a uniform droplet of sample. However, the high fluorescence intensity (Fig. 7) indicates that there is still aggregation characterized by β-sheet character.

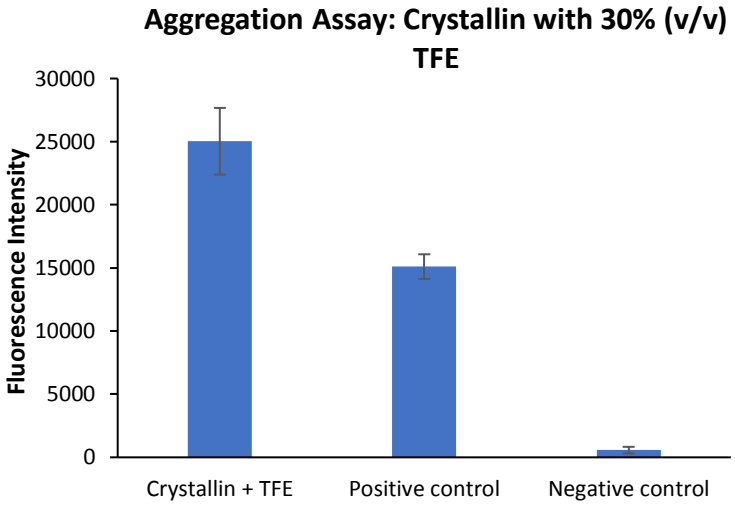


Figure 7: Aggregation Assay of αA-66-80 Crystallin Peptide with TFE
Excitation wavelength was 550 nm and the emission wavelength was 600 nm.

Transmission Electron Microscopy (TEM)

After a one-hour incubation period, the 0.25 mM αA-66-80 crystallin peptide solution with 30% (v/v) TFE was applied to a grid. Filaments of varied length and thickness were detected (Fig. 8). The thicker filaments may be a result of filaments associating with each other. The addition of TFE altered the morphology of aggregates, but did not minimize overall aggregation. The bubbles seen in the background of the images (Fig. 8) are believed to be a result of the TFE.

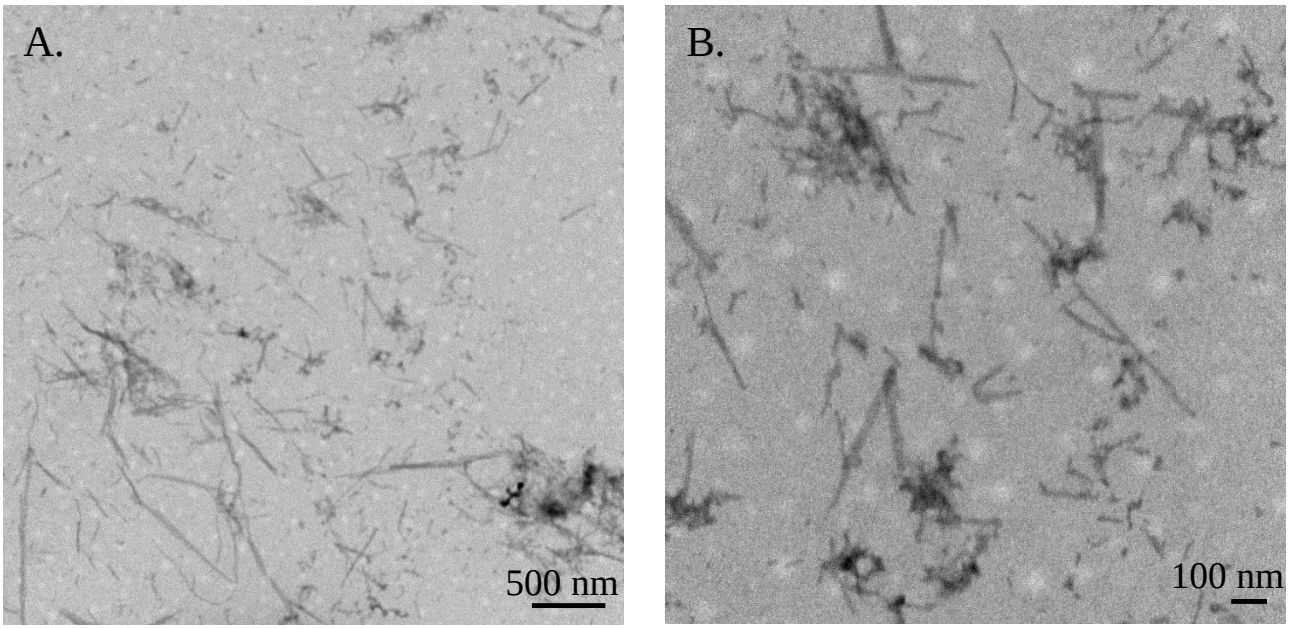


Figure 8: TEM Images of Crystallin Peptide with TFE
A. and B. αA-66-80 crystallin peptide and TFE at different magnification.

Characterization of αA-94-116 Crystallin Peptide

Transmission Electron Microscopy (TEM)

Unlike αA-66-80 crystallin peptide, αA-94-116 crystallin peptide does not contain the hydrophobic FVIFLD sequence which is associated with amyloid fibril formation. Therefore, this peptide is not likely to aggregate. A 24-hour incubation of 0.25 mM αA-94-116 crystallin peptide solution at room temperature in ambient light did not cause the formation of any filaments (Fig. 9A). The αA-94-116 crystallin peptide contains several histidine residues that may coordinate metals and this metalation may induce aggregation. When 3.4 mM of copper perchlorate (Fig. 9B) or 3.4 mM zinc perchlorate (Fig. 9C) were added to the peptide, no filaments were detected in either case. Another method of inducing aggregation will need to be identified.

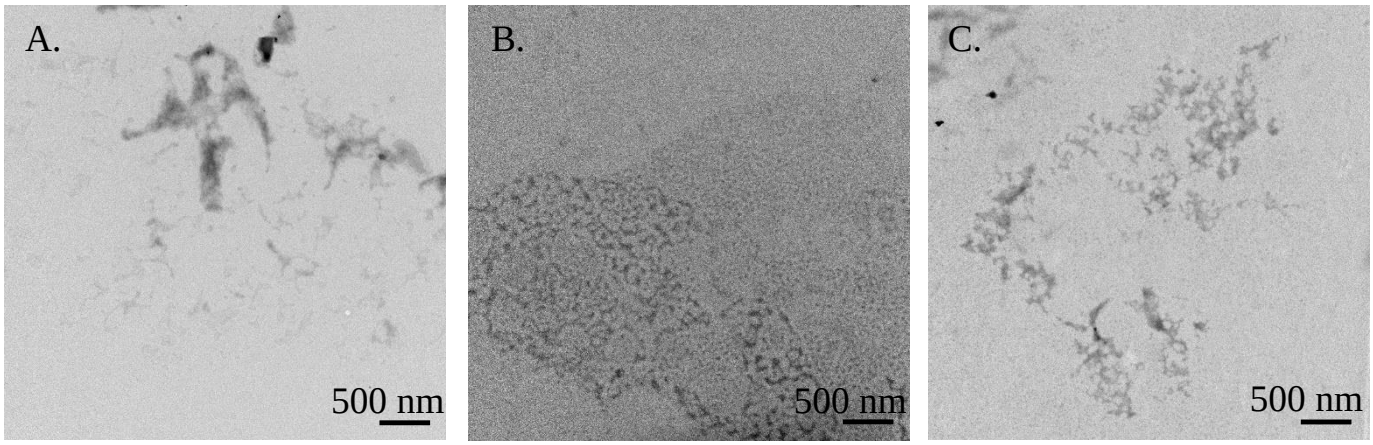


Figure 9: αA-94-116 Crystallin Peptide and Metal Ions
A. αA-94-116 crystallin peptide B. αA-94-116 crystallin peptide and copper perchlorate C. αA-94-116 crystallin peptide and zinc perchlorate

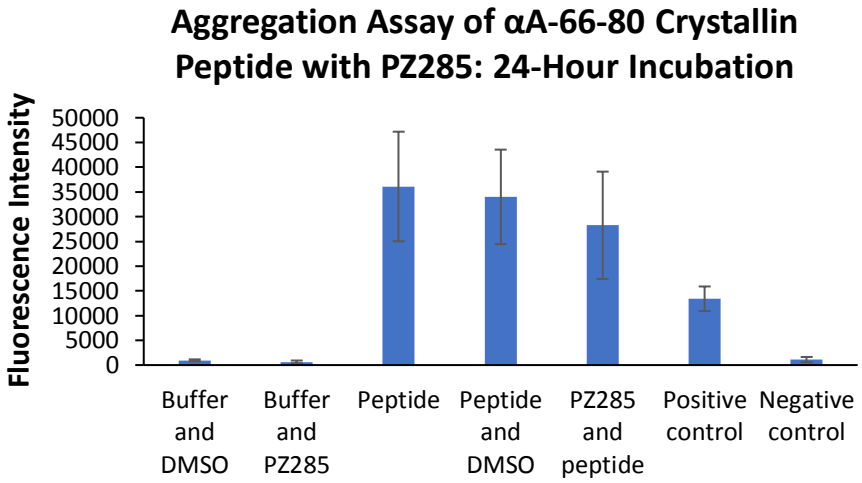
Inhibition Studies with PZ285 Compound

Fluorescence Proteostat Aggregation Assay

The solutions prepared for the fluorescence proteostat aggregation assay had a final αA-66-80 crystallin peptide concentration of 0.25 mM and a final PZ285 concentration of 0.05 mM. A lower fluorescence reading for the crystallin peptide with inhibitor than the crystallin peptide alone would indicate suppression of aggregation. The readings that were taken with an excitation wavelength of 600 nm and emission wavelength of 625 nm and at an excitation wavelength of 300 nm and emission wavelength of 625 nm suggest that PZ285's fluorescent properties were not interfering with the assay (results not shown). Readings taken after a 24-hour incubation (Fig. 10A) and readings taken after a 48-hour incubation (Fig. 10B) were similar. High fluorescence readings greater than 20,000 a.u. indicated peptide aggregation. PZ285 exhibited poor inhibition given the similar fluorescence intensities when compared to uninhibited sample (Fig. 10). This is believed to be due to PZ285's limited solubility in aqueous solutions. PZ285 was dissolved in dimethyl sulfoxide (DMSO). The limited solubility made it difficult to administer equal quantities of PZ285 for each trial and may have interfered with the compound's

ability to interact with the crystallin peptide. At this juncture, other phthalocyanine compounds that are more water soluble were investigated.

A.



B.

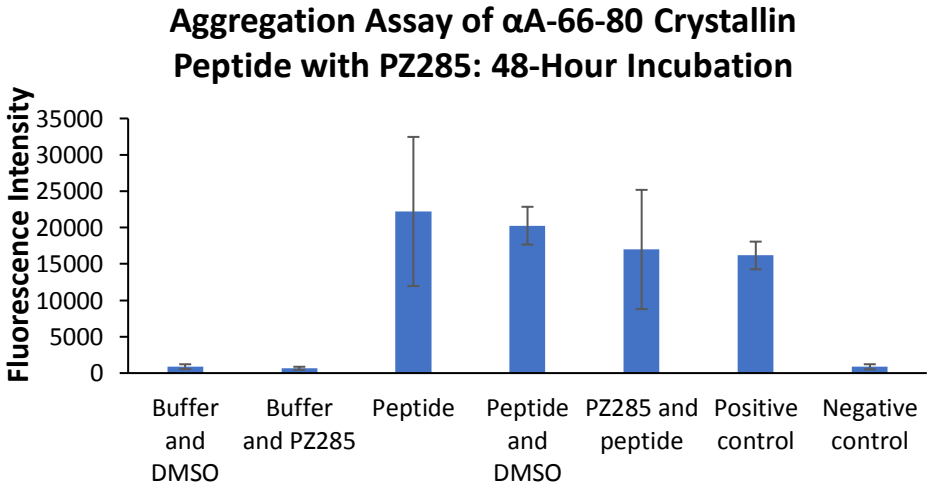


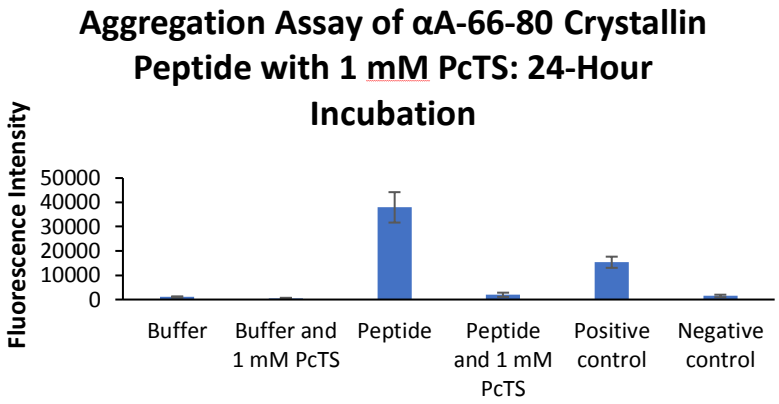
Figure 10: Aggregation Assays of a Single Concentration of PZ285 and αA-66-80 Peptide A. shows the average reading for the 24-hour incubation. B. shows the average reading for the 48-hour incubation. Excitation wavelength was 550 nm and the emission wavelength was 600 nm.

Inhibition Studies with PcTS Compound

Fluorescence Proteostat Aggregation Assay: Single PcTS Concentration

The solutions prepared for the fluorescence proteostat aggregation assay had a final αA-66-80 crystallin peptide concentration of 0.25 mM and a final PcTS concentration of 0.074 mM. High fluorescence intensity was associated with aggregation of peptide via β-sheet formation. The readings that were taken with an excitation wavelength of 600 nm and emission wavelength of 625 nm and at an excitation wavelength of 300 nm and emission wavelength of 625 nm suggest that PcTS's fluorescent properties had not affected fluorescence readings (results not shown). The crystallin peptide solution with 0.074 mM PcTS had a dramatic decrease in fluorescence intensity as compared to the peptide solution without inhibitor (Fig. 11). This result was consistent for the 24-hour incubation (Fig. 11A) and the 48-hour incubation (Fig. 11B). This decrease in fluorescence intensity may indicate reduction in β-sheet content, i.e. peptide aggregation. These promising results spurred more investigation of PcTS as an inhibitor of crystallin aggregation.

A.



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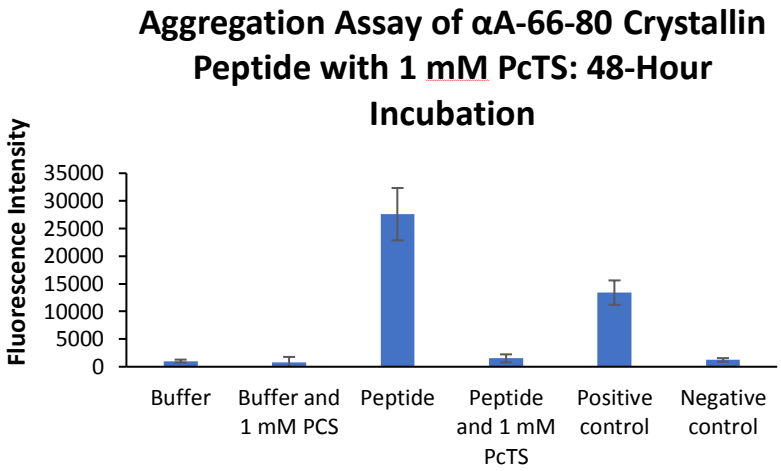


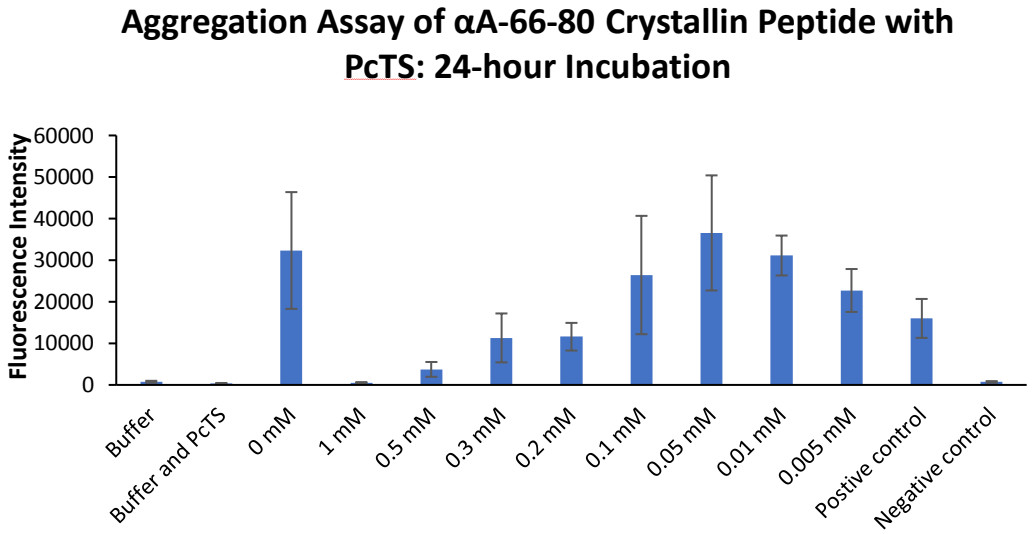
Figure 11: Aggregation Assays of a Single Concentration of PcTS and αA-66-80 Peptide
A. shows fluorescence readings taken after 24-hour incubation while B. shows fluorescence readings taken after 48-hour incubation. Excitation wavelength was 550 nm and the emission wavelength was 600 nm. 1 mM PcTS is the concentration of PcTS added; 0.074 mM is the final concentration.

Fluorescence Proteostat Aggregation Assay: PcTS Concentration Studies

To further characterize PcTS's effectiveness as an inhibitor of crystallin aggregation, a range of concentrations of PcTS were added to 0.25 mM αA-66-80 crystallin peptide. The following solutions were prepared (the final concentration is in parentheses): 1 mM (0.074 mM), 0.5 mM (0.037 mM), 0.3 mM (0.022 mM), 0.2 mM (15 μM), 0.1 mM (7.4 μM), 0.05 mM (3.7 μM), 0.01 mM (0.74 μM), and 0.005 mM (0.37 μM). The concentration curve generated from

this data (Fig. 12) is sigmoidal and can be used to calculate the IC_{50}, the concentration of PcTS at which the fluorescence intensity is reduced by 50% as compared to the fluorescence intensity of the uninhibited sample. Using excel and the data taken at 24 hours (Fig. 12A), the IC_{50} was calculated to be approximately 13 μM. The concentration-based profiles indicate that PcTS concentrations above 7.4 μM may reduce aggregation in 0.25 mM αA-66-80 crystallin peptide given the magnitude of the decrease in fluorescence intensity (Fig. 12). The readings taken after 24-hour incubation (Fig. 12A) were consistent with the readings taken after 48-hour incubation (Fig. 12B).

A.



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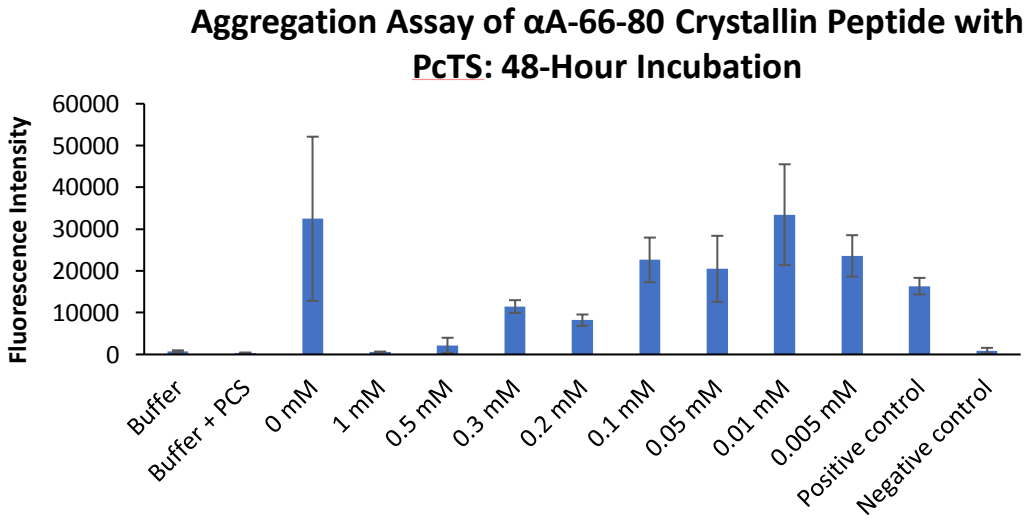

Figure 12: Aggregation Assays of Multiple Concentrations of PcTS and αA-66-80 Peptide A. shows the readings taken after 24-hour incubation and B. shows the readings taken after 48-hour incubation. Excitation wavelength was 550 nm and the emission wavelength was 600 nm. Concentrations of PcTS listed are the concentration of PcTS added, not the final concentrations.

Transmission Electron Microscopy (TEM)

Samples made for TEM were prepared 24 hours prior to preparation of the grids and allowed to incubate at room temperature in ambient light. Representative images of 0.25 mM

αA-66-80 crystallin peptide (Fig. 13A, B), 0.25 mM αA-66-80 crystallin peptide and 0.074 mM PcTS (Fig. 14A, B), and 0.074 mM PcTS (Fig 14C) are shown. αA-66-80 crystallin peptide aggregates into fibrils (Fig. 13A, B). The presence of PcTS slightly reduced filament formation (Fig. 14A, B). The filaments that appear in the presence of PcTS (Fig. 14A, B) appear to be shorter on average than the fibrils that were formed in the absence of PcTS (Fig. 13A, B), but measurements will need to be taken using a program like ImageJ to determine that in a more scientifically rigorous manner.

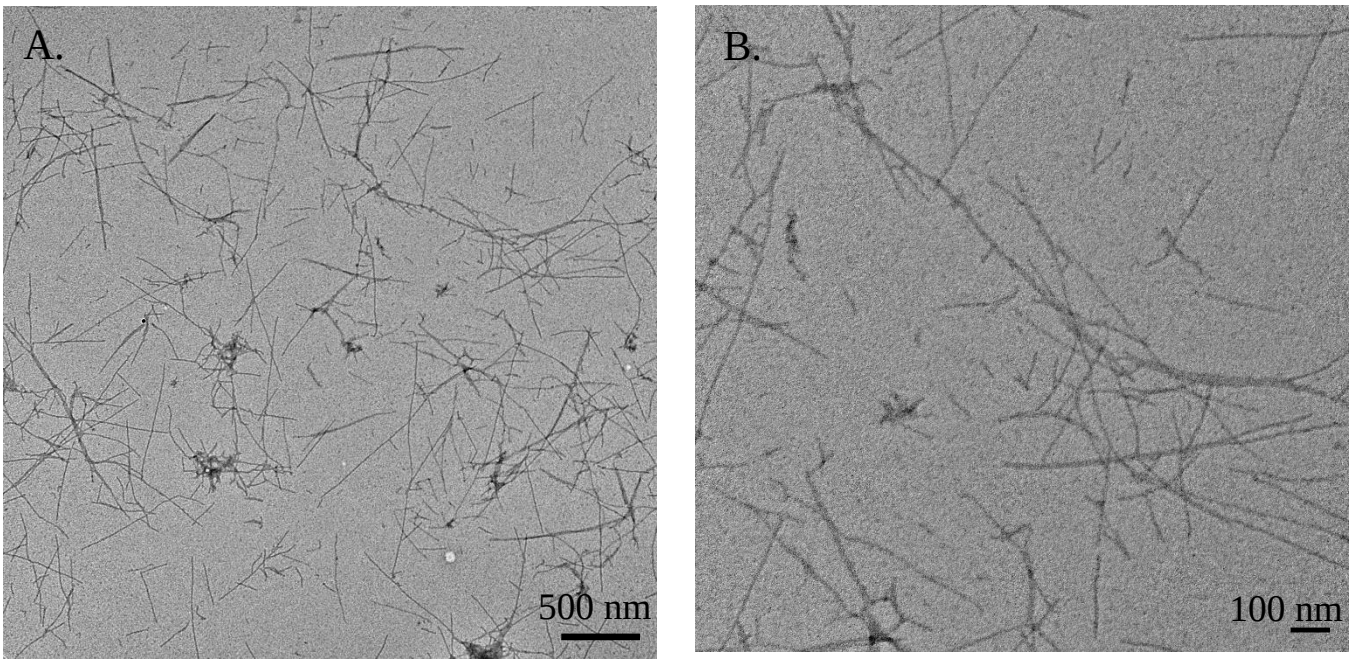

Figure 13: TEM Images of Crystallin Peptide After 24 Hours at Room Temperature
A. and B. αA-66-80 crystallin peptide at different magnification.

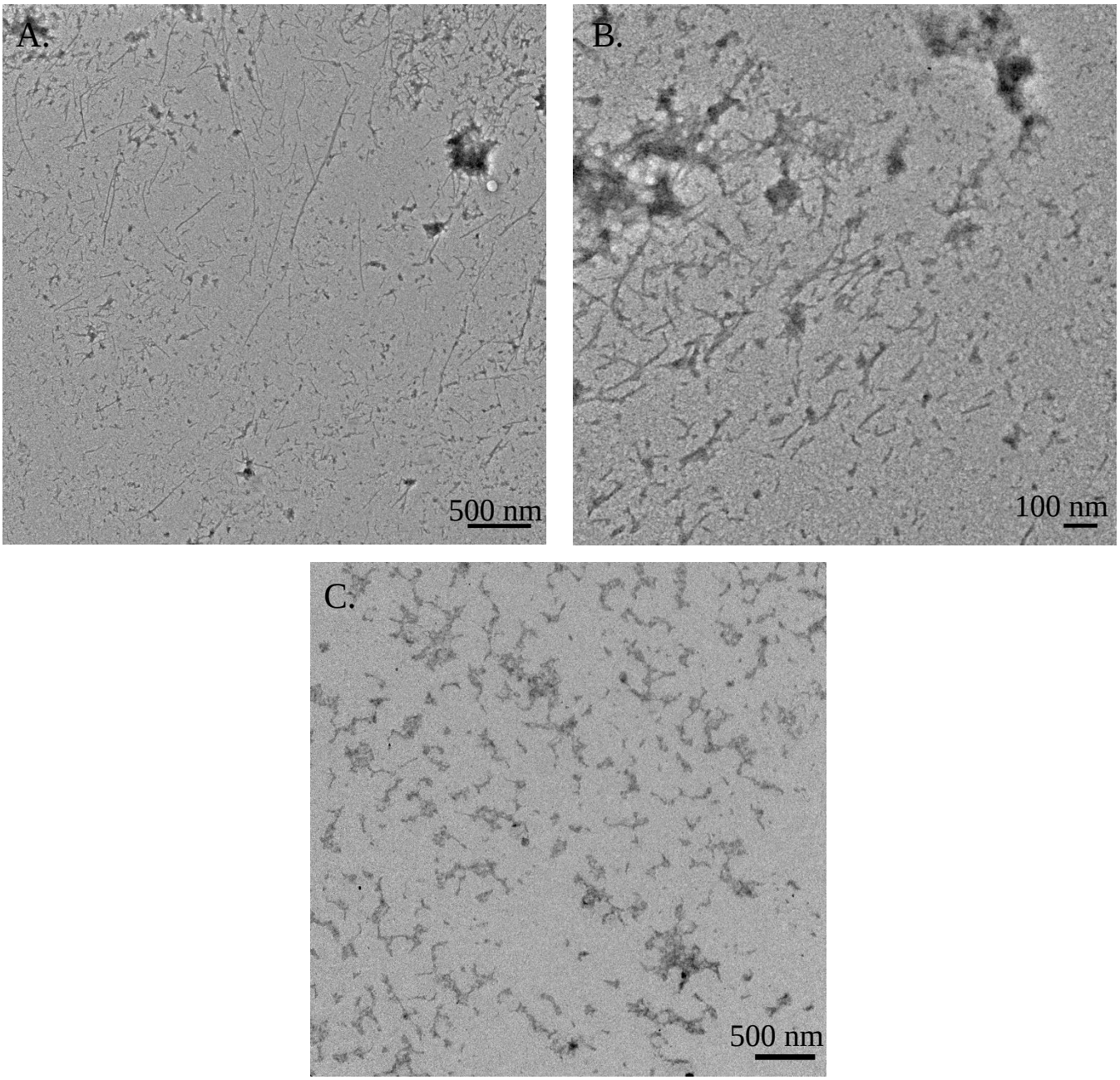

Figure 14: TEM Images of Crystallin Peptide with PcTS Inhibitor
A. and B. αA-66-80 crystallin peptide and PcTS at different magnification. C. PcTS

Discussion

PZ285's minimal solubility in aqueous solutions made it a poor candidate as an inhibitor of crystallin aggregation. PcTS is soluble in aqueous solutions as a result of its four sulfonate groups and was a better candidate. The fluorescence proteostat aggregation assays suggested that PcTS inhibits crystallin aggregation in a concentration-dependent manner as a decrease in fluorescence intensity occurred. High intensities of fluorescence indicate aggregation via β-sheet formation while a reduction in fluorescence intensity as compared to the peptide alone indicate aggregation inhibition. The fluorescence data suggest that PcTS concentrations above 7.4 μM are capable of reducing crystallin aggregation. TEM images showed that the αA-66-80 crystallin peptide aggregates into fibrils and that there is some reduction in filament formation when PcTS is present. Fluorescence data and TEM images of αA-66-80 crystallin peptide without incubation revealed that the peptide is already aggregated. Given this, the data collected reflect the effect of PcTS on aggregated crystallin peptide rather than native peptide regardless of when PcTS is added to crystallin solutions. As of yet, aggregation has not been induced in the αA-94-116 crystallin peptide and thus the peptide has not been utilized in aggregation assays.

Future Plans

In the future, the role of metal ions on the inhibitory activity of PcTS will also be evaluated. We also plan to design and synthesize a number of PcTS-based derivatives by tuning the hydrophobicity and electrostatic charges. The interactions between phthalocyanines and proteins are believed to be primarily π-π interactions with aromatic residues and hydrophobic interactions. Dr. Evan Trivedi's lab in the Department of Chemistry synthesizes asymmetric phthalocyanines and this provides an opportunity to optimize PcTS's ability to interact with proteins while maintaining solubility in aqueous solutions.

Acknowledgements

Special thanks go to Martic Lab, the Department of Chemistry, the Honors College, and OU. Dr. Evan Trivedi's involvement in the conception of the project, his providing of the PZ285 compound, and his future involvements in phthalocyanine synthesis are much appreciated. Thanks also go out to Dr. Victoria Kimler of Oakland University's Eye Research Institute for imaging of the TEM grids and to Dr. David Szlag for use of his Biotek plate reader.

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