“ESI-MS-Bioinformatics Studies on Crosslinking of α A-Crystallin and Lysozyme using a New Small Aryl Azido-N-HydroxySuccinimidyld Heterobifunctional Crosslinker based on a Metabolite of the Alternative Kynurenine Pathway”

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Abstract

The use of a new small aryl azido-N-Hydroxysuccinimidyl heterobifunctional crosslinker for crosslinking of αA-crystallin and lysozyme is described here. The crosslinker is based on the small molecule, 3-hydroxyanthranilic acid (3HAA) a part of the kynurenine pathway in Tryptophan metabolism. Enhanced amounts of 3HAA are found in disease states in the human body. The new crosslinker contains a photo labile azido group and an amine reactive, N-hydroxy succinimide (NHS) group. Small crosslinkers capture interacting protein interfaces better, while the larger ones are more useful for identifying interacting partners. Our earlier work has shown that aryl azides in this series lead to ‘long lived’ transients allowing for increased intermolecular reaction rates, otherwise difficult to achieve. Using this crosslinker, successful crosslinking of αA-Crystallin & lysozyme has been demonstrated in two steps i.e. incubation followed by photolysis (366 nm, 6W UV lamp). Previous studies on αA-Crystallin have mostly used only homobifunctional crosslinkers. As hypothesized by us, the use of a heterobifunctional crosslinker has indeed led to more efficient crosslinking. This has been confirmed using SDS-PAGE, ESI-MS/MS (following trypsinization of the homo and hetero ‘dimer’ bands) and use of StavroX 3.6.0.1, the bioinformatics software especially suited for analyzing intermolecular crosslinking. These investigations are expected to lead to a better understanding of the role of αA-Crystallin in chaperoning mechanism and in cataractogenesis.

Keywords: 3-Hydroxyanthranilic acid; Kynurenine; ESI-MS; StavroX 3.6.0.1

Introduction

Alpha-Crystallin the transparent, heat stable, water soluble protein of the human eye lens has been studied earlier exhaustively [1], α-Crystallin consists of aA (173 amino acids; Mw; 19909) and aB (175 amino acids; Mw; 20159), “in a molar ratio which is variable among species”. aA-Crystallin and aB-Crystallin play a very important role in keeping the human eye lens transparent and to prevent aggregation of these water soluble proteins leading to opaqueness and cataract. However, only very recently their role as therapeutics, for treating not only eye diseases, but also other major diseases has been demonstrated [2-9]. It is pertinent to note that even in cases of concussion of the brain, treatment with Crystallins has helped in recovery from the neurodegenerative injuries [10]. However, since Crystallins also cause diseases, it is a trade-off between their activity as possible therapeutics and simultaneously as disease causing agents, makes it necessary to tread the path with great caution. It is also known that metabolites of the alternative Kynurenine pathway (Supplementary Figure 1) of the Tryptophan catabolism have an important role in disease states. These include Huntington’s disease, Parkinson’s disease, HIV-AIDS, and cerebral malaria. Thus, the ratio of 3-Hydroxyanthranilic acid (3HAA) to Anthranilic acid (AA) in the human brain differentiates a patient from a normal person [11]. 3-Hydroxykynurenine (3HK) and 3HAA oxidize a-A-Crystallin, which leads to the production of hydrogen peroxide in the human eye and has been implicated in cataractogenesis [12]. Earlier work has shown that 3HK and 3HAA reduce Cu (II) and Fe (III) and generate superoxide and H2O2, when the kynurenine pathway is activated, which could be relevant in Cataractogenesis. These workers [13] even carried out cyclic voltammetry studies showing loss of Cu (II) by complexation and/ or reduction with 3HAA being the most effective. Srivastava et al. [14,15] have carried out mass spectral studies of the proteins of human eye lens and correlated it with age; larger number of proteins being found with increasing age. The D. Balasubramanian group at the L.V Prasad Eye Hospital, Hyderabad, India [16,17] has done pioneering work on crystallins. For example, these workers showed that transglutaminase mediated dimerization of alpha Crystallin decreases its chaperone like activity with considerable loss of tertiary structure and decrease in its secondary structure based fluorescence. The effect of alpha Crystallin on the refolding of the denatured-disulphide intact and denatured-lysozyme was studied. However, no refolding of disulphide intact enzyme occurred but alpha crystallin inhibited the aggregation and...
oxidative renaturation of denatured-reduced lysozyme” [18,19].

“Crystallin is known to prevent the heat induced aggregation of the protein by forming a stable complex.” These workers chose Lysozyme as it “is one of the most extensively studied enzyme for its refolding properties.” Peschek et al. [20] (a) demonstrated the chaperoning function of αA-Crystallin in binding with lysozyme, leading to soluble and insoluble proteins. These workers “studied the chaperoning activity of αA-Crystallin in aggregation assays using Lysozyme as a substrate”. Said Abgar et al. [20] (b) studied the chaperoning function with αA-Crystallin and Lysozyme and they also stated that “we have chosen Lysozyme because many aspects of its structure have been extensively studied”. Krishna Sharma’s [21-24] group reported the differences between αA-WT and mutant-G98R Crystallins. Using the homobifunctional crosslinker d0/d4(1:1) deuterium labeled BS2G and used the GPMAW software to show that majority of inter subunit crosslinking was clustered in K88 region in αA-WT Crystallin, while in the mutant-G98R Crystallin, crosslinking was seen in the K99 region of the protein. Thus, in the wild type protein, crosslinking is at K88 and in the mutant it shifts to K99. This one difference, according to them reflects the different oligomerization and conformational changes in the mutant that contribute to its aggregation, making the mutant αA-Crystallin more prone to cataract formation. Their studies have helped identify the Alpha Crystallin Domain (ACD), which is common to most known HSPs. The ACD of αA-Crystallin is now referred to as the ‘Mini α crystallin Chaperons’ (MαCs), which is represented by the 70°KFVIFLDVKHFSP82 sequence [25-27]. 3-D representation of MACs using protein Swiss server in αA-Crystallin where MAC (represented as a mesh shadow is shown in Supplementary Figure 2. Chaperons may work as “holdase”, “foldase” and “unfoldase” functions for stabilizing the non-native/native state to prevent protein aggregation and to make misfolded state conformations by to regain the original conformation [28].

Many crosslinkers are known in literature and are commercially available [29,30]. Thus one has moved away from the days when formaldehyde and glutaraldehyde were used as crosslinkers which brought about indiscriminate crosslinking. Nowadays, zero length, isotope labeled, MS cleavable, homo and heterobifunctional crosslinkers are known. The difficulty is how to differentiate and separate the uncrosslinked peptides from the crosslinked ones, especially when the latter are found only in low abundance. This is referred to as “a needle in the haystack problem”. This has been overcome in recent years, by the use of strong cation exchange (SCX) chromatography which help separate or enrich the crosslinked fragments (which are invariably charged) from the uncross linked fragments which are neutral. It is also known that smaller crosslinkers give better information about interacting interfaces while the larger crosslinkers give better information about the interacting sites. Progress in this field has also been possible due to great advances in the field of mass spectrometry like MALDI-MS, MS/MS, ESI-MS [31-35], which allow detailed investigations even when the amount of sample available is very small. Similarly, great advances have happened in Bioinformatics tools like the M3SD Links, GPMAW, Xlink, Kojac, CLMS vaults, pLink, StarVox & MeroX [36-39]; the latter being especially suitable for cleavable MS. Hagan Bayley [40], had predicted that intermediates during thermolysis/photolysis of pentafuro phenyl azide could lead to efficient photoaffinity labeling agents, as these involve “long-lived” transients. This was subsequently shown to be true by M S Platz et al. [41,42]. Tomioka [43] showed that such ‘long-lived’ transients involve “slippery potential energy surfaces” and could lead to increase intermolecular crosslinking. Computational studies by Borden et al. [44] provided the much needed theoretical basis that there is an increase in the singlet-triplet gap. We have also similarly prepared Aryl Azido-N-hydroxy succinimidyld ( NHS) heterobifunctional crosslinkers based on ‘long-lived’ transients, which do not require any ortho-flanking fluorine atoms [45-51]. The latter is a great advantage as fluorination is both hazardous and toxic and preparation of our new crosslinker does not involve any such hazardous steps. It may be noted that aryl azides are now referred to as “green reagents” [52]. Earlier crosslinking studies on αA-Crystallin have mostly used homobifunctional crosslinkers. In the current study, a new small aryl-azido-NHS- heterobifunctional crosslinker based on 3HAA, a catabolite of Tryptophan in the Kynurenine pathway has been employed. The new crosslinker is based on ‘long-lived’ transients, which could promote efficient intermolecular crosslinking. Crosslinking has been done here using a two-step protocol. The first step involves incubation which is followed by photolysis (366 nm, 6W UV lamp) to crosslink αA-crystallin (19kDa) and lysozyme (14kDa). From the intermolecularly crosslinked (33kDa) band thus obtained, we have identified sites of crosslinking and characterized a previously unidentified and a most significant intermolecularly crosslinked fragment, with a very precise m/z value. This fragment which contains fragments from both αA-Crystallin and Lysozyme provides a positive, confirmatory evidence for the binding of the two proteins. These investigations are expected to lead to a better understanding of the role of αA-Crystallin in chaperoning mechanism and cataractogenesis and for studies on other diseases, as well. This technique of chemical crosslinking-mass-spectrometry-bioinformatics is useful in proteomics, systems and structural biology, antibody drug conjugates (ADCs) and even for refining structures based on cryo-EM [53-64]. It is hoped that this new technique would be amenable to High Throughput Screening (HTS) of large number of patient samples in a rapid, reliable and routine manner.

Materials and Methods

Synthesis of the new crosslinker

The new heterobifunctional crosslinker, 2-Azido-3-Hydroxy-benzoic acid-2, 5-dioxo-pyrrolidin-1-yl ester (135 mg, (II) was prepared from 2-Amino-3-Hydroxy-benzoic acid (I) 200 mg, (I19 mmol) was taken and dissolved in 8 ml of concentrated hydrochloric acid and 2 ml of water and cooled at 0°C, this was diazotized by slow addition of sodium nitrate (140 mg, 1.2mmol) in minimum amount of water required. A solution of sodium azide NaN3 (120 mg, 2mmol.) and sodium acetate (3.36 g, 40mmol) in minimum amount of water was taken and slowly added to the diazotized solution, when an off white solid settled down on the bottom of the vessel. This compound was filtered and thus obtained (yield, 175 mg). 175 mg of compound (II) was dissolved in 10 ml of dichloromethane (DCM) and then sodium acetate (3.36 g, 40mmol) was added and the reaction mixture was stirred at room temperature overnight. The solution was filtered to separate the urea side product. The filtrate was distilled and put in a desiccator with P2O5 when pale white compound (III) was obtained.

The new crosslinker is based on the principle that the crosslinker is a ‘green reagent’ and it has been synthesized in a rapid, reliable and routine manner.
Analysis was undertaken.

...LC-MS/MS until LC-MS/MS peptides were vacuum-dried and stored at –80°C.

The peptides were removed from the gel pieces with 0.4% formic acid in 50% ACN solution and finally with 100% ACN. The extracted acid was dehydrated with 100% ACN, followed by digestion with trypsin (Gold mass-spectrometry trypsin; Promega, Madison, WI) at 37°C for 10–12 h.

The gel bands were subjected to reduction and alkylation using 10 mM iodoacetamide (IAA). The gel sections were dehydrated with 100% ACN, followed by digestion with trypsin (Gold mass-spectrometry trypsin; Promega, Madison, WI) at 37°C for 10–12 h. The peptides were removed from the gel pieces with 0.4% formic acid in 50% ACN solution and finally with 100% ACN.

The extracted peptides were vacuum-dried and stored at –80°C until LC-MS/MS analysis was undertaken.

**SDS-PAGE and in-gel digestion**

The FASTA sequence for Lysozyme (Supplementary Figure 5), αA-Crystallin (Supplementary Figure 6) and for 1:1 mixture of lysozyme and αA-Crystallin Supplementary Figure 7 are given in the supplementary material. The SDS-PAGE standard protocol used for this studies is given in Supplementary Figure 8. 10 micrograms of proteins were incubated with the crosslinker (overnight) and then photolyzed at (366 nm, 6W UVlamp, 30 mins.) and then resolved by SDS-PAGE (Lysozyme, αA-Crystallin and 1:1 mixture of lysozyme and αA-Crystallin Supplementary Figures 9 and 10. The gels were stained with Commassie blue and destained with water. Gel pieces were excised and in-gel digestion was carried out [65]. The excised bands were destained with 40 mM ammonium bicarbonate (ABC) in 40% acetonitrile (ACN). The gel bands were subjected to reduction and alkylation using 5 mM dithiothreitol (DTT) (60°C for 45 min) and alkylation using 10 mM iodoacetamide (IAA). The gel sections were dehydrated with 100% ACN, followed by digestion with trypsin (Gold mass-spectrometry trypsin; Promega, Madison, WI) at 37°C for 10–12 h.

The mass spectral data thus obtained for 28 kDa ‘homodimer’ band of lysozyme (14kDa), with the new heterobifunctional crosslinker based on 3HAA

The mass spectral data thus obtained for 28 kDa ‘homodimer’ band was fed into the StavroX.3.6.0.1 software as a dot (.mgf) file. As a result, 6784 of 7539 spectra were compared to 166544 theoretical candidates out of which 40135 possible crosslinks were identified within 1 minute and 07 seconds of the run. Major fragments identified by StavroX.3.6.0.1 are shown in Supplementary Figure 11. The software also provided the decoy analysis (Figure 1). "Blue bars represent the number of candidates from the real experimental set, while red bars represent the positive false candidates from the inverted sequence of the FASTA file. More enriched real data set candidates indicate toward better crosslinking".

The cross-linked candidate spectrum gives us details about the peptides that are involved in the process of cross-linking also and “N-2” major crosslinking site thus being suggested as a link between “K-6” and “N-2”. The cross-linking candidate spectrum gives us details about the peptides that are involved in the process of cross-linking also and “N-2” major crosslinking site thus being suggested as a link between “K-6” and “N-2”.

**Results and Discussion**

**Crosslinking details of the 28 kDa ‘homodimer’ band of lysozyme (14kDa), with the new heterobifunctional crosslinker based on 3HAA**

The mass spectral data thus obtained for 28 kDa ‘homodimer’ band was fed into the StavroX.3.6.0.1 software as a dot (.mgf) file. As a result, 6784 of 7539 spectra were compared to 166544 theoretical candidates out of which 40135 possible crosslinks were identified within 1 minute and 07 seconds of the run. Major fragments identified by StavroX.3.6.0.1 are shown in Supplementary Figure 11. The software also provided the decoy analysis (Figure 1). “Blue bars represent the number of candidates from the real experimental set, while red bars represent the positive false candidates from the inverted sequence of the FASTA file. More enriched real data set candidates indicate toward better crosslinking”. The top ten crosslinked peptide fragments, the intensity of intermolecular crosslinking and efficiency of the crosslinking is shown in Supplementary Figure 12. The highest score for fragment peaks was observed as 116. Figure 2 shows the annotation with the extent of deviation and the identified ‘b’ and ‘y’ ions for the fragment m/z 1802.862. “Less deviation in the annotation points toward better crosslinking”. The analysis of this peak with the score 105 is shown in Figure 2. Some of the most intense intermolecularly crosslinked peptides according to observed mass as well as specified sequences identified by StavroX 3.6.0.1 are shown in Table 1. For the fragment m/z 1802.862 with the score of 105, “K-6” of Peptide 1 (“LAAAmK”) crosslinks via “N-2” of Peptide 2 (“mNAWVAWR”), the major crosslinking site thus being suggested as a link between “K-6” and “N-2”. The cross-linked candidate spectrum gives us details about the peptides that are involved in the process of cross-linking also and shows the annotation with the extent of deviation and the identified ‘b’ and ‘y' ions. "Less deviation in the annotation points toward better crosslinking” [The modified fragment ions along with ‘b’ and ‘y’ ions, for the highest score 105 with the peak value of m/z 1802.862 is shown in Supplementary Figures 13 and 14].

**Crosslinking details of the 38 kDa ‘homodimer’ band of αA Crystallin (19kDa), with the new heterobifunctional crosslinker based on 3HAA**

The mass spectral data thus obtained for 38 kDa ‘homodimer’ band was fed into the StavroX.3.6.0.1 software as a dot (.mgf) file. As a result, 2935 of 3541 spectra were compared to 157302 theoretical candidates out of which, 35037 possible crosslinks were identified as a result, 2935 of 3541 spectra were compared to 157302 theoretical candidates out of which, 35037 possible crosslinks were identified.
Figure 1: Screen shot of the decoy Analysis for the 28 kDa ‘homodimer’ band of Lysozyme fragment m/z 1146.540 obtained from StavroX 3.6.0.1. (“Blue bars represent the number of candidates from the real experimental set, while red bars represent the positive false candidates from the inverted sequence of the FASTA file. More enriched real data set candidates indicate toward better crosslinking.”).

Figure 2: The annotation spectrum with the extent of deviation and identified peaks for the fragment m/z 1802.862 for 28kDa ‘homodimer’ band obtained from StavroX 3.6.0.1 “Less deviation in the annotation points toward better crosslinking”.

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<th>Peptide 2</th>
<th>Score</th>
<th>Sequence</th>
</tr>
</thead>
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<td>[BELAA]</td>
<td>116</td>
<td>K1-A5+CXL</td>
</tr>
<tr>
<td></td>
<td>1802.862</td>
<td>[LAAAmK]</td>
<td>[mNAWVWR]</td>
<td>105</td>
<td>K6-N2+CXL</td>
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<td>[TPGSRN]</td>
<td>[GMNAWVWR]</td>
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<tr>
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<td>1655.821</td>
<td>[ELAAAMK]</td>
<td>[KIVSDGNG]</td>
<td>74</td>
<td>E1-S4+CXL</td>
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</table>

Table 1: The intermolecularly crosslinked fragments with high scores identified by the software.
within 1 minute and 16 seconds of the run. Major fragments identified by StavroX 3.6.0.1 is shown in Supplementary Figure 15. The software also provided the decoy analysis (Figure 3). As stated above, “Blue bars represent the number of candidates from the real experimental set, while red bars represent the positive false candidates from the inverted sequence of the FASTA file. More enriched real data set candidates indicate toward better crosslinking”. The top ten crosslinked peptide fragments, the intensity of intermolecular crosslinking and efficiency of the crosslinking is shown in Supplementary Figure 16. The highest score for fragment peaks was observed as 66. Figure 4 shows the annotation with the extent of deviation and the identified 'b' and 'y' ions for the fragment m/z 1286.540. "Less deviation in the annotation points toward better crosslinking”. The analysis of this peak with the score 66 is shown in Figure 4. Some of the most intense intermolecularly crosslinked peptides according to observed mass as well as specified sequences identified by StavroX 3.6.0.1 are shown in Table 2. For the fragment m/z 1286.640 with the score of 66, "S-1" of Peptide 1 ("SAPSS") crosslinks via "D-5" of Peptide 2 ("VIFLDV"), the major crosslinking site thus being suggested as a link between "S-1" and "D-5". The cross-linked candidate spectrum gives us details about the peptides that are involved in the process of cross-linking, the annotation with the extent of deviation and the identified 'b' and 'y' ions. The modified fragment ions along with 'b' and 'y' ions, for the highest score 66 with the peak value of m/z 1286.640 is shown in Supplementary Figure 17.

Crosslinking details of the 33 kDa 'heterodimer' band of αA-Crystallin (19kDa) and lysozyme (14kDa), with the new heterobifunctional crosslinker based on 3HAA

The mass spectral data thus obtained for 33 kDa ‘heterodimer’ band was fed into the StavroX 3.6.0.1 software as a dot (. ) mgf file. As a result, 2011 of 2012 spectra were compared to 14460479 theoretical candidates out of which, 1759 possible crosslinks were identified within 1 minute and 02 seconds of the run. Major fragments identified by StavroX 3.6.0.1 are shown in Supplementary Figure 18. The software also provided the decoy analysis (Figure 5). As stated above, “Blue bars represent the number of candidates from the real experimental set, while red bars represent the positive false candidates from the inverted sequence of the FASTA file. More enriched real data set candidates indicate toward better crosslinking”. The top ten crosslinked peptide fragments, the intensity of intermolecular crosslinking and efficiency of the crosslinking is shown in Table 3. The highest score for fragment peaks was observed as 86. Figure 6 shows the annotation with the extent of deviation and the identified 'b' and 'y' ions for the fragment m/z 1290.597. "Less deviation in the annotation points toward better crosslinking”. The analysis of this peak with the score 86 is shown Figure 6. Some of the most intense intermolecularly crosslinked peptides according to observed mass as well specified sequence identified by StavroX 3.6.0.1 are shown in Table 4. A most significant intermolecularly crosslinked fragment with a peptide each from αA-Crystallin and from Lysozyme was observed with the value of m/z 1290.597. For this fragment with m/z 1290.597 and the score of 86, "S-1" of Peptide 1 ("SALSB") crosslinks via "L-1" of Peptide 2 ("LAAMK"), the major crosslinking site thus being suggested as a link between "S-1" and "L-1". The cross-linked candidate spectrum. Figure 7 gives the details about the peptides that are involved in the process of cross-linking and also shows the annotation with the extent of deviation and the identified 'b' and 'y' ions. The modified fragment ions along with 'b' and 'y' ions, for the highest score 86 with the peak value of m/z 1290.597 is shown in Supplementary Figure 19.

Additional experiments done, using reduced lysozyme, mutant αA-Crystallin and with lysozyme and αA-Crystallin using the crosslinker (ATFB, SE) are included in Supplementary Figures 19-22 as these did not give significant results.

![Figure 3](image-url)

Figure 3: Screen shot of the decoy Analysis for the 38 kDa 'homodimer' band of αA-Crystallin fragment m/z 1286.540 obtained from StavroX 3.6.0.1. ("Blue bars represent the number of candidates from the real experimental set, while red bars represent the positive false candidates from the inverted sequence of the FASTA file. More enriched real data set candidates indicate toward better crosslinking.”).

**Figure 4:** The annotation spectrum with the extent of deviation and identified peaks for the fragment m/z 1286.640 of 38kDa 'homodimer' band of αA-Crystallin obtained from StavroX 3.6.0.1. "Less deviation in the annotation points toward better crosslinking".

**Table 2:** The intermolecularly crosslinked fragments with high scores identified by the software.

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**Figure 5:** Screen shot of the decoy Analysis for the 33 kDa 'heterodimer' band of αA-Crystallin and lysozyme fragment m/z 1290.597 obtained from StavroX 3.6.0.1. ("Blue bars represent the number of candidates from the real experimental set, while red bars represent the positive false candidates from the inverted sequence of the FASTA file. More enriched real data set candidates indicate toward better crosslinking.").
Table 3: Top ten intermolecularly crosslinked fragments as given by software StavroX 3.6.0.1.

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Table 4: The intermolecularly crosslinked fragments with high scores identified by the software.

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Conclusion

α-Crystallins have become important not only for understanding diseases of the human eye but are also being considered as therapeutics for treatment of other diseases. Instead of the whole protein the Alpha Crystallin Domain (ACD) or Mini Alpha-crystallin Chaperons (MACs) could themselves serve as therapeutics. These ACDs are conserved across most HSPs. It is thus important to understand the role of Crystallins in chaperoning mechanism and treatment/ cure and onset of other diseases. Crosslinking studies on αA-Crystallin have been carried out previously. However, most of these studies were restricted to use of homobifunctional crosslinkers, which could not elicit as much information about intermolecular crosslinking. The work described here deals with crosslinking of lysozyme, αA-Crystallin, and a 1:1 mixture of αA-Crystallin and lysozyme, using a small Aryl...
Azido-N-hydroxy succinimide (NHS) heterobifunctional crosslinker. Using a two-step protocol, i.e., an initial incubation step followed by photolysis (366 nm, 6W UV lamp), SDS-PAGE, excision of 'homo and hetero Dimer' bands, trypsinization, ESI-MS, MS/MS investigations and analysis of the MS data using StavroX 3.6.0.1, a bioinformatics software especially suited for identifying intermolecular crosslinking. Our results have identified many significant intermolecular crosslinks not previously identified. Many of these are in the ACD region and thus could be important for understanding the chaperoning mechanism of αA-Crystallin and cataractogenesis. The new crosslinker could also find application for treating keratoconus, a disease affecting the human cornea [67].

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Conflict of Interest

The authors have no conflict of interest to declare.

Author Contributions


References


66. “Crosslinker for karetocorus” https://www.youtube.com/watch?v=sQHDWgEhQs