Exploring Therapeutic Approaches for Targeted Delivery of Cabazitaxel in Breast Cancer Therapy

Thesis submitted in partial fulfilment for the Award of Degree

Doctor of Philosophy

By

Abhishek Jha, M. Pharm

Department of Pharmaceutical Engineering & Technology
Indian Institute of Technology
(Banaras Hindu University)
Varanasi-221005, India

Roll No. 19161002 **Year: 2024**

Department of Pharmaceutical Engineering & Technology
Indian Institute of Technology
(Banaras Hindu University)
Varanasi-221005

CERTIFICATE

It is certified that the work contained in the thesis titled "**Exploring Therapeutic Approaches for Targeted Delivery of Cabazitaxel in Breast Cancer Therapy**" by **Mr. Abhishek Jha** has been carried out under my supervision and that this work has not been submitted elsewhere for a degree.

It is further certified that the student has fulfilled all the requirements of Comprehensive Examination, Candidacy and SOTA for the award of Ph.D. Degree.

Prof. Brahmeshwar Mishra
(Supervisor)
Dr. B. Mishra
Professor of Pharmaceutics
Department of Pharmaceutical
Engineering And Technology
Indian Institute of Technology
(Banaras Hindu University)
Varanasi-221005
Date: 27.03.2025
Place: IIT (BHU), Varanasi

Department of Pharmaceutical Engineering & Technology
Indian Institute of Technology
(Banaras Hindu University)
Varanasi-221005

DECLARATION BY THE CANDIDATE

I, **Abhishek Jha**, certify that the work embodied in this Ph.D. thesis is my own bonafide work and carried out by me under the supervision of **Prof. Brahmeshwar Mishra** from **July, 2019 to December, 2024** at the **Department of Pharmaceutical Engineering & Technology, Indian Institute of Technology (Banaras Hindu University), Varanasi.** The matter embodied in this Ph.D. thesis has not been submitted for the award of any other degree/diploma. I declare that I have faithfully acknowledged and given credit to the research workers wherever their works have been cited in my work in this thesis. I further declare that, I have not willfully copied any other's work, paragraphs, text, data, results, etc. reported in the journals, books, magazines, reports, dissertations, theses, etc., or available on websites and have not included them in this Ph.D. thesis and have not cited as my own work.

Date: 27.3.25
Place: IIT (BHU), Varanasi

Ajha
Abhishek Jha

CERTIFICATE BY THE SUPERVISOR(S) AND HEAD OF THE DEPARTMENT

It is certified that the above statement made by the student is correct to the best of our knowledge.

Prof. Brahmeshwar Mishra
(Supervisor)
Dr. B. Mishra
Professor of Pharmaceutics
Department of Pharmaceutical Engineering And Technology
Indian Institute of Technology
(Banaras Hindu University)
Varanasi-221005

27/3/25

Prof. Sairam Krishnamurthy
(Head of the Department)
विभागाध्यक्ष / Head
भैषजकीय अभियांत्रिकी एवं प्रौद्योगिकी विभाग /
Department of Pharmaceutical Engineering & Technology
भारतीय प्रौद्योगिकी संस्थान / INDIAN INSTITUTE OF TECHNOLOGY
(बनारस हिन्दू विश्वविद्यालय) / (BANARAS HINDU UNIVERSITY)
वाराणसी–२२१००५ / Varanasi-221005

Department of Pharmaceutical Engineering & Technology

Indian Institute of Technology
(Banaras Hindu University)
Varanasi-221005

COPYRIGHT TRANSFER CERTIFICATE

Title of the Thesis: Exploring Therapeutic Approaches for Targeted Delivery of Cabazitaxel in Breast Cancer Therapy

Candidate's Name: Mr. Abhishek Jha

Copyright Transfer

The undersigned hereby assigns to the Indian Institute of Technology (Banaras Hindu University), Varanasi all rights under copyright that may exist in and for the above thesis submitted for the award of the Ph.D. degree.

Date: 27.3.25

Place: IIT (BHU), Varanasi

Abhishek Jha

Note: However, the author may reproduce or authorize others to reproduce material extracted verbatim from the thesis or derivative of the thesis for author's personal use provided that the source and University's copyright notice are indicated.

Acknowledgement

I am deeply grateful to Bharat Ratna Pandit Madan Mohan Malviya ji for his unwavering dedication in establishing the esteemed institution, Banaras Hindu University. His tireless efforts have created this sacred space of learning, and I feel privileged to be a part of it.

I extend my sincere gratitude to my supervisor, Prof. Brahmeshwar Mishra, for his unwavering support and guidance throughout my Ph.D. research journey. His extensive knowledge, vast research experience, and invaluable suggestions have been instrumental in shaping the trajectory of my work. His insightful feedback challenged me to refine my thinking and elevate the quality of my research. I am truly thankful for the opportunity to be his student, and for the enduring faith he has shown in me over the years. I find it challenging to express adequately my appreciation for his role in shaping me into not only a better researcher but also a better person.

I extend my sincere thanks to Prof. Sairam Krishnamurthy, Head of the Department of Pharmaceutical Engineering & Technology, I.I.T. (B.H.U.), Varanasi, for his invaluable support in providing the necessary infrastructure facilities throughout the course of my work.

I would like to extend my sincere thanks to RPEC member Dr. Ruchi Chawla from the Department of Pharmaceutical Engineering & Technology), and Dr. Abha Mishra from the School of Biochemical Engineering. Their valuable input and constructive criticism have played a pivotal role in encouraging me to broaden the scope of my research from various perspectives.

I would like to thank Prof. M. S. Muthu, Dr. Ashish Agarwal, Dr. Shreyans K Jain, and Dr. Dinesh Kumar for proving access to their lab facilities. Also, Dr. Vinod Tiwari for providing the necessary resources and guidance to carry out in vivo experiments.

I extend my heartfelt thanks to all the esteemed faculty members of the department, including Prof. Sushil K Singh, Prof. Sushant K. Srivastava, Prof. S. Hemalatha (Former Head), Prof. A. Senthil Raja, Dr. Ashok Kumar, Dr. Alakh. N Sahu, Dr. Sunil. K. Mishra, Dr. Prasanta Kumar Nayak, Dr. Gyan Prakash Modi, Dr. Deepak Kumar, and Dr. Jairam Meena. Their unwavering support and encouragement have been instrumental during the progress of my research, and I am sincerely grateful for their kindness and guidance.

The support and the resources provided by Central Instrument Facility (CIF), IIT(BHU), Varanasi and Sophisticated Analytical & Technical Help Institute, Banaras Hindu University (SATHI-BHU) are gratefully acknowledged.

I pay sincere thanks to Mr Yashwant Singh, Mr Atul Gupta, Mr Anand and all the departmental non-teaching staff for their co-operation and help during my research work.

I am thankful to the Ministry of Education, GoI, for providing support in the form of a teaching assistantship.

I want to express my heartfelt thanks to my amazing friends. Your constant encouragement and support have played a crucial role in making this work a reality. Having friends like you by my side has made the journey not only memorable but also achievable.

I would like to extend my deepest gratitude to my family. Without their unwavering support, encouragement, and love, achieving this would not have been possible. Their belief in me has been my source of strength and motivation throughout this journey. Thank you for being my pillars of support and for making this achievement a reality.

Date: 27.3.25

Place: IIT(BHU) Varanasi

Jha

Abhishek Jha

Table of Contents

1 Introduction 2

1.1 Biopolymers for the design of tumor microenvironment responsive systems 3

2 Literature Review 6

2.1 Biopolymers with pH-responsive cleavage bonds 7

2.2 Biopolymers with pH-responsive protonated chemical groups 10

2.3 Biopolymers with Cleavable ROS responsive bond 10

2.4 Biopolymers with amphiphilicity transition-ROS responsive bond 13

2.5 Biopolymers with enzyme responsive bonds 16

2.6 Biopolymers with hypoxia activatable groups 18

2.7 Cleavable-bond based targeting 23

2.8 pH-dependent Protonation mediated targeting 26

2.9 Size dynamic targeting 27

2.10 Charge reversal targeting 27

2.11 Redox responsive nanomedicine 30

2.12 Enzyme responsive nanomedicine 33

2.13 Hypoxia responsive nanomedicine 35

2.14 Dual stimuli-responsive nanomedicine 37

2.15 Stimuli activated surface ligand-mediated targeting 38

2.16 Limitations 45

2.17 Carriers for localized delivery of TME-Responsive nanomedicine 46

3 Objectives and Plan of Work 50

3.1 Objectives 50

3.2 Plan of Work 50

3.2.1 Cetuximab functionalized chitosan/hyaluronic acid-based nanoparticles loaded with cabazitaxel for enhancing anti-tumor efficacy in DMBA-induced breast cancer model in rats through spatial targeting 50

3.2.2 Cabazitaxel-loaded redox-responsive nanocarrier based on D-alpha-tocopheryl-chitosan and hyaluronic acid loading for improved anti-tumor efficacy in DMBA-induced breast cancer model 51

3.2.3 Hyaluronic acid-oleylamine and chitosan-oleic acid conjugate-based hybrid nanoparticle delivery via. dissolving microneedles for enhanced treatment efficacy in localized breast cancer 52

4 Cetuximab functionalized chitosan/hyaluronic acid-based nanoparticles loaded with cabazitaxel to improve the anti-tumor efficacy in DMBA-induced breast cancer models in rats 54

4.1 Introduction 54

4.2 Materials and Methods 56

4.2.1 Materials 56

4.2.2 Synthesis and characterization of succinylated TPGS 57

4.2.3 Preparation of chitosan/hyaluronic acid-based nanoparticles 58

4.2.4 Physicochemical characterization of nanoparticles 60

4.2.5 Analytical method development 61

4.2.6 Encapsulation efficiency, drug loading, and degree of conjugation 61

4.2.7 Drug release study 62

4.2.8 In vitro cell line studies 63

4.2.9 In vivo studies in female Sprague Dawley rats 66

4.2.10 Statistics 67

4.3 Results and Discussion 67

4.3.1 Characterization of succinylated TPGS 67

4.3.2 Characterization of nanoparticles 79

4.3.3 Drug release study 86

4.3.4 In vitro assessment of nanoparticle efficacy 88

4.3.5 In vivo studies 96

5 Cabazitaxel-loaded redox-responsive nanocarrier based on D-alpha-tocopheryl-chitosan and hyaluronic acid for improved anti-tumor efficacy in DMBA-induced breast cancer model 106

5.1 Introduction 106

5.2 Materials and Methods 109

5.2.1 Materials 109

5.2.2 Synthesis and Characterization of TPGS-COOH and CSVE 110

5.2.3 Formulation of CSVE-based nanoparticles 111

5.2.4 Characterization of CSVE-based nanoparticles 112

5.2.5 Drug release Study 114

5.2.6 Stability of lyophilized nanoparticles 115

5.2.7 In vitro cell line studies 115

5.2.8 In vivo Studies 118

5.2.9 Statistical Analysis 121

5.3 Results and Discussion 121

5.3.1 Synthesis of TPGS-COOH and CS-VE 121

5.3.2 Formulation and characterization of CSVE-based nanoparticles 125

5.3.3 Drug release Study 133

5.3.4 In vitro Study 139
5.3.5 In vivo Studies 151
6 Hyaluronic acid-oleylamine and chitosan-oleic acid conjugate-based hybrid nanoparticle delivery via. dissolving microneedles for enhanced treatment efficacy in localized breast cancer 162
6.1 Introduction 162
6.2 Materials and methods 164
6.2.1 Materials required 164
6.2.2 Synthesis of CS-OA, HA-OA, and TPGS-COOH 165
6.2.3 Characterization of CS-OA, HA-OA, and TPGS-COOH 167
6.2.4 Synthesis of HA-OA/CS-OA based nanoparticles 167
6.2.5 Fabrication of Microneedle 169
6.2.6 Characterization of HA-OA/CS-OA based nanoparticles 171
6.2.7 In vitro drug release of HA-OA/CS-OA based nanoparticles 173
6.2.8 Cell culture studies 173
6.2.9 Evaluation of MN 175
6.2.10 In vivo efficacy: tumor regression, survival analysis, and histological studies 177
6.2.11 Statistical analysis 177
6.3 Results and discussion 178
6.3.1 Synthesis and characterization of CS-OA, HA-OA, and TPGS-COOH 178
6.3.2 Formulation and characterization of nanoparticles 182
6.3.3 In vitro drug release study 189
6.3.4 Cell culture studies 190

6.3.5 Fabrication and evaluation of dissolving MN 196

6.3.6 In vivo studies 201

7 Summary and conclusions 208

7.1 Future perspective 210

References 212

8. Appendices……256

List of Figures

Figure 1.1 Graphical illustration of the precise therapeutic delivery mechanism of biopolymer-based nanomedicine tailored to exploit tumor microenvironment stimuli.___ 4

Figure 2.1 Mechanism of pH-responsive behavior of Dimethylacrylamide-Trimethyl chitosan; At acidic pH, DMMA-TCM exhibits imine bond cleavage resulting in dissociation of dimethylacrylamide and Trimethyl-chitosan [2]**.** _____ 7

Figure 2.2 Synthetic route of N-succinyl-CS and Al-CS [4]. _____ 8

Figure 2.3 Synthesis of dual pH and redox-sensitive acrylic polymer conjugated thiolated chitosan [5]._____ 9

Figure 2.4 Synthesis of pH and redox-sensitive DOX/α-TOS-HMSN-TK-CMCH-GRP78P [15]**.**_____ 12

Figure 2.5 Synthesis of ROS sensitive amphiphilic Poly(propylene sulfide) grafted Chondroitin sulfate (ChS) polymer ChS-g-PPS [16]**.**_____ 14

Figure 2.6 Synthesis of ROS-sensitive thioether-bearing polymer and mechanism of ROS mediated increased hydrophilicity [10]**.** _____ 15

Figure 2.7 Synthesis of redox-sensitive and CD44 receptor-targeted HASF polymer [21]**.** _____ 16

Figure 2.8 Synthesis of hypoxia-sensitive nitroimidazole modified chitosan (Cs-NA) [31]**.** _____ 19

Figure 2.9 Synthesis of a 2-nitroimidazole modified carboxymethyl dextran by EDC/NHS mediated carbodiimide chemistry [33]**.** _____ 20

Figure 2.10 Synthesis of pH-sensitive adamantane-PEG _____ 24

Figure 4.1 (**A**) Conjugation of SA with TPGS. (**B**) Schematic illustration of preparation of CS-HA-NP and CS-HA-Cmab-NP. _____ 58

Figure 4.2 HPLC Chromatogram of Cabazitaxel (RT- 5.98 min) and Docetaxel (RT-11.23) 61

Figure 4.3 Succinylation of TPGS 68

Figure 4.4 (A) FTIR, **(B)** DSC, and **(C)** XRD spectra of TPGS and TPGS-COOH 68

Figure 4.5 ^{1}H NMR spectrum of TPGS Deuterated Methanol. 69

Figure 4.6 ^{1}H NMR spectrum of TPGS-COOH Deuterated Methanol. 69

Figure 4.7 ^{13}C NMR spectrum of TPGS Deuterated Methanol. 72

Figure 4.8 ^{13}C NMR spectrum of TPGS-COOH in Deuterated Methanol. 72

Figure 4.9 Mass spectra of TPGS at {m/z (Da) = 300-1200} 74

Figure 4.10 Mass spectra of TPGS at {m/z (Da) = 600-1800} 75

Figure 4.11 Mass spectra of TPGS-COOH at m/z (Da) in the range of 300-1200 76

Figure 4.12 Mass spectra of TPGS-COOH at m/z (Da) in the range of 600-1800 77

Figure 4.13 Morphological assessment of CS-HA-NP and CS-HA-Cmab-NP by TEM, SEM and AFM. 80

Figure 4.14 (A) FTIR and **(B)** DSC spectra of neat CBT, CS, HA, TPGS-COOH, CS-HA-NP, and CS-HA-Cmab-NP. 82

Figure 4.15 XRD spectra of neat CBT, CS, HA, TPGS-COOH, CS-HA-NP, and CS-HA-Cmab-NP. 83

Figure 4.16 XPS spectra of CS-HA-NP and CS-HA-Cmab-NP. 85

Figure 4.17 The drug release profile of CS-HA-NP and CS-HA-Cmab-NP in Phosphate Buffered Saline pH 7.4, Phosphate Buffer pH 6.8, and Acetate Buffer pH 5.5. Comparative graph of release profiles of CS-HA-NP and CS-HA-Cmab-NP at various pH. Data presented as Mean ± SD (vertical bars); n=6. 86

Figure 4.18 Normalized cell viability of bCS-HA-NP, Neat CBT, CS-HA-NP, and CS-HA-Cmab-NP at various concentrations calculated by MTT assay. Data presented as Mean ± SD (vertical bars); n=6. _____ 89

Figure 4.19 Microscopic images of cells imaged using fluorescence microscope after treatment with Free C6, c6CS-HA-NP, and c6CS-HA-Cmab-NP for 6 h. Cells were counter stained with DAPI. Scale bar = 100 µm. _____ 90

Figure 4.20 Hoechst33342/PI-stained MDA-MB-231 cells after 24 h treatment with Neat CBT, bCS-HA-NP, CS-HA-NP, CS-HA-Cmab-NP. The yellow and red arrows represent early apoptotic and late apoptotic cells respectively. Cells emitting blue fluorescence are Hoechst positive while those with red fluorescence are PI positive. Scale bar= 100 µm. 91

Figure 4.21 Microscopic images of JC-1-stained MDA-MB-231 cells after treatment with bCS-HA-NP, neat CBT, CS-HA-NP, and CS-HA-Cmab-NP. The control and bCS-HA-NP treated cells showed no signs of depolarization with cationic JC-1 binding to negatively charged polarized mitochondria to spontaneously form J-aggregates (red fluorescence). The CBT and CBT loaded nanoparticles show presence of JC-1 monomer (green fluorescence) due to depleted mitochondrial membrane potential **Scale bar = 100 µm**. 92

Figure 4.22 Images of MDA-MB-231 cells incubated with DCFH-DA after receiving various treatments for 24 h. The green fluorescence is due to the reduction of DCFH-DA to DCF in the presence of ROS (Scale bar = 400 µm). _____ 94

Figure 4.23 Cell cycle analysis; graphs showing population distribution of cells in different phases after incubation with various treatment for 24 h. _____ 95

Figure 4.24 Comparative Plasma concentration vs time curve of Neat CBT, CS-HA-NP, and CS-HA-Cmab-NP. Data presented as Mean ± SD (vertical bars); n=12._____ 97

Figure 4.25 Change in tumor volume in various treatment groups during 21-day treatment period. Data presented as Mean ± SD (vertical bars); n=5 _____ 98

Figure 4.26 Images of tumors collected from animals after 21-day treatment. In-image cm-scale on left side. _____99

Figure 4.27 Average body weight of various treatment groups during the 21-day treatment period. Data presented as Mean ± SD; n=5. *** (p-value < 0.001), **** (p-value < 0.0001), ns (p-value > 0.05)_____99

Figure 4.28 Kaplan Meier survival plot of various treatment groups (up to 120 days). _101

Figure 4.29 H&E-stained histological images of normal and tumor tissue after 21-day treatment period (images shown are captured with 4X lens). _____101

Figure 4.30 H&E-stained histological images of organs collected from various treatment groups after 21-day treatment period (images shown are captured with 4X lens). _____102

Figure 4.31 Graphical Summary of Objective 1 _____104

Figure 5.1 Schematic representation of Synthesis of CSVE **(A)** and TPGS-COOH **(B)**; Preparation of CSVE/HA-based redox responsive nanoparticles **(C)**. _____110

Figure 5.2 FTIR spectra of TPGS, TPGS-COOH, CSO, and CSVE. _____122

Figure 5.3 The ^{1}H NMR spectra of CSO (black) and CSVE (blue)._____123

Figure 5.4 The ^{13}C NMR spectra of CSO (black) and CSVE (blue). _____124

Figure 5.5 Morphological assessment (TEM, SEM, and AFM) of CSO/HA NP, CSVE/HA NP, CSVE/HA/DTPA NP, and CSVE/HA/DTPA/Cmab NP._____128

Figure 5.6 The FTIR spectra of CBT, CSVE, TPGS, HA, CSVE/HA NP, CSVE/HA/DTPA NP and CSVE/HA/DTPA/Cmab NP. _____129

Figure 5.7 The XRD spectra of CBT, CSVE, TPGS, HA, CSVE/HA NP, CSVE/HA/DTPA NP and CSVE/HA/DTPA/Cmab NP. _____130

Figure 5.8 The DSC spectra of CBT, CSVE, TPGS, HA, CSVE/HA NP, CSVE/HA/DTPA NP and CSVE/HA/DTPA/Cmab NP. _____132

Figure 5.9 The XPS spectra of CSO/HA NP, CSVE/HA NP, CSVE/HA/DTPA NP and CSVE/HA/DTPA/Cmab NP. 132

Figure 5.10 Drug release profile of CSVE/HA/DTPA NP and CSVE/HA/DTPA/Cmab NP in pH 7.4 phosphate buffer saline, pH 5.5 acetate buffer, and pH 5.5 acetate buffer and Glutathione (10 mM). 134

Figure 5.11 Time-dependent change in free thiol concentration in CSVE/HA/DTPA NP suspension after incubation with Acetate buffer (pH 5.5) and GSH 134

Figure 5.12 Effect of the storage of Lyophilized nanoparticles on Hydrodynamic size, PDI, Zeta Potential, and Entrapment Efficiency Data presented as Mean ± SD (vertical bars); n=6. ns (p-value > 0.05) 138

Figure 5.13 *In vitro* cell viability of MDA-MB-231 cells after treatment with Cabazitaxel-loaded formulations for 24 h. Data presented as Mean ± SD (vertical bars); n=6. 139

Figure 5.14 *In vitro* cell viability of HCT116 cells after treatment with Cabazitaxel-loaded formulations for 24 h. Data presented as Mean ± SD (vertical bars); n=6. 140

Figure 5.15 *In vitro* cell viability of T47D cells after treatment with Cabazitaxel-loaded formulations for 24 h (n=6). 141

Figure 5.16 Fluorescence microscopy images of MDA-MB-231 cells after 6 h incubation with the fluorescent CM6-loaded formulations. The blue fluorescence from DAPI channels shows Hoechst 33342 stained nuclei while the third column shows an overlay image of Hoechst and GFP. The fourth column represents an overlay image of phase contrast, GFP, and Hoechst 33342. Each scale bar represents 100 μm. GFP channel showing higher level of green fluorescence in CM6 loaded CSVE/HA/DTPA/Cmab NP treated cells compared to free CM6 and CM6-loaded CSVE/HA/DTPA NP treated cells due to receptor-mediated cellular uptake of CM6 in the cytoplasm. The CM6-loaded CSVE/HA NP also exhibited superior intracellular localization of CM6 compared to CM6-

loaded CSO/HA NP which may be attributed to the amphiphilic nature of the CSVE conjugate. The Cmab pretreated cells exhibited reduced uptake of CM6 which may be due to the competitive binding of Cmab to the EGFR. _____142

Figure 5.17 Quantitative assessment of intracellular uptake of green fluorescent CM6 in MDA-MB-231 cells treated with free CM6, CSO/HA NP, CSVE/HA NP, CSVE/HA/DTPA NP, CSVE/HA/DTPA/Cmab NP, Cmab pre-treated CSVE/HA/DTPA/Cmab NP at 0.2 μg ml^{-1} concentrations of CM6 for 6 h using flow cytometry. The blue peak shows the highest cellular uptake of CM6 in CM6-loaded CSVE/HA/DTPA/Cmab NP treated cells compared to free CM6, CSO/HA NP, CSVE/HA NP, CSVE/HA/DTPA NP, and CSVE/HA/DTPA/Cmab NP treated cells. _____143

Figure 5.18 Assessment of mitochondrial membrane potential by JC-1 dye in control and treated MDA-MB-231 cells after treatment with Cabazitaxel, CSVE/HA/DTPA NP, CSVE/HA/DTPA/Cmab NP, and Cmab pretreated CSVE/HA/DTPA/Cmab NP. The CSVE/HA/DTPA/Cmab NP treated group showed maximum depolarization (green fluorescence of JC-1 monomer) in cells. In contrast, the control group exhibited an accumulation of JC-1 monomers in negatively charged and energized normal mitochondria to spontaneously form J aggregates (Red fluorescence). _____144

Figure 5.19 Quantitative detection of mitochondrial membrane potential in MDA-MB-231 after treatment with the formulations through flow cytometry. The upper right and lower right quadrants represent cells with normal mitochondrial membrane potential and depolarized mitochondria respectively. _____145

Figure 5.20 Pattern of mitochondrial distribution in MDA-MB-231 cells using Mitotracker™ Red after treatment with Cabazitaxel, CSVE/HA/DTPA NP, CSVE/HA/DTPA/Cmab NP, and Cmab pretreated CSVE/HA/DTPA/Cmab NP. The uniform distribution of mitochondria was seen in control cells represented by the yellow

arrow while aggregated mitochondria were observed in the treatment group represented by the white arrows. The CSVE/HA/DTPA/Cmab treated cells showed the highest mitochondrial aggregation. _____ 147

Figure 5.21 Combined images of Hoechst33342/PI-stained MDA-MB-231 cells after treatment with the formulations. The yellow, white, and red arrows represent early apoptotic, late apoptotic, and necrotic cells. Cells emitting blue fluorescence are Hoechst positive while those with red fluorescence are PI positive. _____ 148

Figure 5.22 Quantitative assessment of apoptosis by AnnexinV/PI dual staining through Flow cytometry in treated MDA-MB-231 cells. AnnexinV/PI dual staining discriminates the percentage of live (lower left quadrant), early apoptotic (lower right quadrant), late apoptotic (upper right quadrant), and necrotic or dead cells (upper left quadrant). ____ 149

Figure 5.23 Concentration of Cabazitaxel in plasma, tumor, liver, lung, spleen and kidney after intravenous administration of Cabazitaxel, CSVE/HA/DTPA NP, and CSVE/HA/DTPA/Cmab NP. Data presented as Mean ± SD (vertical bars); n=12. ____ 153

Figure 5.24 Body weight and tumor volume of normal SD rats, tumor-bearing SD rats, and formulation treated tumor-bearing SD rats over the 28 days treatment period. Data presented as Mean ± SD (vertical bars); n=5._____ 155

Figure 5.25 Images of the tumors harvested from various treatment groups after end of 28-day treatment period. The line marked on the left side of tumor represents a cm-scale. _____ 155

Figure 5.26 Survival rates of tumor-bearing SD rats after treatment with formulations, day 0 represents the day of administration of first dose after the tumor volume reached ~ 500 mm^3. _____ 156

Figure 5.27 (A) Histological assessments of tumors isolated from rats after tumor regression study using H&E stain (magnification 4×; scale bar 100 μm) **(B)** Histological

images of organ tissues after 28 days of treatment regimen. Invasive carcinoma (IC), stromal tissue (ST), proliferated and expanded terminal lobular units (HLU), dilated ducts with inspissated secretions (D), Ductal Carcinoma (DC), Mucin (yellow arrow), Fibroadenoma (F) and breast hyperplasia (H). _____157

Figure 5.28 Graphical Summary of Objective 2 _____159

Figure 6.1 Schematic representation of the a) Succinylation of TPGS, b) synthesis of hyaluronic acid–oleylamine conjugate (HA-OA), and c) synthesis of chitosan–oleic acid conjugate (CS-OA). _____166

Figure 6.2 Schematic representation of the preparation of non-targeted HA-OA/CS-OA based nanoparticles (HA-OA/CS-OA NPT), targeted HA-OA/CS-OA based nanoparticles (HA-OA/CS-OA NPT), free Cabazitaxel drug loaded microneedles (CBT-MN) and targeted HA-OA/CS-OA based nanoparticles loaded microneedles (HA-OA/CS-OA NPT – MN). _____170

Figure 6.3 ^{1}H NMR spectra of Hyaluronic acid, oleylamine (OA), and HA-OA. _____179

Figure 6.4 ^{1}H NMR spectra of Chitosan (CSO), Oleic acid (OA), and CS-OA. _____180

Figure 6.5 FTIR spectrum of CS, CS-OA, HA, HA-OA, TPGS, and TPGS-COOH ___181

Figure 6.6 Characterization of HA-OA/CS-OA based hybrid nanoparticles; (**A)** TEM image of HA-OA/CS-OA NP (**B**) AFM image of HA-OA/CS-OA NP, (**C**) TEM image of HA-OA/CS-OA NPT, and (**D**) AFM image of HA-OA/CS-OA NPT. _____184

Figure 6.7 (A) FTIR and **(B)** XRD spectra of CBT, HA-OA/CS-OA NP, HA-OA/CS-OA NPT, and HA-OA/CS-OA NPT MN. _____185

Figure 6.8 (A) DSC thermogram of CBT, HA-OA/CS-OA NP, HA-OA/CS-OA NPT, and HA-OA/CS-OA NPT-MN. **(B)** Surface characterization by XPS analysis of HA-OA/CS-OA NP and HA-OA/CS-OA NPT. **(C)** *In vitro* drug release study of HA-OA/CS-OA NP and HA-OA/CS-OA NPT at pH 5.8 and pH 7.4. _____186

Figure 6.9 *In vitro* cytotoxicity of HA-OA/CS-OA NP and HA-OA/CS-OA NPT in MDA-MB 231 breast cancer cell line after 24 hours of treatment. Data presented as Mean __ 189

Figure 6.10 Fluorescence microscopy images of MDA-MB-231 cells after 6 hours incubation with free C6 and various HA-OA/CS-OA NP, HA-OA/CS-OA NPT, and Cmab-pretreated HA-OA/CS-OA NPT. GFP channel showing a higher level of green fluorescence in HA-OA/CS-OA NP and HA-OA/CS-OA NPT treated cells as compared to freeC6. Cmab-pretreated HA-OA/CS-OA NPT treatment showed diminished fluorescence than HA-OA/CS-OA NPT treatment. Images were captured through an inverted fluorescence microscope at 400X magnification and 100 µm scale bar. _____ 191

Figure 6.11 Combined microscopic images of Hoechst33342 / PI-stained MDA-MB-231 cells after 24 hours of treatment with free CBT, HA-OA/CS-OA NP, HA-OA/CS-OA NPT, and Cmab-pretreated HA-OA/CS-OA NPT. The white, yellow, and red arrows represent early apoptotic, late apoptotic, and necrotic cells. Cells emitting blue fluorescence (DAPI channel) are Hoechst positive while those with red fluorescence (PI channel) are PI positive._____ 192

Figure 6.12 The impact of free-CBT, HA-OA/CS-OA NP, and HA-OA/CS-OA NPT treatment on the mitochondrial membrane potential of MDA-MB-231 cells was assessed using JC-1 staining after 24 hours of treatment. The increase in green fluorescence emission indicates a decrease in mitochondrial membrane potential upon treatment, with the highest decline seen in HA-OA/CS-OA NPT-treated cells. Red fluorescence indicates normal potential. Cells were examined using an inverted fluorescence microscope at 400X magnification. _____ 194

Figure 6.13 Plot denotes the flow cytometric data of free-CBT, HA-OA/CS-OA NP, and HA-OA/CS-OA NPT treated MDA-MB-231 cells. PI staining was done to determine the percentage of cells in different phases of the cell cycle. HA-OA/CS-OA NPT treated cells

showed a significant (***p < 0.001) increase in cell population in the G2-M phase in comparison to the control. A significant difference between various treatment groups was also observed. Free-CBT and HA-OA/CS-OA NP (***p < 0.001), free-CBT and HA-OA/CS-OA NPT (***p < 0.001), and HA-OA/CS-OA NP and HA-OA/CS-OA NPT (**p < 0.01) was observed. 195

Figure 6.14 Photograph and Scanning Electron Microscopy (SEM) images of HA-OA/CS-OA NPT MN 196

Figure 6.15 Microscopic images of HA-OA/CS-OA NPT MN after exposure to PBS pH 7.4 and dissolution of microneedles in PBS pH 7.4 (calculated in terms of percentage therapeutic payload release w.r.t. time). 197

Figure 6.16 Images of parafilm layers after insertion of HA-OA/CS-OA NPT MN. The histogram represents the percentage of holes created in each layer of parafilm by microneedles after manual insertion 199

Figure 6.17 (**A**) Microscopic image of rat skin after HA-OA/CS-OA NPT MN insertion. (**B**) Graph representing ex vivo drug permeation across rat skin determined using a Franz diffusion cell. HA-OA/CS-OA NPT-MN showed significant improvement in drug permeation in comparison to CBT-MN and HA-OA/CS-OA NPT loaded PVA/HA blend (****p value < 0.001) **(C)** Representation of percentage drug retention in skin after 48 hours of drug permeation study. 200

Figure 6.18 (A) Tumor regression analysis in Tumor-bearing female SD rats; Tumor volume of control, CBT (i.v.), CBT-MN, and HA-OA/CS-OA NPT-MN treated groups during a 14-day treatment period. **(B)** Image of tumors collected from treatment groups after 14 days of treatment. 203

Figure 6.19 (A) Body weight of healthy female SD rats and tumor-bearing female SD rats receiving CBT (i.v.), CBT-MN, and HA-OA/CS-OA NPT-MN treatment for a 14-day

treatment regimen. **(B)** The Kaplan-Meier survival probability plot (120 days) of Healthy and tumor-bearing female SD rats treated with CBT (i.v.), CBT-MN, and HA-OA/CS-OA NPT-MN treated groups. 203

Figure 6.20 Histological images of H&E stained healthy/tumor tissues before and after 14-day treatment with CBT (i.v.), CBT-MN and HA-OA/CS-OA NPT-MN. 205

Figure 6.21 Graphical Abstract of Objective 3 206

List of Tables

Table 2.1 Biopolymer for tumor microenvironment responsiveness for site-specificity _21

Table 2.2 Targeted nanomedicine responsive to the tumor microenvironment and their drug release mechanisms _____39

Table 4.1 Composition of CS-HA-NP and CS-HA-Cmab-NP nanoparticles_____59

Table 4.2 Interpretation of ^{1}H NMR Spectra of TPGS and TPGS-COOH _____69

Table 4.3 Interpretation of ^{13}C NMR Spectra of TPGS and TPGS-COOH_____71

Table 4.4 Interpretation of the mass (TOF) spectra of TPGS and TPGS-COOH _____78

Table 4.5 Characterization of CS-HA-NP and CS-HA-Cmab-NP nanoparticles _____81

Table 4.6 XPS surface atomic percentage of N1s, O1s, C1s, S2p, and P2p in CS-HA-NP and CS-HA-Cmab-NP _____85

Table 4.7 Correlation coefficients of Cabazitaxel release profile from CS-HA-NP and CS-HA-Cmab-NP at various pH in various fitted release kinetic models _____87

Table 4.8 Pharmacokinetic parameters of Neat CBT, CS-HA-NP, and CS-HA-Cmab-NP after I.V. administration (6.5 mg/kg IV, n = 12) in female Sprague Dawley rats. _____96

Table 5.1 Composition, particle size, polydispersity index (PDI), zeta potential, and entrapment efficiency of various formulations. _____127

Table 5.2 R-square values for mathematical models to determine the goodness of fit. _137

Table 5.3 Pharmacokinetic parameters of various formulations determined from plasma concentration vs time plot _____151

Table 6.1 Composition and properties of various HA-OA/CS-OA NPs prepared during optimization _____168

Table 6.2 Composition of the various formulations used to prepare MNs _____169

Table 6.3 Particle size, PDI, zeta potential, EE, and DL of prepared nanoparticles.____183

Table 6.4 Fitted release kinetic model for in-vitro drug release of nanoparticles. _____188

LIST OF ABBREVIATIONS

EPR : Enhanced permeation and retention effect

i.v. : Intravenous

VES : Vitamin-E succinate

CSO : Chitosan oligosaccharide

CS : Chitosan

HA : Hyaluronic acid

NaTPP: Sodium tripolyphosphate

EDAC / EDC : 1-Ethyl-3-(3-Dimethylaminopropyl)carbodiimide Hydrochloride

NHS : N-hydroxysuccinimide

Sulfo-NHS: N-hydroxysulfosuccinimide

DTPA : 3,3`-dithiodipropionic acid

CBT : Cabazitaxel

PDI : Polydispersity Index

DLS : Dynamic Light Scattering

FTIR : Fourier Transform Infrared Spectroscopy

SEM : Scanning Electron Microscope

SPM : Scanning Probe Microscope

AFM : Atomic Force Microscope

HR-TEM: High-Resolution Transmission Electron Microscope

XRD : Powder X-ray diffraction

DSC : Differential Scanning Calorimetry

XPS : X-ray Photoelectron Spectroscopy

TPGS : Tocopherol polyethylene glycol succinate

PBS : Phosphate Buffer Saline

FBS : Fetal Bovine Serum

MTT : (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide)

DCFDA: 2′− 7′- dichlorodihydrofluorescein diacetate

JC-1 : 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimi- dazolylcarbocyanine iodide

PI : Propidium Iodide

DAPI : 4′,6-diamidino-2-phenylindole

DMBA: 7,12-Dimethylbenz[a]anthracene

H&E : Hematoxylin and eosin staining

AUC : Area Under Curve

MRT : Mean Residence Time

GSH : Glutathione

ROS : Reactive Oxygen Species

IC_{50} : Half maximal inhibitory concentration

C6 : Coumarin-6

Cmab : Cetuximab

Preface

Cancer remains one of the leading causes of mortality worldwide, posing significant challenges to healthcare systems and researchers alike. Despite numerous advancements in cancer therapy, the effective delivery of therapeutic molecules to cancer cells remains a significant hurdle. Traditional chemotherapeutics often suffer from poor aqueous solubility and limited availability at the intended region. Recognizing these challenges, researchers are delving into developing tumor microenvironment (TME)--responsive nanomedicine as a promising strategy for breast cancer therapy. This approach leverages the unique characteristics of the TME, such as lower pH, reactive oxygen species (ROS), redox imbalance, hypoxia, and enzymatic activity, to achieve targeted and controlled drug release, thereby enhancing treatment efficacy while minimizing systemic toxicity.

Chapter 1 provides an introduction to the biopolymer-based therapeutic approaches for targeted delivery of chemotherapeutics in breast cancer.

Chapter 2 comprehensively discusses various strategies employed in recent years to develop targeted therapeutic carriers that selectively deliver the drugs to cancerous cells.

Chapter 3 outlines the objectives and plan of work.

Chapter 4 focuses on preparing and characterizing HA-stabilized chitosan (CS) nanoparticles using the ionic gelation method. These nanoparticles were designed to entrap cabazitaxel (CBT), a potent chemotherapeutic agent, and were functionalized with Cetuximab (Cmab) to target epidermal growth factor receptors (EGFR) overexpressed on cancer cells. The nanoparticles were evaluated for various physicochemical characteristics, followed by *in vitro* and *in vivo* evaluation. *In vitro* studies demonstrated enhanced cytotoxicity, cellular uptake, and cellular responses, including mitochondrial depolarization and nuclear condensation, due to receptor-mediated endocytosis. The nanoparticles also showed improved pharmacokinetic performance and in vivo anti-tumor

efficacy, with minimal organ toxicity, highlighting their potential as effective drug carriers for targeted cancer therapy.

Chapter 5 covers the synthesis of chitosan oligosaccharide and vitamin E succinate conjugate, its evaluation, and its application in the development of a novel GSH-responsive Cetuximab-anchored chitosan/vitamin-E succinate conjugate-based hybrid nanoparticle. The chapter subsequently covers the characterization and evaluation of these spherical, sub-200 nm, cationic, and redox-responsive nanoparticles. *In vitro* evaluations showed significantly higher cytotoxicity against MDA-MB-231 cells, enhanced cellular uptake, and reduced mitochondrial membrane potential compared to free CBT. The *in vivo* pharmacokinetic studies indicated better performance of the nanoparticles, with improved half-life, area under the curve (AUC), and mean residence time (MRT). Further, the chapter discussed the tumor regression, survival, and histological data confirming these nanoparticles' efficacy and safety, demonstrating their potential as a targeted anticancer drug delivery platform.

Chapter 6 delves into the fabrication of targeted nanoparticles-loaded microneedles for localized drug delivery of cabazitaxel in DMBA-induced tumor-bearing SD rats. The article focuses on developing chitosan and hyaluronic acid-based amphiphilic conjugates with similar hydrophobic fragments. The fabrication of deformable HA-OA/CS-OA-based nanoparticles and loading of these particles in PVA/HA-based dissolving microneedles, followed by its *in vitro* and *in vivo* evaluation, forms the basic premise of this chapter.

Chapter 7 outlines the summary, conclusions, shortfalls, and future perspectives of all the objectives undertaken.

Chapter 8 lists all the references utilized to conduct this research.

Appendices provide a list of key publications from the work done during the doctoral research, and the first page of the publications from the work discussed in the thesis has been included.