

**INSIGHTS INTO THE INTRICACY OF PROTEIN AGGREGATION IN
CROWDED ENVIRONMENTS**

SANDIP KARMAKAR



**DEPARTMENT OF CHEMISTRY
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**INSIGHTS INTO THE INTRICACY OF PROTEIN AGGREGATION IN
CROWDED ENVIRONMENTS**

by

SANDIP KARMAKAR

Department of Chemistry

Submitted

in fulfillment of the requirements of the degree of Doctor of Philosophy

to



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Dedicated to

My

Parents

CERTIFICATE

This is to certify that the thesis titled “**Insights into the Intricacy of Protein Aggregation in Crowded Environments**” being submitted by **Mr. Sandip Karmakar** to the Indian Institute of Technology Delhi for the award of degree of **Doctor of Philosophy** is a record of bonafide research work carried out by him. Mr. Sandip Karmakar has worked under my guidance and supervision and has fulfilled the requirements for the submission of this thesis, which to my knowledge has reached the requisite standard.

The results contained in this thesis are original and have not been submitted, in part or full, to any other University or Institute for the award of any other degree or diploma.

Prof. Pramit K. Chowdhury

Professor

Department of Chemistry

Indian Institute of Technology Delhi

Hauz Khas, New Delhi 110016, India

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ABSTRACT

The thesis entitled **“Insights into the Intricacy of Protein Aggregation in Crowded Environments”** describes the different ways of probing the effects of macromolecular crowding agents on different aspects of aggregation of bovine serum albumin (BSA), hen-egg white lysozyme (HEWL) and bovine β -lactoglobulin in varying aggregation conditions.

Chapter 1 entitled **“Introduction”** describes the basics of protein aggregation and the effects of various macromolecular crowding agents on the aggregation of different proteins. It also describes in detail the different ways of monitoring/probing protein aggregation using various extrinsic and intrinsic probes and employing different techniques including FRET, solvation dynamics, antibody dot assay etc.

Chapter 2 entitled **“Materials and Methods”** describes all the chemicals and experimental techniques used in this thesis for studying protein aggregation and related characterisation such as fluorescence spectroscopy, time-resolved fluorescence spectroscopy, circular dichroism spectroscopy, Raman spectroscopy, scanning electron microscopy etc. This chapter also describes in detail the protein labelling and purification processes.

Chapter 3 entitled **“Exploring the potency of the naturally occurring polyphenol curcumin to probe protein aggregation in crowded environments”** shows that the polyphenolic dye curcumin can also probe the aggregation of BSA in an effective manner similar that of the widely used aggregation probe thioflavin T. The distinct spectral profile of curcumin in response to the varying aggregation conditions is a further indication of the sensitivity of the same towards detection of aggregates. Moreover, curcumin was also found

to be highly effective in determining the underlying heterogeneity of the aggregation medium at any point of time even in highly crowded environments. SEM images of BSA aggregates formed in presence of different macromolecular crowding agents indicated that morphology of the aggregates became independent of the solvent conditions on prolonged incubation.

Chapter 4 entitled “Crowder induced structural modulation of a multi-domain protein during the early stages of aggregation: A FRET-based and protein solvation study”

describes the conformation modulation of domains I & II and dynamics of domain I in particular of the multi-domain protein BSA in the early stages of its aggregation in buffer and in presence of macromolecular crowding agents. Conformational modulations of BSA at the early stages of its aggregation were monitored by measuring Förster resonance energy transfer (FRET) between the donor tryptophans (W134 & W213) and acceptor IAEDANS, the latter being labelled at the free –SH group of Cys34 at domain I of BSA. Domain I dynamics of BSA at the early stages of aggregation was also followed with the help of solvation dynamics, for which the same free –SH group of Cys34 was labelled with another dye BADAN. FRET measurements showed that there was an initial compaction in the domain I size during the early time points of aggregation followed by an increase in the distance between the donor-acceptor pair implying elongation of the same. Analysis of the solvent correlation function of BADAN labelled BSA revealed that the domain I became rigid initially subsequent to which the domain lost its rigidity gradually with concomitant increase in the flexibility.

Chapter 5 entitled “Is the effect of macromolecular crowding on protein aggregation universal in nature? A study to unveil the complex nature of crowding” describes the effect of macromolecular crowding agents of different size, shape and molecular weight on the

aggregation properties of two proteins, lysozyme and β -lactoglobulin having different structural dispositions. Lysozyme is mostly α -helical while β -lactoglobulin is rich in β -sheets. Lysozyme followed nucleation dependent polymerisation irrespective of the nature and concentration of crowding agents. On the other hand the kinetic profiles of the aggregation of β -lactoglobulin were found to be dependent on the nature and concentration of the crowding agents. The switch from the nucleated to non-nucleated polymerisation of β -lactoglobulin aggregation kinetic profile in presence of different macromolecular crowding agents shed light into the crossover from the soft interactions to the excluded volume effects of the crowding agents. Secondary structural evolution during aggregation showed that lysozyme accumulated β -sheet structure at the later time points of incubation. However, β -lactoglobulin formed intermediates that were rich in α -helical content which subsequently got transformed into disordered structure. SEM studies suggest the presence of multiple aggregation pathways co-existing together for both the proteins thereby forming aggregates with different morphology in presence of different macromolecular crowding agents. These results therefore suggest that the effects macromolecular crowding agents are rather protein dependent as opposed to being universal in nature.

Chapter 6 entitled “**Conclusions and Future Prospects**” include the conclusions drawn from chapter 3 to chapter 5 and the future scope of the work reported in this thesis.

सार

"भीड़ भरे वातावरण में प्रोटीन एकत्रीकरण की गहनता में अंतर्दृष्टि" शीर्षक थीसिस अलग-अलग एकत्रीकरण की स्थिति में गोजातीय सीरम एल्ब्यूमिन (बीएसए), लाइसोजाइम और बीटा-लैक्टोग्लोब्युलिन के एकत्रीकरण के विभिन्न पहलुओं पर मैक्रोमोलेक्यूलर भीड़ एजेंटों के प्रभाव की जांच करने के विभिन्न तरीकों का वर्णन करता है।

अध्याय १ "परिचय" हकदार प्रोटीन एकत्रीकरण की मूल बातें और विभिन्न प्रोटीनों के एकत्रीकरण पर विभिन्न मैक्रोमोलेक्यूलर भीड़ एजेंटों के प्रभाव का वर्णन करता है। यह विभिन्न बाहरी और आंतरिक जांचों का उपयोग करके प्रोटीन एकत्रीकरण की निगरानी / जांच करने के विभिन्न तरीकों का विस्तार से वर्णन करता है, जिसमें एफआरईटी, सॉल्वेशन डायनेमिक्स, एंटीबॉडी डॉट परख आदि सहित विभिन्न तकनीकों को नियोजित किया जाता है।

अध्याय २ का शीर्षक "सामग्री और विधियाँ" इस थीसिस में प्रोटीन एकत्रीकरण और संबंधित लक्षण वर्णन जैसे प्रतिदीप्ति स्पेक्ट्रोस्कोपी, समय-हल प्रतिदीप्ति स्पेक्ट्रोस्कोपी, परिपत्र द्विवर्णता स्पेक्ट्रोस्कोपी, रमन स्पेक्ट्रोस्कोपी, स्कैनिंग इलेक्ट्रॉन माइक्रोस्कोपी आदि के लिए इस थीसिस में उपयोग किए गए सभी रसायनों और प्रयोगात्मक तकनीकों का वर्णन करता है। अध्याय में प्रोटीन लेबलिंग और शुद्धि प्रक्रियाओं के बारे में विस्तार से वर्णन किया गया है।

अध्याय ३ शीर्षक "भीड़ भरे वातावरण में प्रोटीन एकत्रीकरण की जांच करने के लिए स्वाभाविक रूप से होने वाली पॉलीफेनोल करक्यूमिन की शक्ति का पता लगाना" से पता चलता है कि पॉलीफेनोलिक डाई करक्यूमिन भी बीएसए के एकत्रीकरण की प्रभावी तरीके से जांच कर सकता है जो व्यापक रूप से उपयोग किए गए एकत्रीकरण जांच थियोफ्लेविन टी। अलग-अलग एकत्रीकरण की स्थिति के जवाब में करक्यूमिन की अलग वर्णक्रमीय प्रोफाइल समुच्चय का पता लगाने की दिशा में उसी की संवेदनशीलता का एक और

संकेत है। इसके अलावा, करक्यूमिन को अत्यधिक भीड़ भरे वातावरणों में भी किसी भी समय एकत्रीकरण माध्यम की अंतर्निहित विषमता के निर्धारण में अत्यधिक प्रभावी पाया गया। विभिन्न मैक्रोमोलेक्यूलर भीड़ एजेंटों की उपस्थिति में गठित बीएसए समुच्चय की SEM छवियों ने संकेत दिया कि समुच्चय की आकृति विज्ञान लंबे समय तक ऊष्मायन पर विलायक स्थितियों से स्वतंत्र हो गई।

अध्याय ४ "एकत्रीकरण के शुरुआती चरणों के दौरान एक मल्टी-डोमेन प्रोटीन के क्राउड प्रेरित संरचनात्मक मॉड्यूलेशन: ए FRET-आधारित और प्रोटीन सॉल्वेशन अध्ययन" को डोमेन १ और २ के डोमेन मॉड्यूलेशन और विशेष रूप से मल्टी के डोमेन की गतिशीलता का वर्णन करता है। डोमेन प्रोटीन बीएसए बफर में अपने एकत्रीकरण के शुरुआती चरणों में और मैक्रोमोलेक्यूलर भीड़ एजेंटों की उपस्थिति में। इसके एकत्रीकरण के शुरुआती चरणों में बीएसए के संचलन संबंधी संयोजनों की निगरानी डोनर ट्रिप्टोफैन (डब्ल्यू-१३४ तथा डब्ल्यू-२१३) और स्वीकर्ता IAEDANS के बीच फोरस्टर रेज़ोनेंस एनर्जी ट्रांसफर (FRET) को मापकर की गई थी, जिसे बाद में डोमेन १ पर Cys34 के फ्री -SH ग्रुप में लेबल किया गया। बीएसए के। एकत्रीकरण के शुरुआती चरणों में बीएसए के डोमेन १ गतिकी का भी उत्कीर्णन गतिशीलता की मदद से किया गया था, जिसके लिए Cys34 के समान मुक्त-समूह को एक अन्य डाई BADAN के साथ लेबल किया गया था। एफआरईटी मापन से पता चला कि एकत्रीकरण के शुरुआती समय बिंदुओं के दौरान डोमेन १ आकार में एक प्रारंभिक संघनन था, जिसके बाद दाता-स्वीकर्ता जोड़ी के बीच की दूरी में वृद्धि के साथ उसी के बढ़ाव को लागू किया गया था। बीएसए द्वारा लेबल किए गए BADAN के विलायक सहसंबंध समारोह के विश्लेषण से पता चला कि डोमेन १ शुरू में कठोर हो गया था जिसके बाद डोमेन ने लचीलेपन में सहवर्ती वृद्धि के साथ धीरे-धीरे अपनी कठोरता खो दी।

अध्याय ५ शीर्षक "प्रकृति में प्रोटीन एकत्रीकरण सार्वभौमिक पर मैक्रोमोलेक्यूलर भीड़ का प्रभाव है? भीड़ की जटिल प्रकृति का अनावरण करने के लिए एक अध्ययन "दो प्रोटीन, लाइसोजाइम और बीटा-

लैक्टोग्लोबुलिन के एकत्रीकरण गुणों पर विभिन्न आकार, आकृति और आणविक भार के मैक्रोलेक्युलर कौलिंग एजेंटों के प्रभाव का वर्णन करता है। लाइसोजाइम मस्तिष्कीय अल्फा-पेचदार होता है जबकि बीटा-लैक्टोग्लोबुलिन बीटा-चादरों से भरपूर होता है। लाइसोजाइम प्रकृति और भीड़ एजेंटों की एकाग्रता के बावजूद केंद्रक निर्भर पोलीमराइजेशन का पालन किया। दूसरी ओर बीटा-लैक्टोग्लोबुलिन के एकत्रीकरण के गतिज प्रोफाइल को भीड़ एजेंटों की प्रकृति और एकाग्रता पर निर्भर पाया गया। विभिन्न मैक्रोमोलेक्युलर मुकुट एजेंटों की उपस्थिति में बीटा-लैक्टोग्लोबुलिन एकत्रीकरण काइनेटिक प्रोफाइल के गैर-न्यूक्लियेटेड पोलीमराइजेशन के लिए केन्द्रक से स्विच, भीड़ एजेंटों के बहिष्कृत मात्रा प्रभावों के लिए नरम बातचीत से क्रॉसओवर में प्रकाश बहाया। एकत्रीकरण के दौरान माध्यमिक संरचनात्मक विकास से पता चला कि ऊष्मायन के बाद के समय में लाइसोजाइम ने संरचना-शीट संरचना जमा की। हालांकि, बीटा-लैक्टोग्लोबुलिन ने मध्यवर्ती का गठन किया जो अल्फा-पेचदार सामग्री में समृद्ध थे जो बाद में अव्यवस्थित संरचना में बदल गए। एसईएम अध्ययन दोनों प्रोटीनों के लिए सह-विद्यमान एक साथ कई एकत्रीकरण मार्गों की उपस्थिति का सुझाव देता है, जिससे विभिन्न मैक्रोमोलेक्यूलर क्राउडिंग एजेंटों की उपस्थिति में विभिन्न आकृति विज्ञान के साथ समुच्चय बनता है। इन परिणामों का सुझाव है कि प्रभाव मैक्रोमोलेक्यूलर मुकुट एजेंटों प्रोटीन बल्कि प्रकृति में सार्वभौमिक होने के लिए विरोध के रूप में निर्भर हैं।

"निष्कर्ष और भविष्य की संभावनाएं" अध्याय ६ में अध्याय ३ से अध्याय ५ तक के निष्कर्ष और इस थीसिस में रिपोर्ट किए गए कार्य के भविष्य के दायरे शामिल हैं।

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