TOWARDS NOVEL APPROACHES FOR ENCOUNTERING AGGREGATION OF BIOLOGICALLY RELEVANT PROTEINS

SAURABH GAUTAM

DEPARTMENT OF CHEMISTRY

INDIAN INSTITUTE OF TECHNOLOGY DELHI

OCTOBER 2016

TOWARDS NOVEL APPROACHES FOR ENCOUNTERING AGGREGATION OF BIOLOGICALLY RELEVANT PROTEINS

by

SAURABH GAUTAM

Department of Chemistry

Submitted

in fulfilment of the requirements of the degree of Doctor of Philosophy

to the

INDIAN INSTITUTE OF TECHNOLOGY DELHI

OCTOBER 2016

“Knowledge can only be got in one way, the way of experience; there is no other way to know”

— Swami Vivekananda

CERTIFICATE

This is to certify that the thesis titled "**Towards Novel Approaches for Encountering Aggregation of Biologically Relevant Proteins**" being submitted by **Mr. Saurabh Gautam** 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. Saurabh Gautam 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.

Dr. Pramit K. Chowdhury
Associate Professor
Department of Chemistry
Indian Institute of Technology Delhi
Hauz Khas, New Delhi 110016, India

ACKNOWLEDGEMENTS

It is a great pleasure for me to write the acknowledgement for my Ph.D. thesis which arose out of years of research work carried out at IIT Delhi. As is the case with any accomplishment, I have also worked for this thesis with different people whose contribution in various ways deserves special mention. I feel honoured while conveying my gratitude to all of them in my humble acknowledgement.

In the first place I would like to express my special appreciation and thanks to my supervisor ***Dr. Pramit K. Chowdhury****, he has been a tremendous mentor for me. I would like to thank him for encouraging my research and for allowing me to grow as a researcher. His advice on both research as well as on my career have been priceless. Without his constant guidance, I could not have finished this thesis. I am highly indebted to him for providing help, support and all the opportunities not only during research but also during critical phase of my career and countless other times.*

I would also like to thank my research committee members, ***Dr. Nalin Pant, Dr. Shashank Deep*** *and* ***Dr. Bishwajit Kundu*** *(Kusuma School of biological Sciences, IIT Delhi) for serving as my committee members, and for their brilliant comments and suggestions.*

I would like to thank all the past and present ***Heads of the Department of Chemistry*** *at IIT Delhi for providing me necessary facilities required for the completion of this research work. I would also like to thank* ***DRC chairperson****, all the* ***faculty****, and* ***staff members*** *of the Department of Chemistry.*

I am thankful to ***Council of Scientific and Industrial Research (CSIR)*** *for the financial support provided to me during the course of research.*

This thesis would be incomplete without words of gratitude to all my lab members who helped me to make this thesis possible. To begin with, I would like to thank my labmates ***Dr. Ashima Malik, Jayanta, Saikat, Sanjib, Priyanka, Sandip, Tripti*** *and all the M.Sc., M.Tech. and project students. Their kind support and guidance have been of great value in this work.*

I would also like to thank the lab members of Dr. Shashank Deep and Dr. Bishwajit Kundu's groups for always being supportive and helpful.

I am also extremely grateful to ***Dr. Anurag Sharma*** *(Amity University, Gurgaon) and* ***Dr. Gulam Mohmad Rather*** *(Robert Wood Johnson Medical School, New Jersey) for their help during my early days at IIT Delhi.*

A line of appreciation of course goes to some of my dearest friends including ***Yogendra, Sumit, Satyendra*** *and all my batchmates for their invariable support and indispensable help in achieving this feat.*

I would also like to thank all the members of our own ***Ph.D. football club AAFC****. We always found time out of our research work and busy schedules to play football which always used to refresh us. This is the right moment to mention about my antagonizing (fans of FC Barcelona and Real Madrid are always rivals in football!!) but constructive discussions about football with* ***Soumen*** *and* ***Tanmoy****. These discussions always cheered me up.*

A special thanks to my family. Words cannot express how grateful I am to my ***mother-in law, father-in-law, mother, father*** *and* ***my siblings*** *for all of the sacrifices that you've made on my behalf. Your prayer for me was what sustained me thus far. At the end I would like to express appreciation to my beloved wife* ***Priyanka*** *who spent sleepless nights with me and was always my support in the moments when there was no one to answer my queries. My sincere apologies to those whom I could not mention due lack of space, but thank them equally.*

Saurabh Gautam

ABSTRACT

The thesis entitled "**Towards novel approaches for encountering aggregation of biologically relevant proteins**" describes various novel approaches for overcoming protein aggregation. Major focus has been on providing therapeutic alternatives for protein aggregation diseases such as Parkinson's disease wherein α-synuclein protein undergoes aggregation and forms amyloid fibrils in brain tissues. The thesis also describes about the strategies for refolding proteins using smart polymers from aggregates known as inclusion bodies formed during recombinant protein expression in research laboratories and industrial settings.

Chapter 1 (Introduction) introduces the problems and challenges in the field of protein aggregation and associated diseases being investigated by researchers globally and in the context of this thesis. The chapter also gives an account of the research work that has been carried out by various research groups to understand and overcome protein aggregation using various strategies. The chapter further discusses macromolecular crowding, that has also been shown to modulate protein aggregation.

Chapter 2 (Materials and Methods) describes various materials and methods used for carrying out experiments described in this thesis. The chapter also provides details on the expression, isolation and purification of α-synuclein and other recombinant proteins producing inclusion bodies. Different techniques and methodologies used for characterizing proteins and protein aggregates have been summarized.

Chapter 3 entitled "**β-Cyclodextrin and curcumin, a potent cocktail for disaggregating and/or inhibiting amyloids: A case study with α-synuclein**" describes the study on the influence of the combination of β-CD (β-cyclodextrin) and curcumin on the aggregation of α-synuclein. Aggregation of α-synuclein has been implicated in Parkinson's disease (PD). While many compounds are known to inhibit α-synuclein aggregation, dissolution of aggregates into their constituent monomers cannot be readily achieved. In this study, using a range of techniques, we have shown that an optimized cocktail of curcumin and β-cyclodextrin, at appreciably low concentrations, not only inhibited aggregation but also broke up the preformed aggregates almost completely. We propose that these compounds exhibit synergy in their action and thus provide us with the exciting prospect of working toward the development of a suitable drug candidate for prevention and treatment of PD.

Chapter 4 entitled "**Polyphenols in combination with β-cyclodextrin can inhibit and disaggregate α-synuclein amyloids under cell mimicking conditions: A promising**

therapeutic alternative" examines the effects of four naturally occurring polyphenols in combination with β-cyclodextrin (β-CD) on the aggregation of α-synuclein in the presence of macromolecular crowding agents. Parkinson's disease is characterized by the presence of insoluble and neurotoxic aggregates (amyloid fibrils) of an intrinsically disordered protein α-synuclein. Our results reveal that even at sub-stoichiometric concentrations of the individual components, the polyphenol–β-CD combination(s) not only inhibited the aggregation of the proteins but was also effective in disaggregating preformed fibrils. Curcumin was found to be the most efficient, followed by baicalein with (–)-epigallocatechin gallate and resveratrol coming in next, the latter two exhibiting very similar effects. Our results suggest that the efficiency of curcumin results from a balanced composition of the phenolic –OH groups, benzene rings and flexibility. The uniqueness of β-CD was reinforced by the observation that none of the other cyclodextrin variants [α-CD and HP-β-CD] used were as effective, in spite of these possessing better water solubility. Moreover, the fact that the combinations remained effective under conditions of macromolecular crowding suggests that these have the potential to be developed into viable drug compositions in the near future. MTT assays on cell viability independently confirmed this hypothesis wherein these combinations (and the polyphenols alone too) appreciably impeded the toxicity of the prefibrillar α-synuclein aggregates on the mouse neuroblastoma cell lines (N2a cells).

Chapter 5 entitled "**Anti-amyloidogenic effect of a chemical chaperone in combination with β-cyclodextrin and polyphenols: Further improving the polyphenol–β-CD combination**" shows the effect of an osmolyte on the modulation of protein aggregation in association with polyphenol–β-CD combination. The aim of this chapter was to further improve the polyphenol–β-CD combination by incorporating sucrose, a well known osmolyte. While sucrose by itself could only inhibit aggregation at a concentration of 500 mM, however in combination with polyphenol–β-CD mixture, its effective concentration came down to 3.5 mM. Sucrose (3.5 mM) with polyphenol–β-CD combination was highly effective in curbing the aggregation of α-synuclein. This study showed that the presence of sucrose is very important in the polyphenol–β-CD combination to enhance their effectiveness and overcome the shortcomings (e.g. high dosage requirement).

Chapter 6 entitled "**Examining the universality of the combination of polyphenols, β-CD and sucrose**" investigates the universality of the combinations of polyphenol–β-CD and polyphenol–β-CD–osmolyte in terms of their effectiveness on other proteins that have definite three dimensional structures in their native states. Studies were performed on two different globular proteins: β-lactoglobulin (from bovine milk) and lysozyme (from chicken

egg white). β-Lactoglobulin showed faster kinetics of aggregation with an increase in the concentration of all the crowding agents up to 200 g/L. On the contrary, lysozyme followed a reversed path and showed a decrease in the aggregation kinetics under similar conditions. The polyphenol–β-CD and osmolyte–polyphenol–β-CD combinations were able to inhibit the aggregation and disaggregate preformed aggregates of these two proteins. However, the concentration of these molecules required was different in case of different proteins. This study thus proves the potency of the concoction and strives to establish its overall effectiveness for protein aggregates in general.

Chapter 7 entitled "**Smart polymer mediated purification and recovery of active proteins from inclusion bodies**" discusses the effect of stimuli sensitive smart polymers as pseudochaperonins on the refolding various highly aggregation prone recombinant proteins forming inclusion bodies. Obtaining correctly folded proteins from inclusion bodies of recombinant proteins expressed in bacterial hosts requires solubilization with denaturants and a refolding step. Aggregation competes with the second step. Refolding of eight different proteins was carried out by precipitation with smart polymers. A high throughput refolding screen based upon fluorescence emission maximum around 340 nm (for correctly folded proteins) was developed to identify the suitable smart polymer. The proteins could be dissociated and recovered after the refolding step. The refolding could be scaled up and high refolding yields in the range of 8 mg/L (for CD4D12, the first two domains of human CD4) to 58 mg/L (for malETrx, thioredoxin fused with signal peptide of maltose binding protein) were obtained. The refolded proteins were characterized using fluorescence spectroscopy, circular dichroism (CD) spectroscopy, melting temperature (T_m) analyses, and surface hydrophobicity measurements using ANS (8-anilino1-naphthalene sulfonic acid) fluorescence. Biological activity assay for thioredoxin and fluorescence based assay in case of maltose binding protein (MBP) were also carried out to confirm correct refolding.

Chapter 8 entitled "**Conclusion and future prospects**" represents the conclusions drawn from all the studies mentioned above and the future prospects of the work carried out so far in this thesis. The studies described in this thesis have opened up opportunities for the follow up work which include: expanding the repertoire of polyphenols and encapsulators; understanding the mechanism of action of such combinations in greater detail; and use of other proteins related with neurodegenerative diseases for studying the effectiveness of such combinations.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES