

CONTROLLED DELIVERY OF PROTEIN VIA FOLIC ACID NANOPARTICLES

RAJAT GUPTA



**DEPARTMENT OF CHEMICAL ENGINEERING
INDIAN INSTITUTE OF TECHNOLOGY DELHI
APRIL 2018**

CONTROLLED DELIVERY OF PROTEIN VIA FOLIC ACID NANOPARTICLES

by

RAJAT GUPTA

DEPARTMENT OF CHEMICAL ENGINEERING

Submitted

in fulfilment of the requirements of the degree of doctor of philosophy

to the



Indian Institute of Technology Delhi

April 2018

**Dedicated to
My Parents**

CERTIFICATE

This is to certify that the thesis entitled, “**Controlled delivery of protein via folic acid nanoparticles**” submitted by **Mr. Rajat Gupta** to Indian Institute of Technology Delhi for the award of the degree of **Doctor of Philosophy** is a record of original bonafide research work carried out by him under my guidance and supervision. The thesis has reached the standards of fulfilling the requirements of the regulations of Indian Institute of Technology Delhi for awarding the degree. The research report and results presented in this thesis have not been submitted, in part or full, to any other university or institute for the award of any degree or diploma.

Date:
New Delhi

Prof. Rajesh Khanna
Professor,
Department of Chemical Engineering,
Indian Institute of Technology Delhi, India



Prof. Sanat Mohanty
Former Associate Professor,
Department of Chemical Engineering, Indian
Institute of Technology Delhi, India

ACKNOWLEDGMENTS

I wish to express my deepest appreciation to Prof. S. Mohanty (Supervisor) for his valuable guidance during my research work. Despite leaving IIT Delhi, he reviewed all the results, reports, journal papers and thesis progress from United States of America. His valuable suggestions and moral support helped me to improve my technical writing skills. I am grateful that his involvement in my research has provided the necessary facilities.

I would like to thank my other supervisor, Prof. R. Khanna for his constant motivation, inspiration and support to work on research project. The supervisor has helped me to complete the research goals of my project. Without his unconditional support, the reported work wasn't easy to finish within the five-year time frame. It was a fortunate and unforgettable experience to work under his reflective and revered guidance. His friendly nature and endless care were an effective tool to eradicate the hurdles during my work.

I am grateful to Prof S. Basu, former Head, Chemical Engineering Department, for providing me all the necessary facilities during the course of my work at IIT Delhi. The encouragement, critical reviews and important suggestions given by Prof. S. Gupta, Prof. A. Mittal, and Prof. A. S. Rathore are greatly acknowledged. I wish to convey my sincere thanks to Prof. A. Shukla, Prof. B. Kundu and Prof. A.K. Gupta for their encouragement and motivation towards the research work. I also wish to thank the other faculty members of the department for their support.

The acknowledgment is incomplete without recognition to Dr. Rahul Misra, Mr. Manish Lonare, Mr. Prasanta Kalita, Mrs. Mili Pathak, Mr. Omkar Patil, Ms. Ekta Jain and all the other colleagues with whom I have worked during my Ph.D. at IIT Delhi. I am grateful to my friends and colleagues of IIT Delhi, Dr. Jogender Singh, Dr. Jay Pandey, Dr. Kishore, Mr. Dinesh Attarde, Dr. Manish Jain, Dr. Tarak Mondal, Ms. Sonal, Mr. Tanmoy Patra and Mr. Chaitanya for their help as well as support during my research work.

I was fortunate to have an excellent work environment in Prof. S. Gupta's laboratory, at Chemical Engineering Department at IIT Delhi which facilitated my work to a great deal. I wish to thank department office staff at IIT Delhi for their constant support. I am highly thankful to Mr. Ramdev, Mr. Gaurav Jhakda and Mr. Dilbagh Singh for their constant help in every possible way to carry forward my research work. I wish to thank library management staff at IIT Delhi for providing all the necessary material (books, e-journals, e-books etc.) to carry forward my research work. I also wish to thank hostel management of Girnar House where I have spent a wonderful time. I am obliged by their hospitality.

I have no words to express motivation and inspiration given by my father and mother to me, their courage and patience for their unshakable stand during the period of studies. Their encouraging words and deeds through these tough physical and emotional times will always remain in my heart and soul. I also wish to say thanks to my elder brother Mr. Amit Gupta, elder sister Mrs. Arti Gupta and brother-in-law Mr. Pradeep Gupta for their help, support, and advice during the period.

Herewith, I would like to thank everyone, who have directly or indirectly supported me to the realization of this thesis. Last but not the least, a note of heartfelt devotion to almighty GOD, who has made me capable of accomplishing this acclivitous task.

New Delhi

Rajat Gupta

January 2018

ABSTRACT

Therapeutic proteins are useful for the treatment of different diseases such as cancer, diabetes, and immune disorders. The short life of proteins encourages the use of carriers for the delivery of therapeutic proteins. An efficient carrier should be capable enough to control the protein release. In addition, a controlled protein delivery system can also reduce the side effects and the high cost of the therapy. High encapsulation and controlled release of cancer and tuberculosis drug using folic acid (FA) as a nanocarrier encourage to develop a FA-based protein delivery system. The larger and complex structure of therapeutic proteins as compared to many other drugs motivate to investigate the FA nanoparticles for protein delivery application. Therefore, the feasibility of FA as a nanocarrier for controlled delivery of protein is explored in this project.

An understanding of the behaviour of FA self-assembly after addition of protein is essential to examine the applicability of FA carrier for the protein delivery. Hence, an investigation of FA self-assembly and FA-protein interaction was carried out before the synthesis of protein loaded FA nanoparticle. Proteins such as bovine serum albumin (BSA) and insulin are used for all experimental work in this project. Fluorescence emission study has been carried out to investigate the behaviour of protein in the presence of FA molecules. This study indicate about the associative interactions between the components in FA-protein mixture. Molecular dynamics simulations confirm FA-protein association and indicate that the aromatic rings in the structure of protein and FA are the probable sites of associative interactions between FA and protein. Ordered structure of FA has also been investigated in the presence of various guest compounds having aromatic moieties in their structure such as insulin, BSA and tryptophan. Studies using X-ray diffraction technique were able to show that FA self-assembly remains unaffected even after the addition of any guest compound.

After interaction studies, protein loaded FA nanoparticles were prepared using the emulsification process followed by crosslinking using ZnCl_2 salt. First, studies with FA particles loaded with model protein (BSA) has been carried out, then insulin (therapeutic protein) encapsulated FA particles have been prepared and characterized. The studies with model protein show that the size of the BSA loaded FA nanoparticles was in the range of 200 to 300 nm. These nanoparticles were further characterized by studying the release behaviour of BSA from FA formulations. The release study of BSA from FA nanoparticles was performed in 0.08% NaCl, 0.8% NaCl, and phosphate buffer saline. The quantification of BSA has been carried out using high-performance liquid chromatography during the release study. The release analysis suggests that more than 90% of the encapsulated BSA under the nanoparticles (having BSA loading as high as 57% (wt/wt)) is released within 48 hours of release study when phosphate buffer saline was used as release medium. Release results indicate that cation concentration in the release medium plays an important role in the release mechanism. Crosslinked cations on the nanoparticles are found to be the key parameters to control the protein release. Thus, FA nanoparticles were found to be an efficient carrier for protein encapsulation and controlled release with minimum drug loss.

Insulin-loaded folate nanoparticles were synthesized using the same synthesis process as in the case of BSA loaded FA nanoparticles. The particle size study shows that the size of these nanoparticles was in the range of 100 to 250 nm. The size of insulin-loaded nanoparticles remains smaller than BSA-loaded nanoparticles due to the smaller size of insulin as compared to that of BSA. More than 90% of insulin was encapsulated in these nanoparticles, with protein loading levels up to 73% (wt/wt) of the FA used during synthesis. Crosslinking salt and FA concentration are found to be the key parameters to control the insulin release. More than 95% of the total FA and protein was released in the release medium within 24 hours of the study.

A separate investigation of FA release along with insulin/BSA release from the nanoparticles reveals that the particles are formed through the folic acid-protein complex. The similarity between FA release pattern from BSA and insulin loaded FA nanoparticles indicate that the drivers responsible for protein loaded FA particle formation remain same irrespective of the protein structure. The insulin release results fitted well to reciprocal-powered-time model. The investigation of release rate from modeling reconfirms that the protein release from the particles depend on the FA molecules in the surrounding of protein. Thus, the release results from this project are sufficient to prove that controlled release of the protein is achievable through FA nanoparticles with minimum loss of protein.

सार

चिकित्सीय प्रोटीन विभिन्न रोगों जैसे कैंसर, मधुमेह, और प्रतिरक्षा विकारों के इलाज के लिए उपयोगी हैं। प्रोटीन का लघु जीवन उपचारात्मक प्रोटीन की डिलीवरी के लिए वाहकों के उपयोग को प्रोत्साहित करता है। प्रोटीन रिलीज को नियंत्रित करने के लिए एक कुशल वाहक पर्याप्त सक्षम होना चाहिए। इसके अलावा, एक नियंत्रित प्रोटीन वितरण प्रणाली दुष्प्रभावों और चिकित्सा की उच्च लागत को भी कम कर सकती है। एक नैनोकैरियर के रूप में फोलिक एसिड (एफए) का उपयोग करके कैंसर और तपेदिक दवा के उच्च encapsulation और नियंत्रित रिहाई एक एफए आधारित प्रोटीन वितरण प्रणाली विकसित करने के लिए प्रोत्साहित करते हैं। कई अन्य दवाओं की तुलना में चिकित्सकीय प्रोटीन की बड़ी और जटिल संरचना प्रोटीन वितरण अनुप्रयोग के लिए एफए नैनोकणों की जांच करने के लिए प्रेरित करती है। इसलिए, इस परियोजना में प्रोटीन की नियंत्रित डिलीवरी के लिए एफए की व्यवहार्यता को नैनोकैरियर के रूप में खोजा जाता है।

प्रोटीन वितरण के लिए एफए वाहक की प्रयोज्यता की जांच करने के लिए प्रोटीन के अतिरिक्त एफए स्व-असेंबली के व्यवहार की समझ आवश्यक है। इसलिए, एफए स्व-असेंबली और एफए-प्रोटीन परस्पर संपर्क की जांच प्रोटीन लोड एफए नैनोपार्टिकल के संश्लेषण से पहले की गई थी। इस परियोजना में सभी प्रयोगात्मक कार्यों के लिए बोवाइन सीरम एल्बुमिन (बीएसए) और इंसुलिन जैसे प्रोटीन का उपयोग किया जाता है। एफए अणुओं की उपस्थिति में प्रोटीन के व्यवहार की जांच के लिए प्रतिदीप्ति उत्सर्जन अध्ययन किया गया है। यह अध्ययन एफए-प्रोटीन मिश्रण में घटकों के बीच सहयोगी बातचीत के बारे में इंगित करता है। आण्विक गतिशीलता सिमुलेशन एफए-प्रोटीन एसोसिएशन की पुष्टि करते हैं और संकेत देते हैं कि प्रोटीन और एफए की संरचना में सुगंधित छल्ले एफए और प्रोटीन के बीच सहयोगी बातचीत की संभावित साइटें हैं। एफए की आदेशित संरचना की जांच इंसुलिन, बीएसए और ट्रायटोफान जैसी संरचना में सुगंधित moieties वाले विभिन्न अतिथि यौगिकों की उपस्थिति में भी की गई है। एक्स-रे विवर्तन तकनीक का उपयोग करने वाले अध्ययन यह दिखाने में सक्षम थे कि एफए स्व-असेंबली किसी भी अतिथि परिसर के अतिरिक्त होने के बावजूद अप्रभावित बनी हुई है।

बातचीत अध्ययन के बाद, प्रोटीन लोड एफए नैनोकणों को emulsification प्रक्रिया का उपयोग करके तैयार किया गया था जिसके बाद $ZnCl_2$ नमक का उपयोग कर क्रॉसलिंग किया गया था। सबसे पहले, मॉडल प्रोटीन (बीएसए) के साथ लोड किए गए एफए कणों के साथ अध्ययन किया गया है, फिर इंसुलिन (उपचारात्मक प्रोटीन) encapsulated एफए कण तैयार और विशेषता है। मॉडल प्रोटीन के साथ अध्ययन से पता चलता है कि बीएसए लोड किया गया एफए नैनोकणों का आकार 200 से 300 एनएम की सीमा में था। इन नैनोकणों को एफए फॉर्मूलेशन से बीएसए के रिलीज व्यवहार का अध्ययन करके आगे की विशेषता थी। एफए नैनोकणों से बीएसए का रिलीज अध्ययन 0.08% NaCl, 0.8% NaCl, और फॉस्फेट बफर नमकीन में किया गया था। रिलीज अध्ययन के दौरान उच्च प्रदर्शन तरल क्रोमैटोग्राफी का उपयोग करके बीएसए की मात्रा का प्रदर्शन किया गया है। रिलीज विश्लेषण से पता चलता है कि नैनोकणों के तहत 90% से अधिक encapsulated बीएसए (57% (wt / wt) के रूप में उच्च बीएसए लोडिंग होने के बाद) रिलीज अध्ययन के 48 घंटे के भीतर जारी किया जाता है जब फॉस्फेट बफर खारा रिलीज माध्यम के रूप में इस्तेमाल किया गया था। रिलीज के नतीजे बताते हैं कि रिलीज माध्यम में कैशन एकाग्रता रिलीज तंत्र में एक महत्वपूर्ण भूमिका निभाती है। नैनोकणों पर क्रॉसलिंकड केशन प्रोटीन रिलीज को नियंत्रित करने के लिए महत्वपूर्ण पैरामीटर पाए जाते हैं। इस प्रकार, एफए नैनोकणों को प्रोटीन encapsulation और न्यूनतम दवा हानि के साथ नियंत्रित रिलीज के लिए एक कुशल वाहक पाया गया था।

बीएसए लोड एफए नैनोकणों के मामले में इंसुलिन-लोड फोलेट नैनोकणों को समान संश्लेषण प्रक्रिया का उपयोग करके संश्लेषित किया गया था। कण आकार के अध्ययन से पता चलता है कि इन नैनोकणों का आकार 100 से 250 एनएम की सीमा में था। बीएसए की तुलना में इंसुलिन के छोटे आकार के कारण इंसुलिन-लोड नैनोकणों का आकार बीएसए-लोड नैनोकणों से छोटा रहता है। इन नैनोकणों में 90% से अधिक इंसुलिन encapsulated था, प्रोटीन लोडिंग के स्तर संश्लेषण के दौरान इस्तेमाल एफए के 73% (wt / wt) तक। क्रॉसलिंग नमक और एफए एकाग्रता इंसुलिन रिलीज को नियंत्रित करने के लिए महत्वपूर्ण पैरामीटर पाए जाते हैं। कुल एफए और प्रोटीन का 95% से अधिक अध्ययन के 24 घंटे के भीतर रिलीज माध्यम में जारी किया गया था।

नैनोकणों से इंसुलिन / बीएसए रिहाई के साथ एफए रिलीज की एक अलग जांच से पता चलता है कि कण फोलिक एसिड प्रोटीन परिसर के माध्यम से बनते हैं। बीएसए से एफए रिलीज पैटर्न और इंसुलिन लोड एफए नैनोकणों से उत्पन्न समानता से संकेत मिलता है कि प्रोटीन संरचना के बावजूद प्रोटीन लोड एफए कण गठन के लिए जिम्मेदार ड्राइवर समान रहते हैं। इंसुलिन रिलीज के परिणाम पारस्परिक-पावर-टाइम मॉडल के लिए अच्छी तरह से फिट किए गए। मॉडलिंग से रिलीज दर की जांच से पुष्टि होती है कि कणों से प्रोटीन रिहाई प्रोटीन के आसपास एफए अणुओं पर निर्भर करती है। इस प्रकार, इस परियोजना से रिलीज के परिणाम साबित करने के लिए पर्याप्त हैं कि प्रोटीन की नियंत्रित रिहाई प्रोटीन के न्यूनतम नुकसान के साथ एफए नैनोकणों के माध्यम से प्राप्त की जा सकती है।

TABLE OF CONTENT

Certificate	i
Acknowledgments	ii
Abstract	iv
Table of Content	vii
List of Figure	xiv
List of Tables	xx
Nomenclature	xxii
CHAPTER 1: INTRODUCTION	1
1.1 Problem statement	1
1.2 Motivation of research	2
1.3 Controlled drug delivery through chromonic molecules	3
1.4 FA as a chromonic compound for drug delivery	5
1.5 Interaction of FA with proteins	7
1.6 Folates as a vehicle for controlled drug delivery	8
1.7 Quantification of protein in the presence of FA	9
1.8 Aims & Objectives	10
1.9 Organization of thesis	11

CHAPTER 2: LITERATURE REVIEW	14
2.1 Introduction	14
2.2 Current status of therapeutic proteins	15
2.2.1 Cell expression systems	15
2.2.2 Protein production from yeast as well as fungi	17
2.2.3 Protein expression from bacteria	17
2.3 Different carriers in protein delivery applications	19
2.3.1 Lipid as a carrier	19
2.3.2 Polymer as a carrier	20
2.3.3 Gel as a carrier	22
2.3.4 Porous silica as a carrier	23
2.4 Status of nanoparticle strategies in drug delivery	24
2.5 Protein loading and encapsulation in nanoparticles	25
2.6 The role of particle size in drug delivery	25
2.7 Influence of surface properties of a nanocarrier	27
2.8 Controlled release of drug	28
2.9 Preparation methods and their effect on the particles	29
2.10 Application of FA in drug delivery	30
2.11 Synthesis of protein loaded nanoparticle	30

2.12 Summary	35
CHAPTER 3. INTERACTION OF FA WITH PROTEINS	36
3.1 Introduction	36
3.2 Materials and methods	37
3.2.1 Material used	37
3.2.2 Interaction study using Bradford assay	38
3.2.3 Fluorescence spectroscopy measurements	38
3.2.4 Simulation parameters	38
3.3. Results and discussion	39
3.3.1 Interaction of Bradford assay with BSA in the presence of FA	39
3.3.2 MD simulation to investigate FA-protein interaction at molecular level	40
3.3.3 Interaction between FA and Trp through RDF analysis	42
3.3.4 Interaction of Phe/Tyr with FA	47
3.3.5 Interaction between FA and Phe/Tyr through RDF analysis	48
3.3.6 Fluorescence emission analysis of FA-insulin mixture	52
3.4 Summary	57
CHAPTER 4: CHARACTERIZATION OF FA SELF-ASSEMBLY IN THE PRESENCE DRUG MOLECULES	58
4.1 Introduction	58

4.2 Materials and methods	58
4.2.1 Material used	58
4.2.2 pH study of FA solution	59
4.2.3 FA Self-assembly analysis	59
4.2.4 X-ray diffraction (XRD) measurements	59
4.2.5 Rheology study with MB dye	60
4.3 Result and discussion	60
4.3.1 Effect of pH on FA	60
4.3.2 Structural analysis of FA self-assembly	61
4.3.3 Impact on the crystallinity of FA after the addition of different additives	63
4.3.4 Rheological study of FA with MB	68
4.4 Summary	70
CHAPTER 5: SYNTHESIS AND CHARACTERIZATION OF PROTEIN	71
LOADED FA NANOPARTICLES	
5.1 Introduction	71
5.2 Materials and methods	72
5.2.1 Material used	72
5.2.2 HPLC measurements	72
5.2.3 Synthesis of BSA loaded nanoparticles	74

5.2.4 Cross-linking of nanoparticles	74
5.2.5 Synthesis of insulin-loaded nanoparticles	75
5.2.6 Size distribution study	76
5.2.7 Encapsulation efficiency and drug loss measurement	76
5.3 Results and discussion	77
5.3.1 Separation of FA and protein on HPLC	77
5.3.2 Drug loss analysis	79
5.3.3 Particle size study	80
5.4 Summary	85
CHAPTER 6: RELEASE ANALYSIS OF BSA AS A MODEL PROTEIN FROM FA NANOPARTICLES	85
6.1 Introduction	86
6.2 Materials and methods	86
6.2.1 Material used	86
6.2.2 Measurement of released protein concentration	86
6.3 Result and discussion	87
6.3.1 Mass balance over the particles	87
6.3.2 Impact of release medium on BSA release with time	88
6.3.3 Release behaviour of FA along with BSA	89

6.3.4 Effect of cross-linking salt concentration on protein release	89
6.4 Summary	95
CHAPTER 7: RELEASE ANALYSIS OF INSULIN FROM FA NANOPARTICLES	96
7.1 Introduction	96
7.2 Material and methods	97
7.2.1 Material used	97
7.2.2 Protein quantification using HPLC	97
7.2.3 Synthesis of nanoparticles	97
7.2.4 Release study	98
7.2.5 Model development for release kinetic analysis	98
7.3 Results and discussion	101
7.3.1 Material balance over the nanoparticles	101
7.3.2 Effect of ZnCl_2 loading on insulin release	102
7.3.3 Release behaviour of FA along with insulin	102
7.3.4 Comparison of FA and insulin release	104
7.3.5 Release behaviour with increase in FA concentration	105
7.3.6 Kinetic analysis of insulin release results	109
7.4 Summary	120

CHAPTER 8: CONCLUSIONS	121
References	124
LIST OF PUBLICATIONS	146
Bio-data	147

LIST OF FIGURES

Figure	Title	Page
1.1	Schematic diagram of concentration “peaks” observed in the blood after parenteral administration.	2
1.2	The structure of methyl orange dye.	3
1.3	The diagram of a stack in the chromonic liquid crystal and the disk having tetramer of chromonic compound	4
1.4	The structure of FA.	6
1.5	Randomly oriented stacks made by FA disks having encapsulated drug (on left-hand side) and diagram of single FA stack (on right-hand side).	7
2.1	Diagram of (a) well-distributed drug loaded inside the core of the particle (b) drug loaded on the surface of particle core (c) drug loaded inside the shell of the particle (d) drug loaded on the shell of the particle.	32
2.2	The structure of FA self-assembled stacks (blue bricks) and the red bricks are representing intercalated drug inside FA stacks.	33
2.3	Schematic diagram of FA nanoparticle synthesis procedure.	34
3.1	(a) UV-Vis spectra of FA and FA-BSA mixture in Bradford reagent (b) Variation in the peak intensity at 595 nm in the presence of mixture of BSA (at different concentrations), Bradford reagent with and without FA.	40

3.2	Positions of ten folate ions with (a) two Trp (b) five Trp and (c) ten Trp molecules (encircled) after simulation.	41
3.3	RDF curves from the results after simulation of 10 folate ions with 2, 5 and 10 molecules of Trp.	43
3.4	The chemical structure of indolicidin.	44
3.5	The position of (a) ten folate ions and one indolicidin molecules mixture (b) the space occupied by indolicidin (after removing all other molecules) in a 30 Å cubic cell.	44
3.6	The position of (a) ten folate ions and one indolicidin in the mixture (b) the space occupied by indolicidin (after removing all other molecules) in a 50 Å cubic cells.	45
3.7	RDF values evaluated between the carbon atom of the aromatic ring in Trp and the carbon atom in the aromatic ring of folate ion after simulation (a) in 30 Å cubic cell (b) in 50 Å cubic cell.	46
3.8	RDF values evaluated between the carbon atom of the aromatic ring of five Trp residues in indolicidin and the nitrogen atom of pterin ring in folate ions after simulation (a) in 30 Å cubic cell (b) in 50 Å cubic cell.	46
3.9	RDF values evaluated between (a) amino acid and water (b) Trp and water after simulation in 30 Å cubic cell.	47
3.10	The structure of (a) FA (b) Phe (c) Tyr.	49
3.11	Image shows the configuration of molecules after simulation of (a) two Phe molecules with folate (b) five Phe molecules with folate (c) ten Phe	50

	molecules with folate (d) two Tyr molecules with folate (e) five Tyr molecules with folate (f) ten Tyr molecules with folate.	
3.12	RDF value graph of 10 folate ions with 2, 5 and 10 molecules of (a) Phe (b) Tyr after simulation.	51
3.13	RDF curves in which RDF values were calculated between aromatic rings of (a) Phe and the aromatic ring of folate ion (b) Phe and pterin ring of folate ion (c) Tyr and the aromatic ring of folate ion (d) Tyr and pterin ring of folate ion after simulation.	52
3.14	Emission spectra of (a) mixture of insulin and FA after excitation at 257 nm (b) mixture of insulin and FA after excitation at 274 nm with the respective plot of F_0/F vs. concentration of quencher (FA) at 25 °C.	54
3.15	Emission spectra of (a) pure FA (b) pure insulin (c) mixture insulin and FA (with a plot F_0/F as a function of quencher (FA) concentration) after excitation at 280 nm at 25 °C.	56
4.1	The image shows the change in colour of FA solution from cloudy yellow to transparent brown with an increase in pH from left to right.	61
4.2	Variation in pH with concentration of FA at different molar ratios of FA:1N NaOH.	61
4.3	Probable aggregates of FA molecules and their internal dimensions.	63
4.4	XRD pattern of FA at different concentrations.	64

4.5	XRD profile of (a) FA-BSA mixture at 1:0.1, 1:0.2 and 1:0.3 ratio (FA: BSA (wt/wt)) ratio and (b) FA before and after addition of 1:0.1 (FA: Trp (wt/wt)).	65
4.6	XRD spectra of 1% (w/w) FA with (a) Phe and Tyr (b) insulin (c) MB.	67
4.7	Comparison of (a) 1% FA (b) 5% FA (c) 10% FA at different methylene blue (MB) ratios (wt/wt).	69
5.1	HPLC chromatogram of FA-BSA mixture.	77
5.2	HPLC chromatogram of (a) FA (b) FA-recombinant insulin mixture (c) FA-insulin lispro mixture (d) Insulin calibration curve.	79
5.3	Images of BSA loaded nanoparticles using (a)-(b) TEM, (c) SEM, (d) DLS.	83
5.4	Images of insulin-loaded nanoparticles using TEM image and DLS.	83
6.1	Released amount of BSA with time from 0.1% ZnCl ₂ cross-linked nanoparticles in (a) 0.08% NaCl (b) 0.8% NaCl (c) PBS.	90
6.2	Variation in the released fraction of (a) BSA (b) FA with time in PBS buffer from nanoparticles cross-linked with 0.1% ZnCl ₂ .	91
6.3	Variation in released fraction of (a) BSA (b) FA with time in PBS buffer from nanoparticles cross-linked with 1% ZnCl ₂ .	92
6.4	Variation in the ratio of FA fraction to BSA fraction released with time in PBS buffer from nanoparticles cross-linked with (a) 0.1% ZnCl ₂ (b) 1% ZnCl ₂ .	93

7.1	The amount of released insulin from nanoparticles (a) cross-linked with 0.1% ZnCl_2 (b) cross-linked with 1% ZnCl_2 at different loading of insulin (8.16 mg FA loaded) in PBS buffer.	103
7.2	The amount of released FA from nanoparticles (a) cross-linked with 0.1% ZnCl_2 (b) cross-linked with 1% ZnCl_2 in PBS buffer at different loading of insulin.	104
7.3	Released fraction of (a) insulin from 0.1% ZnCl_2 cross-linked particles (b) FA from 0.1% ZnCl_2 cross-linked particles (c) insulin from 1% ZnCl_2 cross-linked particles (d) FA from 1% ZnCl_2 cross-linked particles loaded with 8.16 mg FA.	107
7.4	Released fraction of (a) insulin (b) FA (c) insulin (d) FA released from 1% ZnCl_2 cross-linked nanoparticles loaded having 16.33 mg FA.	109
7.5	The graph between $\ln [F/(1-F)]$ vs. $\ln t$ for (a) 2 mg insulin loading (b) 3 mg insulin loading (c) 4 mg insulin loading (d) 5 mg insulin loading (e) 6 mg insulin loading in Set1	112
7.6	The graph between $\ln [F/(1-F)]$ vs. $\ln t$ for (a) 2 mg insulin loading (b) 3 mg insulin loading (c) 4 mg insulin loading (d) 5 mg insulin loading (e) 6 mg insulin loading in Set2.	114
7.7	The graph between $\ln [F/(1-F)]$ vs. $\ln t$ for (a) 2 mg insulin loading (b) 3 mg insulin loading (c) 4 mg insulin loading (d) 5 mg insulin loading (e) 6 mg insulin loading in Set3.	116
7.8	The slope of fitted model for each loading based on Method1 and Method2.	117

LIST OF TABLES

Table	Title	Page
1.1	Characterization techniques used for different analysis during various studies.	12
2.1	List of commercialized and under trial therapeutic proteins.	16
2.2	Different drug delivery strategies with their protein encapsulation and loading efficiency.	26
2.3	The role of FA in different drug delivery strategies.	31
5.1	Comparison of FA and BSA encapsulation efficiency in the supernatant at different BSA loading.	81
5.2	Particle size, FA and insulin encapsulation efficiency of insulin-loaded nanoparticles	82
5.3	Particle size of BSA loaded nanoparticles.	82
5.4	Comparison of drug loading and encapsulation efficiency with strategies available in the literature.	84
6.1	Total encapsulated FA and BSA which remain unaccounted during the release study from 0.1%/1% ZnCl ₂ cross-linked nanoparticles.	88
7.1	The average amount of insulin and FA obtained during encapsulation and release study in PBS from 0.1%/1% ZnCl ₂ cross-linked nanoparticles having 8.16 mg FA.	101
7.2	The evaluated value of time required to release 50% of the loaded insulin from the particles using Method1 and Method2 at each insulin loading level.	118

- 7.3 The evaluated value of time required to release 90% of the loaded insulin 118
from the particles using Method1 and Method2 at each insulin loading
level.

Nomenclature

Symbols

h	Hours
F_0	Fluorescence intensity of fluorophore without quencher
F	Fluorescence intensity of fluorophore with quencher
[Q]	Concentration of quencher
K	Binding constant (M^{-1})
Å	Angstrom (10^{-10} m)
θ	Angle (degree)
F	Fraction of protein released
K_1	Constant
x, y	Powers
t	Time

Acronyms

Arg	Arginine
BSA	Bovine serum albumin
CMC	Critical micelles concentration
DI	Deionised
DLS	Dynamic light scattering
E.coli	Escherichia coli

FA	Folic acid
FRET	Fluorescence resonance energy transfer
GI	Gastrointestinal
HPLC	High performance liquid chromatography
HPMC	Hydroxy Propyl Methyl Cellulose
HSA	Human serum albumin
Ile	Isoleucine
Leu	Leucine
Lys	Lysine
MD	Molecular dynamics
MB	Methylene blue
Phe	Phenylalanine
PBS	Phosphate buffer saline
PVA	Poly vinyl alcohol
Pro	Proline
RDF	Radial distribution function
TEM	Transmission electron microscopy
TFA	Trifluoroacetic acid
Trp	Tryptophan
Tyr	Tyrosine

XRD

X-Ray Diffraction