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Amyloid β -Sheet Secondary Structure Identified in UV-Induced Cataracts of Porcine Lenses using 2D IR Spectroscopy

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Abstract

Cataracts are formed by the aggregation of crystallin proteins in the eye lens. Many *in vitro* studies have established that crystallin proteins precipitate into aggregates that contain amyloid fibers when denatured, but there is little evidence that *ex vivo* cataracts contain amyloid. In this study, we collect two dimensional infrared (2D IR) spectra on tissue slices of porcine eye lenses. As shown in control experiments on *in vitro* α B- and γ D-crystallin, 2D IR spectroscopy can identify amyloidogenic β -sheet secondary structure because this structure has exceptionally strong coupling and a high degree of ordering that creates characteristic diagonal and cross peaks. In *ex vivo* experiments of acid treated tissues, characteristic 2D IR features are observed and fibers >50 nm in length are resolved by TEM, consistent with amyloid fibers. In UV-irradiated lens tissues, fibers are not observed with TEM, but amyloidogenic β -sheet secondary structure is identified from the 2D IR spectra. The characteristic 2D IR features of amyloid β -sheet secondary structure are created by as few as 4 or 5 strands, and so identify amyloid secondary structure even if the aggregates themselves are too small to be resolved with TEM. We discuss these findings in the context of the chaperone system of the lens, which we hypothesize sequesters small aggregates, thereby preventing long fibers from forming. This study expands the scope of heterodyned 2D IR spectroscopy to tissues. The results provide a link between *in vitro* and *ex vivo* studies, and support the hypothesis that cataracts is an amyloid disease.

Graphical Abstract

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Conflict of interest:

M.T.Z. is a co-owner of PhaseTech Spectroscopy, which manufactures mid-IR pulse shapers and 2D IR spectrometers.

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Keywords

Two-dimensional infrared spectroscopy; crystallin; lens tissue; amyloid fiber; UV-irradiation

Introduction

Senile cataracts are an aggregation disease caused by the precipitation of lens proteins.¹ Healthy eye lenses are transparent and focus light onto the retina, enabling vision.² The focus is dictated by the refractive index, which is maintained by high protein concentration within the lens capsule. Human lens proteins have long lifetimes (up to 90 years), but eventually precipitate to form aggregates due to aging.¹ These protein aggregates create opaque cataract deposits, resulting initially in blurred vision, and ultimately in blindness if not corrected with surgery.³ Senile cataracts are the leading cause of blindness worldwide. Because of their prevalence, there is great interest in understanding the composition, structure and mechanism of cataract deposits. It is difficult, however, to study molecular structures directly in tissues (*ex vivo*), and so most structural and biophysical experiments are performed *in vitro* on individual families of crystallin proteins.^{4; 5}

One observation from *in vitro* experiments is that crystallin proteins readily form amyloid fibers under common denaturing conditions. More than 90% of the total protein mass in the ocular lens is composed of crystallin proteins.⁶ There are three families of crystallin proteins: α -, β -, and γ -crystallin, and each has several different members in its family. *In vitro*, proteins from all three of these families form amyloid fibers under denaturing conditions.^{7; 8} Extensive structural and mechanistic studies have been performed on γ D- and α B-crystallins, making them models for their families.^{9; 10} γ D-crystallin exclusively forms amyloid fibers when denatured by acid and precipitates as both disordered aggregates and amyloid fibers upon UV-irradiation.^{11; 12; 13; 14} α B-crystallin also forms amyloid when incubated at 60° C with 10% TFE or under other conditions.⁸ Thus, *in vitro*, the crystallin proteins have multiple pathways to aggregation with different propensities and structures. Amyloid fibers is one common aggregate structure found *in vitro* experiments.

Even though there is a preponderance of evidence for amyloid fiber formation by crystallin proteins *in vitro*, there is little evidence for amyloid fibers *ex vivo* in senile cataracts. Fibers were reported in mouse lenses with a point mutation on γ S-crystallin^{15; 16} and γ B-crystallin¹⁷, as observed by TEM and stained with Congo red, which is consistent with amyloid β -sheet fibers. However, the aggregation mechanism of mutated proteins might not be a good model for senile cataracts that are the result of UV exposure. In a study of senile

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cataracts, early biochemical measurements suggested that protein unfolding occurred in cataractous lenses, but no amyloid was observed.¹ FTIR spectra of lenses from patients with familial congenital cataracts, myopia, and glaucoma might contain amyloid features,¹⁸ but the spectra are too spectrally congested to be conclusive. Fibers were observed in the nucleus region in the lens of an OXYS strain of rat,¹⁹ but those fibers do not stain for amyloid. It is well established that proteins *in vitro* can form amyloid that have not been observed to do so *in vivo*. In fact, it has been hypothesized that the global free energy minimum for all protein structures is amyloid.²⁰ Considering the paucity of evidence for amyloid in *ex vivo* lens tissue, it is not clear whether the many structural and mechanistic studies of *in vitro* amyloid formation by the crystallin proteins are relevant to the disease.^{4; 5}

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There are different conventions for what constitutes amyloid.^{21; 22} In the literature, protein amyloid deposits formed extracellularly are often called amyloid fibers or plaques whereas fibers found inside cells are sometimes called intracellular inclusions. From the viewpoint of a structural biologist, the diagnostic for amyloid is a series of β -strands running perpendicular to the fiber axis. The fibers are typically 10 nm in width and stain with ThT or Congo red which binds to the β -sheets. Recently, bacterial peptides have also been shown to form α -helical structural motifs that assemble into fibers.²³

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In this article, we use observables unique to 2D IR spectroscopy to identify amyloid β -sheet secondary structure alongside more standard approaches. 2D IR spectroscopy is sensitive to amyloid secondary structure because of strong coupling between the carbonyl groups (amide I modes, shown as black arrows in Fig. 1A) and long-range order along the protein backbone.²⁴ The couplings delocalize the vibrations, so that multiple amides along and across the β -strands vibrate in unison. Delocalization, in turn, changes the absorption frequencies, absorptivity coefficients, and anharmonicities, creating diagonal peaks and off-diagonal cross peaks (Fig. 1B), all of which are sensitive to secondary structures²⁵. The diagonal peaks in 2D IR spectra are analogous to the absorption features in FTIR spectroscopy, but are more sensitive to secondary structures because they scale to the 4th power of transition dipole, μ (Fig. 1C).²⁶ The transition dipole, and thus absorption coefficient, for amyloid β -sheets are very large, and so their features are enhanced in 2D IR relative to FTIR spectra.²⁷ Cross peaks provide another diagnostic for amyloid β -sheets that is not available from FTIR. They are a very direct measure of coupling, and for amyloids β -sheets, appear in a region of the 2D spectrum with little contribution from other structures. In a slice of lens tissue, no other biomolecules absorb appreciably in the same frequency range as the amide I modes, so we attribute all the signals in our 2D IR spectra to proteins.^{28; 29} As we explain further in the Discussion, only 4 or 5 β -strands in the tight molecular geometry of amyloid fibers are necessary to form the characteristic 2D IR features. Thus, 2D IR spectroscopy provides a diagnostic for amyloid β -sheet secondary structure even if fibers formed by those amyloid β -sheets are in a size regime that may be difficult to detect with other approaches such as TEM. In this article, we measure 2D IR spectra of *ex vivo* cataracts induced with UV-exposure in slices of porcine lens tissues as model for age-related cataracts, looking for the signatures of amyloid β -sheet secondary structure.

Results

Signature of amyloid β -sheet formation in 2D IR spectra of *in vitro* proteins

The crystallin proteins are the major protein component found in senile cataracts.³⁰ To establish the spectroscopic features expected for amyloid β -sheet secondary structure in fibers formed from crystallin proteins, we collected 2D IR spectra of acid- and UV-treated γ D-crystallin (with an S84C mutation) and α B-crystallin in solutions (structures shown in Fig. 2). It was established in previous studies that the S84C mutation on γ D-crystallin does not alter its native or its aggregated amyloid structure.¹⁴ Shown in Fig. 2A is the 2D IR spectrum of γ D-crystallin in its native fold, prior to acid or UV treatments. 2D IR spectra of γ D-crystallin have been previously reported^{11; 31; 32} and computational modeling has helped to interpret these spectra.³³ Spectra of γ D-crystallin are reported here to serve as comparisons to the spectra of α B-crystallin and *ex vivo* tissues.

The primary features in γ D-crystallin 2D IR spectra are a pair of out-of-phase peaks (red and blue) at pump axis (ω_{pump} or y-axis) = 1636 cm^{-1} (Fig. 2A). The 2D IR pulse sequences always creates pairs of out-of-phase peaks, separated in frequency along the probe axis (ω_{probe} or x-axis). 1636 cm^{-1} is a typical frequency for short β -sheets found in many soluble proteins.²⁹ In the case of γ D-crystallin, the 1636 cm^{-1} frequency is formed by 2 Greek Key folds (4 strands of antiparallel β -sheets) found in each of the two domains (Fig. 2).³³ The 2D peaks have elongated shapes that extend up to about 1660 cm^{-1} because of loops and disordered segments, as well as weaker modes of the β -sheets.^{26; 34} Shown in Fig. 2D is a 2D IR spectrum of native α B-crystallin, which also absorbs near 1636 cm^{-1} . Although the complete structure of α B-crystallin is not known, we assign the 1636 cm^{-1} absorption to the core domain because it consists of short stands of antiparallel β -sheets (Fig. 2).^{9; 35} The other two α B-crystallin domains are either disordered or contain a small amount of α -helix. Thus, the 2D IR spectra of γ D- and α B-crystallin are quite similar. The diagonal slices ($\omega_{\text{pump}} = \omega_{\text{probe}}$) of the 2D IR spectra, which are somewhat analogous to FTIR spectra, are shown in Fig. 2G for native γ D- and α B-crystallin to help illustrate the similarity between the spectra.

Shown in Fig. 2B is γ D-crystallin denatured in pD 3.0 buffer overnight (TEM images have been previously published³⁶). After the incubation, we observe a well-defined and narrow set of peaks around $\omega_{\text{pump}} = 1621 \text{ cm}^{-1}$. These features are created by highly-ordered amyloid β -sheets and have been observed in other peptide samples such as human amylin³⁷ and polyQ³⁸. We know from segmentally labeled γ D-crystallin studies that the amyloid peak at 1621 cm^{-1} comes from amyloid β -sheets formed by the C-terminal domain and that the broad peak around 1650 cm^{-1} corresponds to the random coil structure in the N-terminal domain, which is unfolded in the acid induced structure.¹⁴

We also observe two sets of cross peaks in the spectrum for acid denatured γ D-crystallin (red and blue boxes in Fig. 2B), with frequencies $\omega_{\text{pump}} = 1621 \text{ cm}^{-1}$ and 1687 cm^{-1} , respectively. These cross peaks are often observed in the 2D IR spectra of amyloid fibers, and are a second signature for amyloid fiber formation.

According to a previous study,⁸ α B-crystallin also forms highly ordered β -sheets when incubated in acid. Shown in Fig. 2E is a 2D IR spectrum of α B-crystallin incubated in pD 3.0 buffer overnight (same as for γ D-crystallin). The pair of diagonal peaks at $\omega_{\text{pump}} = 1621 \text{ cm}^{-1}$ is observed, as are cross peaks on either side of the diagonal correlating the 1621 cm^{-1} band to about 1687 cm^{-1} (red and blue boxes, respectively, in Fig. 2E). We note that not all amyloid forming proteins exhibit the exact same frequencies or cross peaks, because these features depend on the size of the β -sheet and the amount of structural disorder.^{27; 34; 39; 40} So, while the amyloid β -sheet peak varies from 1619 to 1623 cm^{-1} in the various experiments presented here, we systematically note its frequency as 1621 cm^{-1} .³⁴ The cross peaks are caused by coupling between the 1621 cm^{-1} β -sheet mode to either a higher mode or to a turn or disordered loop.^{26; 40} The 1621 cm^{-1} mode and the corresponding cross peaks at $\omega_{\text{pump}} = 1621 \text{ cm}^{-1}$ are decisive identifications of amyloid – those frequencies cannot be made by any other protein structure; the β -sheets of native proteins are smaller and curved, creating an absorption feature at about 1636 cm^{-1} . We do not attempt to differentiate between anti-parallel and parallel amyloid β -sheets, which requires identifying modes near 1680 cm^{-1} and/or peaks at 1640 cm^{-1} , because those frequencies also overlap with the native crystallin structures. In principle, the pairs of cross peaks above ($\omega_{\text{pump}} = 1687 \text{ cm}^{-1}$, blue box) and below ($\omega_{\text{pump}} = 1621 \text{ cm}^{-1}$, red box) the diagonal should have the same intensity, but in practice the cross peaks below the diagonal are often better pronounced because they have less overlap with the diagonal peaks. Cross peaks below the diagonal give the signature frequency of amyloid β -sheet secondary structure by their positions along ω_{pump} of the 2D IR spectrum. Thus, cross peaks are strong indicators of amyloid formation, even when the diagonal peaks are congested.

We also used UV-irradiation to induce aggregation of γ D- and α B-crystallin. Shown in Fig. 2C is a 2D IR spectrum of γ D-crystallin irradiated with UV light for 18 hours. Two changes are observed as compared to the spectrum of native γ D-crystallin (Fig. 2A). First, a shoulder appears at $\omega_{\text{pump}} = 1621 \text{ cm}^{-1}$ along the diagonal, indicating the formation of amyloid β -sheet secondary structure. The diagonal slice of the spectrum (Fig. 2I, solid line) shows this shoulder more clearly (red arrow). Second, there is the set of cross peaks expected for amyloid β -sheet secondary structure (Fig. 2C, red box). Third, the peaks along the diagonal have intensities above $\omega_{\text{pump}} = 1640 \text{ cm}^{-1}$ and are very broad, consistent with unfolding. This 2D IR spectrum is representative of a situation in which multiple products are formed. Previously, we determined that only the C-terminal domain of γ D-crystallin is responsible for amyloid formation and that UV radiation causes fragmentation of γ D-crystallin into shorter peptides and as well as covalent crosslinking to form higher molecular weight products.¹¹ As a result, the diagonal peaks have overlapping contributions from different UV-irradiated products of γ D-crystallin. Nonetheless, the diagonal peak at $\omega_{\text{pump}} = 1621 \text{ cm}^{-1}$ and the expected cross peaks indicates that amyloid β -sheet secondary structure is produced. The cross peaks are especially useful for identifying amyloid β -sheets because they lie far from the congested diagonal features.

In contrast to our observations for γ D-crystallin, we do not observe amyloid β -sheet formation upon UV-irradiation of α B-crystallin. Shown in Fig. 2F is a 2D IR spectrum of α B-crystallin irradiated with UV light under the same conditions as γ D-crystallin for 18 hours. The diagonal peaks broaden but the maximum absorbance is still at about 1636 cm^{-1}

after 24 hours (Fig. S1C) and there is little to no intensity in the lower cross peak region, even after 48 hours of UV-irradiation (Fig. S1D, red box). Thus, UV-irradiation causes α B-crystallin to lose some of its native structure but it does not create amyloid β -sheet, in contrast to acid treatment which clearly leads to amyloid β -sheet formation (Fig. 2E).

2D IR spectroscopy identifies highly-ordered amyloid β -sheet secondary structure in aggregated α B-crystallin that is not easily classified with TEM

Shown in Fig. 3 is a comparison of 2D IR spectra and TEM images of α B-crystallin treated under conditions that lead to long amyloid fibers. Fig. 3A, C and E presents data on α B-crystallin solvated in 10% TFE phosphate buffer and heated at 60 C° for 35 hours. Large and long amyloid fibers are present in the TEM (Fig. 3E). The corresponding 2D IR spectrum and diagonal slice (Fig. 3A and C) exhibit the sharp 1621 cm⁻¹ diagonal peak and cross peaks between 1621 cm⁻¹ and 1687 cm⁻¹ (red box). Thus, according to both TEM and 2D IR spectroscopy, under this condition α B-crystallin forms amyloid fiber that contains typical amyloid β -sheet secondary structure. Shown in Fig. 3B, D and F, are 2D IR spectra and a TEM image of α B-crystallin when incubated in pD 3.0 phosphate buffer. The TEM image (Fig. 3F) reveals aggregates that are about 50 nm in length. The 2D IR spectra exhibit the 1621 cm⁻¹ peak and the corresponding cross peaks of amyloid β -sheets. Short amyloid morphologies are not uncommon and are observed in TEM images of other proteins with fibrillary,^{41; 42} prefibrillar,^{43; 44} oligomeric,⁴⁵ or amorphous morphologies.⁴⁶ These images and spectra are shown to illustrate the diversity in length that crystallin amyloids can adopt, as measured by TEM, and that 2D IR spectra exhibit characteristic amyloid β -sheet secondary structure even for lengths that are difficult to resolve with TEM. 2D IR spectroscopy is more sensitive to amyloid formation than TEM because only 4 or 5 strands are needed to create the spectroscopic signatures for amyloid β -sheets,³³ as explained below.

TEM images of porcine lens tissue treated with acid and UV-irradiation

Shown in Fig. 4 are TEM images for slices of porcine lens tissues. Porcine eye lenses are sliced, treated with acid or UV-irradiation, chemically fixed, and then sectioned to 100 nm for TEM imaging (see Materials and Methods). As a control experiment, TEM of the native tissue is shown in Fig. 4A. There are amorphous features on the order of 5 nm in size.

Fig. 4B shows TEM of acid treated tissue. With acid treatment, there are much broader features, indicating that the spatial variation of protein content is much less uniform than for the native tissue. Also apparent are sharper features that appear to be long fibers comparable to morphologies of amyloid fibers in other tissues.⁴⁷ Fig. 4D shows a copy of Fig. 4B, in which we have drawn pink lines along linear features that might be fibers. Shown in Fig. 4C are TEM images for tissue exposed to 90 hours of UV-irradiation. Large amorphous features are observed, but there are few, if any, linear features. These images illustrate the difficulty in identifying amyloid in tissues with TEM, which we discuss more below. According to TEM, the acid treated lenses may contain amyloid fiber, but the assignment is not definitive, and there are no features consistent with amyloid fiber in the UV-irradiated tissues. In what follows, we prove using 2D IR spectroscopy that amyloid β -sheet secondary structure is indeed produced in lens by both acid treatment and UV-irradiation.

2D IR spectra identify the secondary structure of amyloidogenic β -sheets in sliced porcine lens tissues upon acid treatment and UV-irradiation

2D IR measurements were made for 25 μm tissue slices taken from the same lens and denatured in the same way as for the TEM studies above (see Materials and Methods). The 2D IR spectra of untreated lens tissue (Fig. 5A and F) are remarkably similar to those of native γD - and αB -crystallin (Fig. 2A and D) i.e. there are no signatures of amyloid β -sheets. The diagonal peaks have a maximum absorbance at about 1636 cm^{-1} and extend up to 1660 cm^{-1} . The similarity between the spectra of the recombinant protein samples and tissues reflects the fact that crystallin proteins account for 90% of the proteins in mammalian lenses.² The exact composition of porcine lenses has not been determined, but is probably similar to humans, according to sequence comparisons.⁴⁸ Thus, we interpret the 2D IR spectra of the tissue using the *in vitro* spectra of γD - and αB -crystallin as references, keeping in mind that all lens proteins contribute to the tissue spectra.

Shown in Fig. 5B–D are 2D IR spectra of lens slices at different time points after acid treatment. There was a dead time of 5 min between the addition of the acid and when the first 2D IR spectrum was measured (Fig. 5B). Prior to acidification, the spectrum of the tissue exhibits a cross peak at $\omega_{\text{pump}} = 1636\text{ cm}^{-1}$, which is the frequency for native crystallin β -sheets, but there is no cross peak within the red box where we expect to see cross peaks from amyloid β -sheet secondary structure (Fig. 5A, red box). Five min after acidification, cross peaks within the red boxes are clearly present, indicating amyloid β -sheet secondary structure formation, along with a diagonal peak initially at about 1625 cm^{-1} (Fig. 5B, red box). As time progresses, the lower frequency diagonal peak grows to become the largest features in the spectrum and drops in frequency to about 1621 cm^{-1} . Both the lower diagonal peak and the cross peaks grow in with about a 20 minute time constant (Fig. S3). Fig. 5E shows the change of 2D IR diagonal slices. Thus 2D IR spectroscopy provides clear evidence that acidification of the lens tissue causes amyloid β -sheets to form.

Shown in Fig. 5F–I are 2D IR spectra of lens tissues irradiated with a UV lamp after 0, 8, 16 and 24 hours of irradiation. The temperature of the sample cell is monitored and kept between 25 to 30 $^{\circ}\text{C}$ throughout the irradiation. UV-irradiation was halted every hour to a collect 2D IR spectrum. After 8 hrs, the tissue was no longer clear, but was cloudy with a white color. In Fig. 5G the peak at 1636 cm^{-1} has broadened compared to the peak width in the native lens spectrum (Fig. 5F), indicating unfolding. A slight shoulder around 1621 cm^{-1} might be present, but there is distinct pair of cross peaks observed (red box), which is strong evidence of amyloid β -sheet secondary structure.¹⁴ After 16 hrs of UV-irradiation (Fig. 5H), the 1636 cm^{-1} peak of the lens slice further broadens and the pair of cross peaks persists. After 24 hours of UV-irradiation, a diagonal peak near 1621 cm^{-1} becomes prominent enough that it is resolvable from the other diagonal features (Fig. 5I). The 2D IR diagonal slices better illustrate the formation of a diagonal peak (Fig. 5J).

Amyloid fibers typically do not dissolve in 2% SDS solution while other protein structures do.⁴⁹ The UV-irradiated lens tissue (24 hrs) were treated with 2% SDS solution. A 2D IR spectrum of the SDS supernatant (soluble portion) shows no amyloid but the SDS pellet (insoluble fraction) shows the 1621 cm^{-1} diagonal peak and cross peaks (Fig. S4), thereby confirming the presence of amyloid β -sheet secondary structure in UV-irradiated lens

tissues. The SDS-PAGE gel (Fig. S5, lane 4–7) of the UV-irradiated tissue also exhibited higher molecular weight species (~16%) than did the gel of the native tissue (Fig. S5, lane 3), indicating that UV-irradiation causes covalent crosslinking which is known to occur *in vitro* studies of γ D-crystallin.^{11; 50; 51} We also note that the UV-irradiated lens tissues becomes pale yellow upon UV-irradiation (Fig. S6) with some photocleavage products (Fig. S5, ~0.8%). The same color change (Fig. S6) was reported previously as a result of oxidization of the tryptophan residues to generate kynurenine.^{11; 50; 51} ImageJ was used to integrate the gels. The bands near 11.5 and 9 KDa, which are lower molecular weights than that of full length γ D-crystallin, contribute 0.8% to the total intensity. Thus, UV-irradiation of lens tissue also creates fragments and covalently linked products.

The kinetics of amyloid β -sheet formation is plotted in Fig. 6A, as measured by the FWHM of the 1636 cm^{-1} peak and by the ratio of intensities at 1621 cm^{-1} and 1636 cm^{-1} . The former metric follows protein denaturation while the latter is a measure of amyloid β -sheet formation. These ratios increase linearly with exposure time up to 20 hours. Interestingly, both plots plateau after 20 hours of exposure, indicating no additional increases in amyloid β -sheet secondary structure. If UV-irradiation is halted at any point, the sample stops evolving until UV is reapplied (measured up to 48 hours later, see Fig. S7B), indicating that the protein structures are arrested until further damage occurs. Control experiments on lens tissue without UV exposure show that amyloid does not form spontaneously even after two days (Fig. S7A).

2D IR is more sensitive to amyloid β -sheet secondary structure than FTIR spectroscopy

Amyloid formation *in vitro* is often studied with FTIR spectroscopy.⁷ Fig. 6B–D shows a comparison between FTIR and 2D IR spectra, collected on the same tissue samples as reported above for the 2D IR studies. Note that the amyloid peak at 1621 cm^{-1} is much more apparent in the 2D IR spectra (Fig. 6B). It is enhanced by a factor of 5–10 over the FTIR absorbance. For example, from the FTIR spectra alone, one could not conclude that UV-irradiation causes amyloid (Fig. 6C). In contrast, the diagonal slices from the 2D IR spectra shows a pronounced intensity near 1621 cm^{-1} (Fig. 6D). This comparison between FTIR and 2D IR spectra is presented to illustrate the improvement that 2D IR spectroscopy makes for diagnosing amyloid formation in complex mixtures. The physics behind this enhancement is explained below.

Thioflavin T (ThT) fluorescence assay of lens tissue is consistent with the formation of amyloid β -sheet fiber by acid treatment and UV-irradiation

ThT is a dye that binds preferentially to amyloid fiber structures and exhibits an enhanced fluorescence upon binding. The ThT fluorescence assay of native, acid treated, and UV-irradiated lens is shown in Fig. 7. The integrated fluorescence of the acid treated tissue is 3-times higher, and the UV-irradiated lens samples is at least 5-fold higher, than that of the native lens samples (Fig. 7). A similar increase of ThT fluorescence was observed in UV-irradiated *in vitro* γ D-crystallin.^{11; 13; 50} Acid-treated *in vitro* α B-crystallin amyloid fiber also stains with ThT.⁸ Native lens tissue has previously been shown to exhibit ThT fluorescence,⁵² which was thought to demonstrate that ThT binds to β -sheets in native crystallin proteins.^{53; 54; 55} That conclusion is consistent with our results here. Moreover,

ThT is quenched in acidic conditions, and thus should be considered a lower bound in the acid treated tissues.⁵⁶ Thus, the ThT assays by themselves are not conclusive, but the increase in fluorescence upon acid or UV treatment is consistent with the conclusions drawn from the 2D IR data.

Discussion

FTIR spectroscopy is often used to study amyloid fiber formation because of the characteristic 1621 cm^{-1} absorption created by amyloid β -sheets.^{7; 57; 58} While a 1621 cm^{-1} band is a clear identification of amyloid β -sheet, FTIR spectra are often congested, especially for *ex vivo* tissues. To identify the amyloid β -sheet mode, deconvolutions are used or spectra are fitted to Gaussians. Deconvolutions are susceptible to noise and Gaussian fits are almost never unique (nor correct representations of secondary structures). Thus, assigning amyloid formation based on FTIR spectroscopy alone can be circumspect unless amyloids are the dominant protein structure.

2D IR spectroscopy provides a much more rigorous and sensitive method for identifying amyloid β -sheet secondary structure, for two reasons. First, 2D IR spectra scale to the 4th power of transition dipole, μ , while FTIR spectra scale to the 2nd power. Beer's Law for absorption, A , is given by $A^{FTIR} = \epsilon \cdot c \cdot l$, where ϵ is the molar absorptivity, c is the concentration, and l is the path length. Each normal mode of the protein, such as β -sheet modes, has a different molar absorptivity. In FTIR, the molar absorptivities are related to the transition dipoles, μ , by $\epsilon \propto \mu^2$, which are created by linear combinations of the individual (local mode) transition dipoles for each carbonyl group that contributes to that normal mode. For a given secondary structure, the couplings dictate the precise linear combination, which sets the frequency and magnitude of ϵ for that normal mode. The linear combination of transition dipoles that creates the 1621 cm^{-1} amyloid mode is illustrated in Fig. 1. This amyloid mode is due mostly to a linear combination of carbonyl groups across β -strands, creating a normal mode that oscillates along the length of the fiber. It takes 4 or 5 β -strands to create the signature 1621 cm^{-1} mode of amyloids, and up to 12 strands will contribute carbonyl groups vibrating in-phase (the delocalization length),²⁷ which produces a very large ϵ . The vibrational frequency and ϵ are unaffected by additional β -strands, putting the sensitivity for IR spectroscopy between about 4 and 12 strands. There are many other β -sheet modes, which are not illustrated here, that have smaller ϵ . An interesting consequence of Beer's Law is that the integrated area of an FTIR spectrum is conserved for any given protein. That means that if a protein transitions from a random coil to a β -sheet, for example, the spectrum will change shape but the area under the curve is the same. There exist more comprehensive discussions for the physics of these modes elsewhere and we refer the reader to review articles for examples of 2D IR spectra of proteins.^{24; 25; 59}

For 2D IR spectroscopy, the analogous equation to Beer's Law is $A^{2DIR} = |\mu|^4 \cdot c \cdot l$. Thus, for a given normal mode, the frequency is the same in a 2D IR spectrum as it is in an FTIR, but the intensities will be different due to the non-linear dependence on $|\mu|$. An interesting consequence of the $|\mu|^4$ scaling is that the integrated area of a 2D IR spectrum is not conserved.^{25; 34} For example, a protein that has an equal number of residues in β -sheets as it does in random coil structures, will have an FTIR spectrum in which these two secondary

structure contribute equivalent areas under the peak. For the same protein measured with 2D IR, the β -sheet feature will dominate the spectrum because $|\mu|$ for β -sheets is between 2 and 6 times larger than for random coils (illustrated in Fig. 1 for a normal mode made from three local modes).²⁷ Thus, the diagonal peaks of 2D IR spectra are much more sensitive to amyloid fiber formation than is FTIR spectroscopy.

The second reason that 2D IR is more sensitive than FTIR spectroscopy for identifying amyloid β -sheets is because of the cross peaks. Couplings create the linear combinations that give the frequencies of the normal modes, and in doing so, also create cross peaks at the frequencies between the normal modes, like schematically shown in Fig. 1. The intensities of cross peaks are proportional to $A^{2DIR}(\text{cross peak}) \propto |\mu_i|^2 \cdot |\mu_j|^2 \cdot c \cdot l$, where μ_i and μ_j are the transition dipoles for two different normal modes. The strongest cross peak in the crystallin amyloids is between the strong 1621 cm^{-1} β -sheet mode, and a weaker normal mode at around 1687 cm^{-1} . Cross peaks appear off of the diagonal and so are better resolved than the diagonal peaks in 2D IR spectra or the corresponding modes in FTIR spectra. Among coupled modes, the amyloid β -sheet cross peaks are particularly well-resolved, because they lie far from the diagonal peaks in a region of the 2D IR spectrum that is largely background free. Thus, a cross peak can be used to determine the frequency of a given mode by its ω_{pump} even if this mode cannot be identified by the diagonal peaks in a 2D IR spectrum or a congested FTIR spectrum.

The *in vitro* control experiments presented here on γ D- and α B-crystallins clearly show that they are capable of forming amyloid fibers (Fig. 2), consistent with prior work.^{7; 8} Acid treatment of γ D- and α B-crystallin creates long amyloid fibers with few other structural products^{7; 8; 60} and has a prominent a diagonal peak at 1621 cm^{-1} and corresponding cross peaks in the 2D IR spectra (Fig. 2). For UV-irradiation *in vitro*, a shoulder along the diagonal (Fig. 2I, red arrow) at 1621 cm^{-1} and a weak cross peak (Fig. 2C, red box) indicate that γ D-crystallin forms amyloid β -sheet although it is not the predominant structure. In contrast, we see no evidence that *in vitro* α B-crystallin forms amyloid β -sheets upon UV-irradiation, because there is no resolvable diagonal feature nor cross peaks (Fig. 2F), even when α B-crystallin is irradiated more than three times as long as γ D-crystallin (48 versus 18 hours, see Fig. S1). Therefore, at least some subset of the crystallin proteins have the capacity to form amyloid β -sheet secondary structures upon UV-irradiation, but not all. These 2D IR spectra of *in vitro* γ D- and α B-crystallin serve as benchmarks against which more complicated spectra can be compared.

TEM imaging is the most common imaging technique for studying amyloid fibers *in vitro*.^{8; 61} *In vitro*, samples are prepared by evaporating solutions containing amyloid forming proteins onto electron microscopy grids. The background is very low, because the dyes binds preferably to the fibers and there is little protein present in regions of the grid with no fibers. The TEM images of the *in vitro* control experiments presented above are consistent with the conclusions drawn from 2D IR spectroscopy. For amyloid to be identified with TEM microscopy, fibers need to be $>25\text{ nm}$ in length so that they appear elongated (using a 10 nm width from *in vitro* TEM), which requires fibers to contain at least 50 β -strands.⁶² Even then, the native tissue has background features in the same size range. In contrast, 2D IR spectroscopy is sensitive to amyloid β -sheets made from only 4 or 5

strands of β -sheets, which is enough to form the delocalized vibrational modes that create the 2D IR spectroscopic signatures of amyloid, and become insensitive to the number of β -strands for sheets longer than the delocalization length of 12 strands (see Discussion above).³³ 4 or 5 strands corresponds to a fiber length of <2 nm.⁶² Assemblies this small might be considered oligomers that contain some fraction of amyloid β -sheet secondary structure. The data might also be consistent with seeds, which are very short fibers that nucleate growth of longer amyloid fibers.

Another difficulty with TEM is contrast, which is especially problematic for tissues. Tissue imaging with TEM requires samples sliced 100 nm thick, so that there is protein everywhere (more so for lens tissues), and thus, a high background. As a result, *ex vivo* imaging has much lower contrast than *in vitro* imaging. While TEM of cataract lens tissues have been reported, few examples of fibers exist, and their assignment as amyloid fibers is not certain.¹⁹ In our TEM results, we do observe a difference between the native and UV-irradiated lens tissues. There may be fiber present in the acid treated lens tissues as we highlighted in Fig. 4, but none can be identified in the TEM images of the UV irradiated tissues. FTIR spectra of these tissues is also inconclusive (Fig. 6C).

While assigning amyloid in these tissues based on TEM images and FTIR spectroscopy are both problematic, amyloid β -sheet secondary structure is clearly identified with 2D IR spectroscopy. Acid treatment of the lens tissues creates 2D IR spectra with a prominent diagonal peak at 1621 cm^{-1} and a cross peak at $\omega_{\text{pump}}=1621\text{ cm}^{-1}$ (red box, Fig. 5B–D), both of which are the indisputable features of amyloidogenic β -sheets.²⁴ The rest of the spectrum broadens and shifts in intensity to 1645 cm^{-1} , consistent with denaturation into disordered or random coil-like structures. Thus, while acidification is not physiological, this experiment establishes that the proteins in lens tissue can form amyloid β -sheets like the individual crystallin proteins do *in vitro*, although we cannot determine the precise proteins that are responsible. UV-irradiation of the lens tissues also results in amyloid β -sheet formation, which we conclude from the signature cross peaks in the 2D IR spectra (Fig. 5G–I). The cross peaks correlate to intensity at 1621 cm^{-1} along the diagonal (Fig. 5J, red) in which a shoulder appears, similar to the *in vitro* control experiment on γ D-crystallin (Fig. 2C and I). The presence of amyloid β -sheet is supported by 2D IR spectrum of a pellet from 2% SDS extraction, which is a method often used to extract amyloid material from tissues (Fig. S4),⁴⁹ and by ThT fluorescence on the lens tissue (Fig. 7).

To quantify the amount of amyloid formed in UV-irradiated lens tissue, we fit the diagonal slices of the 2D IR spectra. The FTIR spectra cannot be used, because the amyloid peak is not well enough resolved for a reliable fit (Fig. 6C). As explained above, the amyloid peaks are better resolved in the 2D IR spectra because of non-linear scaling with the transition dipole to the 4th power, $|\mu|^4$, and thus, 2D IR spectra are more sensitive to amyloid β -sheets than is FTIR. However, converting those fits to secondary-structure content requires knowing transition dipole strength $|\mu|^2$. Transition dipole strengths have been measured for amyloid fibers before,²⁷ but cannot be experimentally verified in these tissues. Taking these considerations into account, in the SI we use two methods for estimating amyloid content from the 2D IR spectrum, one that uses previously measured transition dipole strengths and one that uses an empirically derived scaling factor. The methods are explained in detail.

Controls are performed with native and acid treated tissues. From these methods, we estimate that between 6 and 15% of the protein content is amyloid β -sheet in the lens tissue after 24 hours of UV-irradiation (See Section 3 in the SI for details).

Porcine lenses are often used as a model for humans.⁶³ The soluble proteins in human lenses is estimated to be 40% α -crystallin and 50% β -plus γ -crystallin; a similar distribution is found in porcine lenses.⁴⁸ The X-ray crystallography structures of porcine crystallin proteins have not been reported so we cannot compare the secondary or tertiary structure of porcine to human crystallin proteins, but the protein sequence of porcine and human α A- and α B-crystallin proteins are highly identical (over 91% for α A and 97% for α B); an uncharacterized protein in porcine (gene: LOC100154892) is 82% identical with human γ D-crystallin. Furthermore, the 2D IR spectra of the native porcine lens tissue produces a spectrum that resembles *in vitro* human γ D- and α B-crystallins. The spectra of the tissue are broader along the diagonal than the *in vitro* spectra (see slices in Fig. 2 and Fig. 5), indicating that there is a greater diversity of structures, but they have the same absorbance maximum at $\sim 1636\text{ cm}^{-1}$ and the same general features. Additionally, porcine lenses are very similar to human lens in terms of size. For cataract surgery training, cataracts are often induced in porcine lenses using microwaves or formalin.⁶³ Thus, while pigs and humans are different species, there are macroscopic and molecular reasons to think that pig lenses are a good model to study human cataracts. However, we note that the level of irradiation surpasses the lifetime of pigs. Because pigs have shorter lifetimes, the proteins in porcine lenses may be less stable than human lens proteins, which is presumably the same reason that dogs develop cataracts in a shorter timespan than do humans.

Cataract formation in humans is linked to sunlight exposure. Oxidative stress, diabetic conditions, alcohol consumption, and other factors also contribute, but studies have established a strong correlation between cataract prevalence and the exposure time to UV-irradiation.^{12; 13; 51; 64; 65} A study done in Nepal by Brilliant *et al.* established that people living in an area with 12 hrs of sun exposure are 4 times more likely to develop cataracts than those with 7 hrs of sun exposure.⁶⁵ As a result, UV light has been used to simulate cataract formation *in vitro*.^{11; 12; 13; 51; 66} Here, we use UV light to simulate cataract formation *ex vivo*.

Estimates put the UV exposure for an average American at 30000 minimal erythema doses (MED) for a person's lifetime, which is equivalent to 12 million J/m². Under our experimental conditions, 1 year of natural UV exposure onto the lens corresponds to 29 minutes of irradiation with our UV lamp (for the calculation, see SI). In the plot of UV induced kinetics (Fig. 6A) both the intensity at 1621 cm^{-1} and the HWFM of the entire peak grow as the time period of UV-irradiation increases. At early times, denaturation and amyloid β -sheet secondary structure increases proportionally with UV exposure time, suggesting that natural damage and aggregation may occur in human lenses even with small amounts of UV light.

If amyloid β -sheet secondary structure does indeed exist *ex vivo* as our results support, then why is there not more evidence for amyloid fibers from images of cataractous lens tissues? As mentioned in the Introduction, we find only one report of an image with a structure

resembling an amyloid fiber, reported in OXYS strain of rat.¹⁹ One answer to this question might be the difficulty associated with obtaining high quality TEM images. *In vitro*, there is very little background in TEM signals and so the contrast in TEM images of amyloid fibers can be high enough to visualize periodic twists.⁶⁰ Tissue imaging with TEM usually requires samples sliced about 100–150 nm thick, which creates a background. Nonetheless, TEM of amyloid fiber deposits in tissues have been reported for various parts of the body, including the brain,⁶⁷ the heart,⁶⁸ and the liver.⁶¹ It may be more difficult to observe fibers in the lens, which consists almost entirely of crystallin proteins at a very high concentration in order to retain the refractive index,^{2; 54; 69} and thus has a low contrast because potential fibers are surrounded by tissue proteins.

There may also be a physiological reason for a lack of images of fibers in lens tissues. The α B-crystallins are small heat shock proteins that are molecular chaperones and are found throughout the body; α B-crystallin is found in the heart, brain, and muscle tissue.⁷⁰ α B-crystallin increases the stability of proteins and adheres to damaged proteins.¹⁰ It has also been shown that α B-crystallin slows amyloid formation and adheres to amyloid fibers.⁷¹ Around 40% of lens proteins are α -crystallin. Having a large percentage of chaperone proteins is thought necessary to enable the long-term stability of the eye proteins, since the lens proteins are not regenerated and so need to be stable for the entire human lifetime.² Thus, we hypothesize that the chaperone proteins in the lens bind to amyloid aggregates, but do so while they are very small, thereby inhibiting them from forming microscopic fibers visible in EM microscopy. Unlike ATP-driven chaperones, α B-crystallin is a passive molecular chaperone that can bind to unfolded proteins but cannot refold them.^{71; 72} The UV kinetics data of Fig. 6A is consistent with passive chaperones, because when UV-irradiation is discontinued, amyloid aggregation is arrested but not reversed. The small aggregates might be oligomers, since they could be as small as only 4 or 5 strands, since that is all that is needed to create the characteristic 2D IR features of amyloid. Since the 2D IR spectra cannot differentiate between fibers with more than about 12 strands, the data is also consistent with short segments of mature fiber, too small to observe with TEM, such as seeds that nucleate fiber growth. Either way, the 2D IR signal indicates that there are assemblies of protein that are rich in amyloid β -sheet secondary structure and appear to be sequestered by the high density of chaperones in the lens. Thus, the formation of amyloid β -sheets in lens tissues may be a signature of UV degradation of the chaperone system itself, which has previously been hypothesized as a cause of cataract formation.⁷³ The plateau in the kinetics (Fig. 6) might indicate that amyloid β -sheet secondary structure is created from a subset of crystallin proteins that is more susceptible to damage than others, thereby limiting the final amount of amyloid β -sheet. For example, we know from the *in vitro* control experiments that UV-irradiation causes γ D-crystallin to form amyloid β -sheets but not α B-crystallin (Fig. 2).

CONCLUSION

There exist many *in vitro* experiments showing that crystallin proteins form amyloid fibers, but there is little evidence that cataracts themselves contain amyloid fibers. Using 2D IR spectroscopy and *in vitro* control experiments of γ D- and α B-crystallin, we have shown that tissues of porcine eye lenses, when irradiated with UV light to simulate cataract formation,

contain aggregates with between 6–15% amyloid β -sheet secondary structures. Acidification of lens tissue also creates amyloid β -sheet secondary structure, but with much longer lengths. The population of amyloid β -sheets increases proportionally with UV exposure until about 20 hours (42 years of UV-exposure), but then stops. Amyloid β -sheet formation is also halted if UV-irradiation is stopped (Fig. S7). We attribute these observations to the differences in susceptibility to UV-induced aggregation between different families of crystallin proteins and to the role of chaperones. Chaperones may be responsible for inhibiting growth of small oligomers of seeds, which could explain the lack of amyloid images in cataract tissues. Many of the observations in this report were made possible by the non-linear scaling of 2D IR intensities and cross peaks, which enhances the technique's sensitivity to amyloid β -sheet secondary structure. Similar applications could be applied to identify and image tissues in other amyloid diseases. Our results support many of the conclusions drawn from *in vitro* experiments on the crystallin proteins and suggests that natural cataract formation in humans is an amyloid disease.

Materials and Methods

Full-length γ D-crystallin is expressed in *E. coli* and purified according the method described previously.¹⁴ Human α B-crystallin pAED4 plasmid was kindly gifted by Jonathan King at the Massachusetts Institute of Technology. The plasmid was electroporated into BL21 cells for expression and purified using an established protocol.³⁵ 2D IR spectra were collected as described previously.¹⁴ The contours on 2D IR spectra were plotted exponentially to the third power to emphasize the lower intensity peaks. All buffer solutions used in 2D IR and FTIR studies are deuterated 20 mM NaH_2PO_4 and 100 mM NaCl unless stated otherwise. Porcine eyeballs are supplied by the Meat Science and Muscle Biology Laboratory at UW-Madison. At time-of-death (TOD) the animals were about 6 months old (with life expectancy of 7 years) and were raised indoors. Data on native tissue were collected on 6 different lenses from 3 different pigs, which all gave similar results, and UV exposure was repeated on 3 different lenses. The lenses are extracted from the eyeballs within approximately 2 hours of TOD of the animals. The lenses were stored at -20°C in embedding medium (Tissue-Tek) until they were sliced using a microtome at -26°C to a thickness of 25 microns (or 60 microns for TEM samples). The slices were deposited directly onto a CaF_2 window (or a coverslip for TEM samples) and deuterated with D_2O . For native and UV-irradiation samples, pD 7.4 phosphate buffer was added to the samples. For acid treated tissue samples, pD 2.0 phosphate buffer were used (instead of pD 3.0 for the protein solutions as previous published¹⁴ because the high protein concentrations in lens tissues served as additional buffering system). 5 μL of phosphate buffer was added to one slice of lens tissue (25 μm thick and about 0.5 cm^2) to make 2D IR and FTIR samples. The volume ratio of tissue to added buffer is around 1:4. After the addition of buffer, the sample cell was assembled with a 56-micron Teflon spacer. For UV-irradiation, the samples cells were exposed to four 8 W medium pressure mercury lamps at a distance of 30 cm, which corresponds to a power intensity of 35 W/m^2 measured by a power meter. For TEM, the 60-micron lens tissues were treated under the same conditions as for the 2D IR samples on a coverslip. The tissues were then fixed and sectioned to 100 nm for imaging. For ThT fluorescence assays, 25-micron lens slices were deposited onto coverslips with a

hybridization chamber. Samples were irradiated with UV light or treated with pD 2.0 buffer before phosphate buffer (pD 7.4) containing 25 μ M ThT was added. A 405 nm laser and a 482 nm filter were used for the measurement. Additional details are given in SI Materials and Methods (SI, Section 1).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Research highlights

- UV exposure causes cataracts, a protein aggregation disease that occurs in lenses.
- *In vitro* the lens proteins form amyloid fibers as identified by TEM and 2D IR spectroscopy.
- In the lens, UV irradiation causes amyloid structure to form as identified using signature peaks in 2D IR spectra.
- Senile cataracts may be an amyloid disease

Fig. 1. Cartoon illustration of amide I modes of (amyloid) β -sheets

(A) Amide I vibrational modes in β -sheets (parallel). Local modes are shown with black arrow. Vibrational coupling results in the redistribution of the transition dipole creating a normal mode along the fiber axis (red arrow). (B) A cartoon representation of a typical 2D IR spectrum with two pairs of diagonal peaks that are coupled to each other, creating two pairs of cross peaks below and above the diagonal. (C–D) Cartoon illustration of the different peak intensity and resolution of FTIR and 2D IR diagonal slices.

Fig. 2. 2D IR spectra of γ D- and α B-crystallin proteins in deuterated buffer

(A) Native, (B) acid denatured and (C) UV-irradiated (18 hrs) γ D-crystallin (30 μ M); and (D) Native, (E) acid denatured and (F) UV-irradiated (18 hrs) α B-crystallin (30 μ M) 2D IR spectra. The structure illustration of α B shows only the core structure and the N-terminal domain. (G–I) The corresponding diagonal slices of the 2D IR spectra (solid for γ D-crystallin and dashed for α B-crystallin).

Fig. 3. 2D IR spectra and TEM images of aggregated α B-crystallin

2D IR spectra of (A) α B-crystallin in 10% TFE phosphate buffer (pD 7.4), incubated at 60 °C for 35 hrs. (B) α B-crystallin in phosphate buffer (pD 3.0), incubated at room temperature overnight. (C–D) 2D IR diagonal slices of corresponding 2D IR spectrum on the left. (E–F) TEM images of corresponding 2D IR samples on the left. Scale bars are 100 nm. The concentration of α B-crystallin is 30 μ M in all the solutions.

Fig. 4. TEM images of porcine lens tissues upon acid and UV treatment

(A) Untreated lens tissue. (B) Lens tissue treated with acid overnight. (C) Len tissue treated with 90 hours of UV-irradiation. (D) Duplicate image of (B) with pink highlights of amyloid fibers. Scale bars are 200 nm.

Fig. 5. 2D IR spectra of porcine lens tissues upon acid and UV treatment

(A and F) Untreated porcine lens tissue in pD 7.4 phosphate buffer. (B) 5 min, (C) 45 min, and (D) 145 min of lens tissues after addition of pD 2.0 phosphate buffer. (E) Overlay of diagonal slices of the 2D IR spectra above for (A) in black, (B) in blue, (C) in green and (D) in red. (G) 8 hrs, (H) 16 hrs, and (I) 24 hrs of UV-irradiation in pD 7.4 phosphate buffer. (J) Overlay of diagonal slices of the 2D IR spectra above for (F) in black, (G) in blue, (H) in green and (I) in red. Pump slices of these spectra through $\omega_{\text{pump}} = 1621 \text{ cm}^{-1}$ are in Fig. S2 further visualize amyloid cross peaks.

Fig. 6. FTIR and 2D IR diagonal slices of porcine lens tissues

(A) Kinetics of amyloid formation plotted as the FWHM (blue circles, left y-axis) and as the ratio between the 1621 cm^{-1} and the 1636 cm^{-1} intensity of the 2D IR diagonal slices (red triangles, right y-axis). (B) Overlay of FTIR spectrum of untreated (black solid) and acid denatured (black dashed) lens tissue, and 2D IR diagonal slice (gray) of acid denatured lens tissues. (C) FTIR spectra of native and UV-irradiated lens tissues. (D) 2D IR diagonal slices of native and UV-irradiated lens tissues collected on the same samples as in (C).

Fig. 7. Histogram of ThT fluorescence assay of native, acid treated and UV-irradiated lens tissue Phosphate buffer with ThT (25 μ M) is added to 25 μ m lens tissues. The fluorescence is measured with a 405 nm excitation laser and a 482 nm filter. Histogram shows the integrated intensity over the camera spots averaged over several samples. Error bars are calculated from the standard deviation throughout different samples. The fluorescence images are examples of the ThT fluorescence assay.