Summary of the Thesis

Synthesis of Nanocarriers for Stimuli responsive Drug Delivery Applications

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By

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The thesis is divided into 5 chapters

Chapter I: Introduction

Drug delivery systems are associated with administration of drug at the site of malady in appropriate concentration to exert the desired therapeutic effect. However easier said than done, this process is much more complicated as bottleneck lies in the prediction of release rates of drug as well as their stability and targeting the specific cells/tissues. Overcoming these limitations has been the focus of the researchers working in this field by devising various strategies to produce efficacious drug delivery systems (DDS). The DDS are now synthesized in such a way that the formulations are stable in-vivo and the drug release is made more effective by controlling the release thus localizing their effects. The DDS are designed to suit not only the physicochemical features of the drug but also to intended administration mode.

Amongst various diseases that need interventions via employing a drug delivery system for its management, cancer holds a specific priority. This is due to a constant surge in cancer cases globally despite availability of various facets for its management. Drug delivery systems can be efficacious over conventional treatment strategies as they can be designed to cause the reversal of the disease by employing tumor specific mechanisms.

The detailed insight of the structural constitution of tumor microenvironment leads to the conclusion that various properties can be exploited to devise therapeutic strategies for effective cancer therapy. For instance the vascular irregularities can allow the passage of nanoparticles.

The revelation of the fact that, nanoparticles have the ability to penetrate the leaky vasculature had led to the exploration of the employment of nanotechnology for drug delivery applications.

Nanotechnology has played a crucial role in this field as a result of its reach in resolving the following mentioned pressing issues:

1. **An improvement in the delivery of drug having poor water-solubility and thus poor bioavailability.**
2. **The potential of delivering drugs to specific cells/organs in a targeted manner.**

3. **The transport of drugs across tight epithelial and endothelial barriers via transcytosis.**
4. **The delivery of macromolecular drugs to site of action at the cellular level.**
5. **The ability of combinatorial therapy via co-delivery of more than one drug or therapeutic modalities.**
6. **The functionalization of delivery system with probes capable of imaging allowing visualization of drug delivery site.**
7. **A real-time readability of the therapeutic efficacy *in vivo.***

These features make the nanocarriers promising candidates for both diagnostic and therapeutic interventions especially for disease like cancer. Various nanomaterials with varying compositions, shapes, sizes, and functionalities have been developed and shown to have application as therapeutic agents and theranostic. Various parameters that are of importance while designing nanocarriers for drug delivery include its morphology, size, type of functionalization, biocompatibility, toxicity, and biodegradability.

Stimuli responsive smart polymers have become increasingly important for designing such systems as they are known to provide greater control over drug dosage as well as targeting. Stimuli responsive drug carriers are those "smart materials" that can respond to the external changes in environment. Several such systems are reported in literature which are made sensitive to various stimuli such as pH, light, temperature, magnetic field, enzymes, redox potentials etc (as shown **in figure 1**).

Such systems can be developed by various strategies, for instance by combination of biocompatible biomaterials with appropriately functionalized materials which can self-assemble or form supramolecular complexes. The carrier thus produced is able to transport therapeutic molecules to specific tissues depending on the abnormal physiological conditions that they demonstrate.

This strategy can be employed for a targeted and sustained drug release to improve the specificity of drug delivery systems. Various categories of stimuli responsive nanocarriers that have been employed for cancer therapy include drug nanoconjugates, liposomes, polymersomes, micelles, liquid crystals, niosomes and dendrimers.

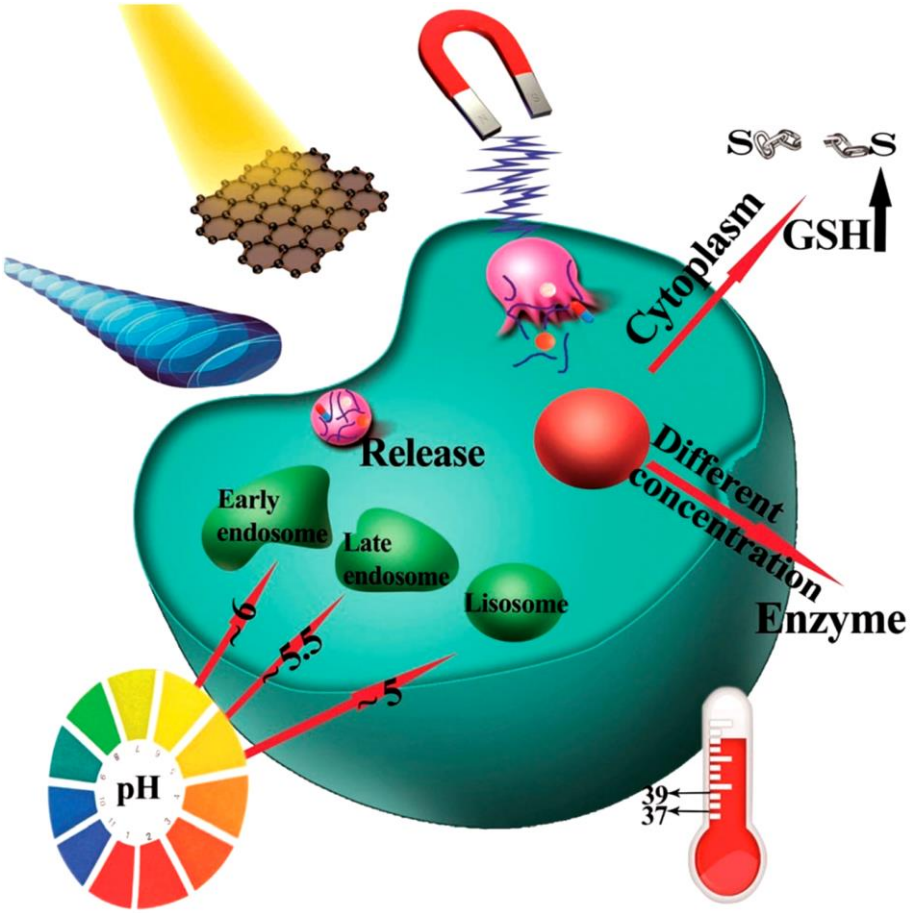


Figure 1: Various stimuli that can be exploited for triggering smart nanocarriers to exhibit a specific and controlled delivery of drugs for cancer therapy. (Adapted from Karimi et. al., *Chem. Soc. Rev.*, 2016, *45*, 1457-1501)

1. Surface functionalized multifunctional nanoconjugates

Nanoconjugates are tailored macrostructures harbouring covalently-bound biologically active modules like drugs that target specific tissues and cells. They are generally derived by surface functionalization of magnetic nanoparticles, graphene, mesoporous silica, carbon nanotubes, carbon or gold nanoparticles.

2. Amphiphilic polymeric nanoassemblies.

Polymersomes like vesicular and micellar assemblies, inspired by liposomes are promising drug carriers since they resemble the structure of the cell membrane. Supramolecular interaction using dynamic covalent bonds can help to construct the vesicles/micelles. Self-

assembled amphiphilic polymers are capable of loading both water insoluble and water soluble anticancer drugs simultaneously in the hydrophobic layer and hydrophilic core respectively for combinatorial therapy. Stimuli-responsive characteristic can be introduced by suitable chemical modifications.

3. Self-therapeutic nanocarriers:

The self-therapeutic nanocarriers are pro-drug nanoassemblies formed by self-assembly of rationally synthesized block co-polymers which disassemble eventually releasing the therapeutic in its active form.

A development of multidrug resistance and lack of targeted drug delivery are major challenges in cancer management that has withdrawn the focus of researchers. Targeted delivery of chemotherapeutic drugs specifically towards cancer cells is one of the apparent solutions to overcome the limitations of selectivity.

Chapter 2: β-cyclodextrin based dual-responsive multifunctional nanotheranostics for cancer cell targeting and dual drug delivery.

Drug resistance and mutations induced by drugs can be overcome by adapting to combination chemotherapy wherein combination of two or more drugs is employed to enhance efficacy of the therapy. Thus for the researchers associated with development of drug delivery vehicles, it is imperative to design an efficient system capable of targeting multiple drugs specifically to tumor site. This approach can assist in improving the therapeutic efficacy along with a reduction in side effects. Bearing this fact in mind, multifunctional nanoconjugates possessing an assortment of various functionalities such as magnetism, florescence, cell-targeting, pH and thermo-responsive features were developed for dual drug delivery **(figure 2).**

The novelty of this work lies in a careful conjugation of each of the functionality with magnetic iron oxide nanoparticles by virtue of urethane linkages in a simple one pot synthetic approach. β-cyclodextrin (CD), an oligosaccharide having amphiphilic properties was utilized to carry hydrophobic as well as hydrophilic drug. The ultimate goal is simplified synthesis of multifunctional nanoconjugates with therapeutic and diagnostic capabilities equipped with features for targeted stimuli responsive release of multiple drugs with following features:

1. Several β-cyclodextrin (CD) units to carry high payload of both hydrophilic and hydrophobic anticancer drugs and dual delivery for use in combination chemotherapy.

2. CD modified by Maleic anhydride and Poly (N-Isopropylacrylamide) (NIPAM) for pH and thermo-responsive drug release.

3. Fluorescein for monitoring cellular uptake.

4. Folic acid for targeted drug delivery.

5. Superparamagnetism for control of intracellular movements for final clearance from the body.

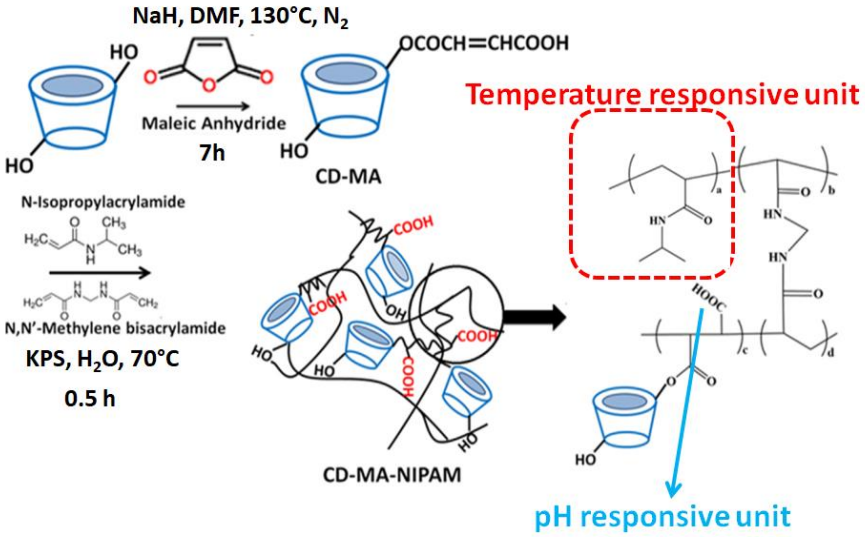


Figure 2: Schematic for synthesis of B-cyclodextrin derived pH thermal dual responsive polymer

The synthesized nanocarriers were characterized as shown in **table 1**.

Table 1: Various properties of the carriers assessed via different techniques

Properties	**Technique/Instrument**
Structural Elucidation	IR Spectroscopy, EDAX
Morphology and Size	SEM, HRTEM, DLS
Optical	UV-Vis & Fluorescence Spectroscopy
LCST of thermoresponsive polymer	Variable Temperature DLS
Thermal	TGA
Magnetic	VSM, Magnetic Hyperthermia experiments
Drug Loading and Release	UV-vis Spectroscopy
Preclinical Evaluation	*In-vitro* and *In-vivo* studies

Curcumin and DOX.HCl were used as model drugs to assess the dual drug delivery by the nanoconjugates **(figure 3).** The carriers demonstrated stimuli responsive release of both the drugs.

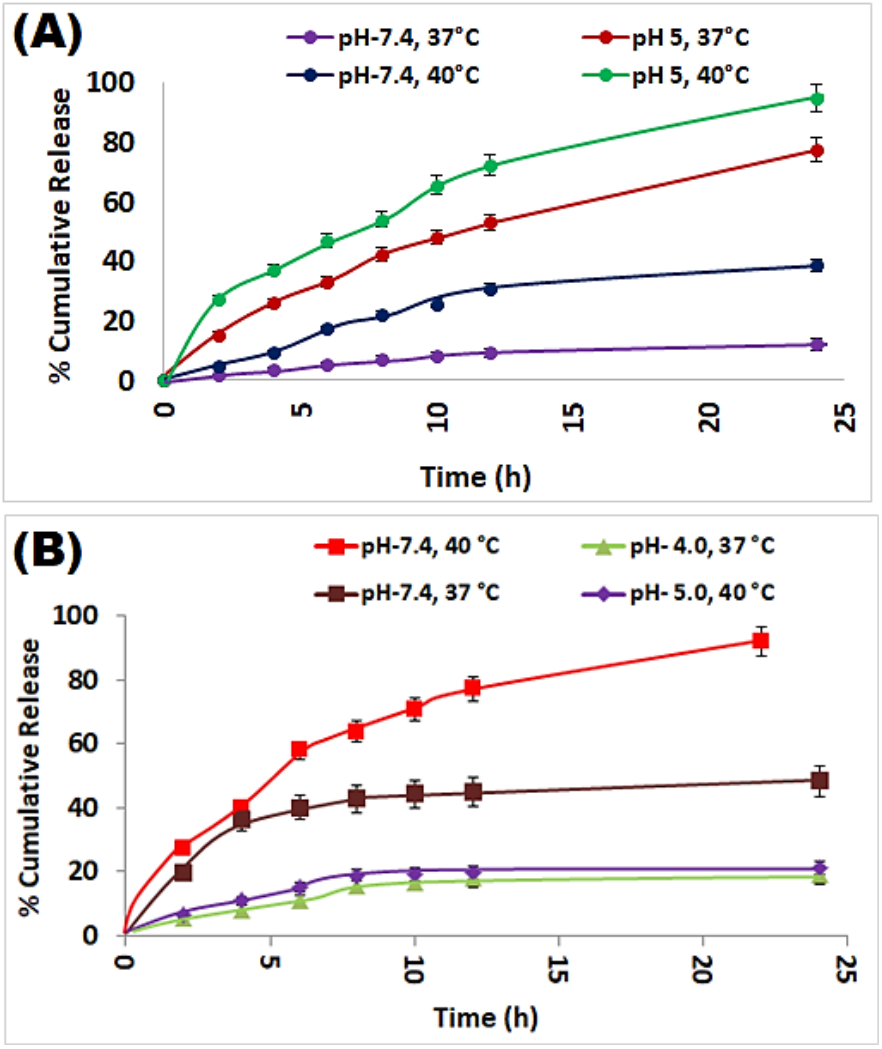


Figure 3: Percent cumulative drug release profile of (A) doxorubicin hydrochloride and (B) curcumin from the nanoconjugates

Curcumin is a potent anticancer agent devoid of side effects but is hydrophobic in nature and hence has poor bioavailability. Encapsulation in the hydrophobic cavity of cyclodextrin can enhance its bioavailability as proved in the *in-vitro* investigations carried out using cancer cell lines. The DOX loaded nanoconjugates on the other hand demonstrated an improved tumor regression on nude mice model **(figure 4)**.

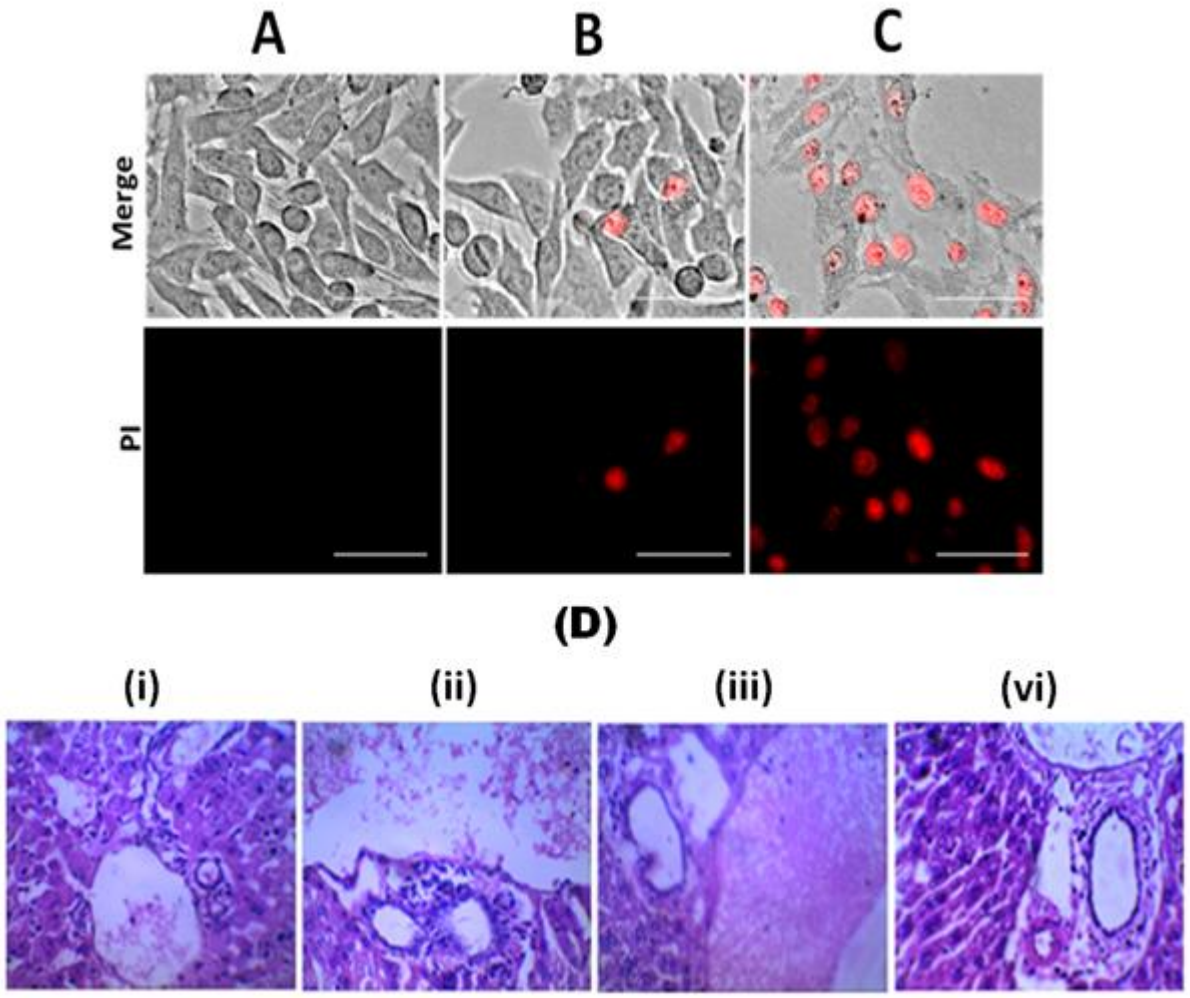


Figure 4: PI staining of curcumin loaded nanoconjugate internalized Hela cells. Cells undergoing necrosis were stained by PI. Here, (A) Control (B) Curcumin (C) Curcumin loaded nanoconjugates (D) Liver Sections of (i) Control and animals dosed with (ii) HEP-G2 (iii) DOX and (iv) DOX loaded nanoconjugates at 40X magnification.

Chapter 3: Carbon nanotube (CNT) embedded cyclodextrin polymer derived injectable nanocarrier: A multiple faceted platform for stimulation of multi-drug resistance reversal

A combination of cocktail chemotherapy (CCT), photothermal therapy (PTT) and inhibition of angiogenesis was investigated as an effective approach to combat major challenges of multidrug resistance and non-targeted drug delivery encountered in conventional cancer therapy. For this the dual stimuli responsive polymer was conjugated to CNTs as shown in **figure 5**.

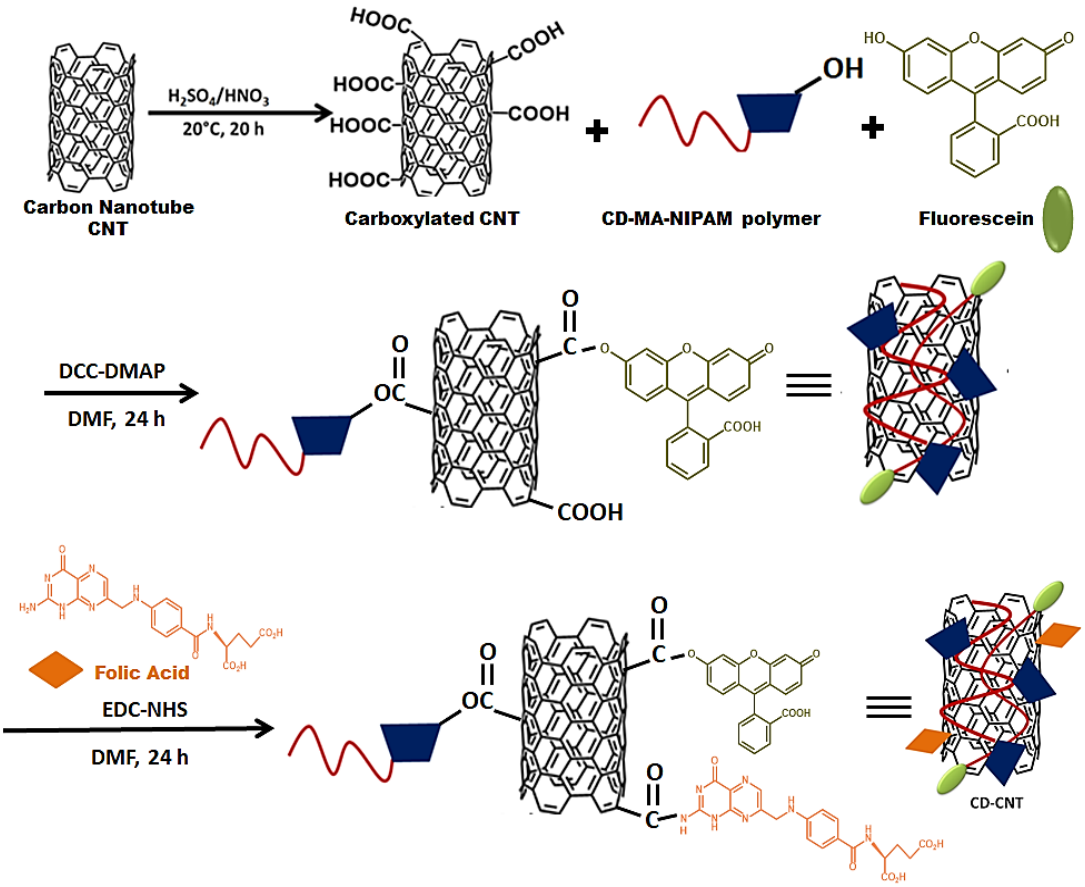


Figure 5: Schematic for the synthesis of CNT derived multifunctional nanoconjugates.

The resulting nanocarriers were characterized by various spectroscopic and microscopic techniques as shown in **table 2**

Table 2: Various properties of the carriers assessed via different techniques

Properties	**Technique/Instrument**
Structural Elucidation	FTIR Spectroscopy, EDAX
Morphology and Size	SEM, HRTEM, DLS
Optical	UV-Vis & Fluorescence Spectroscopy
LCST of thermoresponsive polymer	Variable Temperature DLS
Thermal	TGA
Photothermal	Measurement of heating capacity post NIR laser irradiation
Drug Loading and Release	UV-vis Spectroscopy
Antiangiogenic Potential	Ex-ovo CAM Assay
Preclinical Evaluation	*In-vitro* and *In-vivo* studies

The carriers exhibited multidrug delivery with high magnitude of drug encapsulation efficiency (>90%) (DOX- Curcumin drug pair) and a sustained release over 30 h under tumor microenvironment stimulations and having potential of mild photothermal therapy. The photothermal response of the modified CNTs on exposure to the irradiation was assessed. It was observed that on exposure to Laser for 5 minutes the temperature of the solution in the cuvette reaches 52 °C. The capability of inducing photothermal effect helps to achieve the anticipated therapeutic effect with reduced amount of drug. This helps to abate the potential toxic effects that can arise due to either drug or CNTs. The presence of a stimuli responsive polymer attached to the CNTs aids in maximum drug release under the conditions of tumor microenvironment. This helps in drug retention and any potential loss of drug before the target site is reached thus enhancing the therapeutic efficacy of the drugs. On exposure to NIR irradiation the drug release from the carriers is enhanced thus showing a better possibility of tumor regression[34]. A synergistic effect of chemotherapy and photothermal therapy was observed in the drug release profiles and in-vitro studies **figure 6**.

The excellent antiangiogenic potential of the carriers loaded with both DOX and Curcumin has been demonstrated ex-ovo by CAM (Chorioallantoic membrane Assay) for the first time. The ex-ovo studies showed significant decrement in branching points and vessel density formed during angiogenesis **(figure 7)**. A pronounced decrease total vessel network length and segment numbers was also observed [73]. Further a down regulation of growth factor genes (FGF2 and VEGF) supported the induction of antiangiogenic effect of the drug loaded carriers.

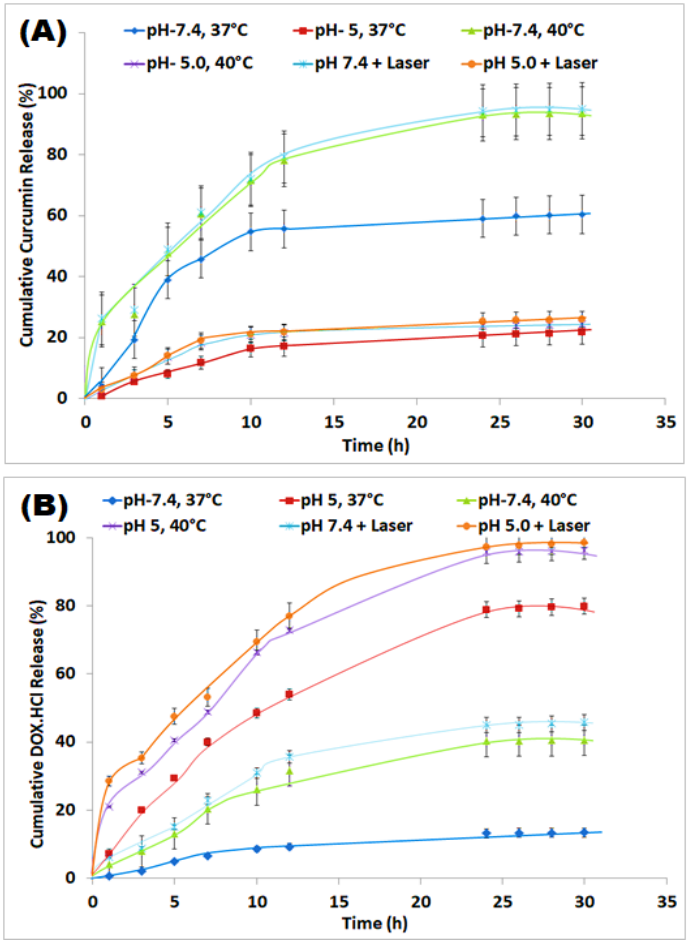


Figure 6: Release profiles of (A) Curcumin and (B) DOX.HCl from nanocarrier under dynamic conditions of pH, temperature and photothermal trigger using NIR laser of 808 nm.

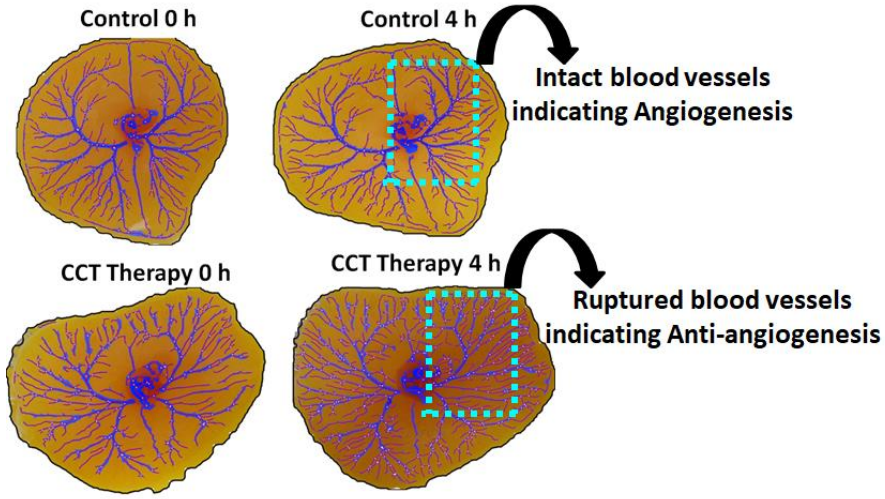


Figure 7: Ex-ovo CAM Assay performed on chick embryo after treatment with DOX-curcumin dual drug pair showing antiangiogenic potential of the drug carriers.

To the best of our knowledge, the strategy of chemo-photothermal combination therapy in amalgamation with angiogenesis inhibition has not been reported.

The in-vivo experiments demonstrate that the injectable formulation of these carriers have a profound influence on the decrement of MMP-9 which is associated with tumor progression and metastasis. This shows the potential of the carriers to combat cancer by the synergistic effect of combinatorial CCT-PT **(figure 8)** and anti-angiogenesis .

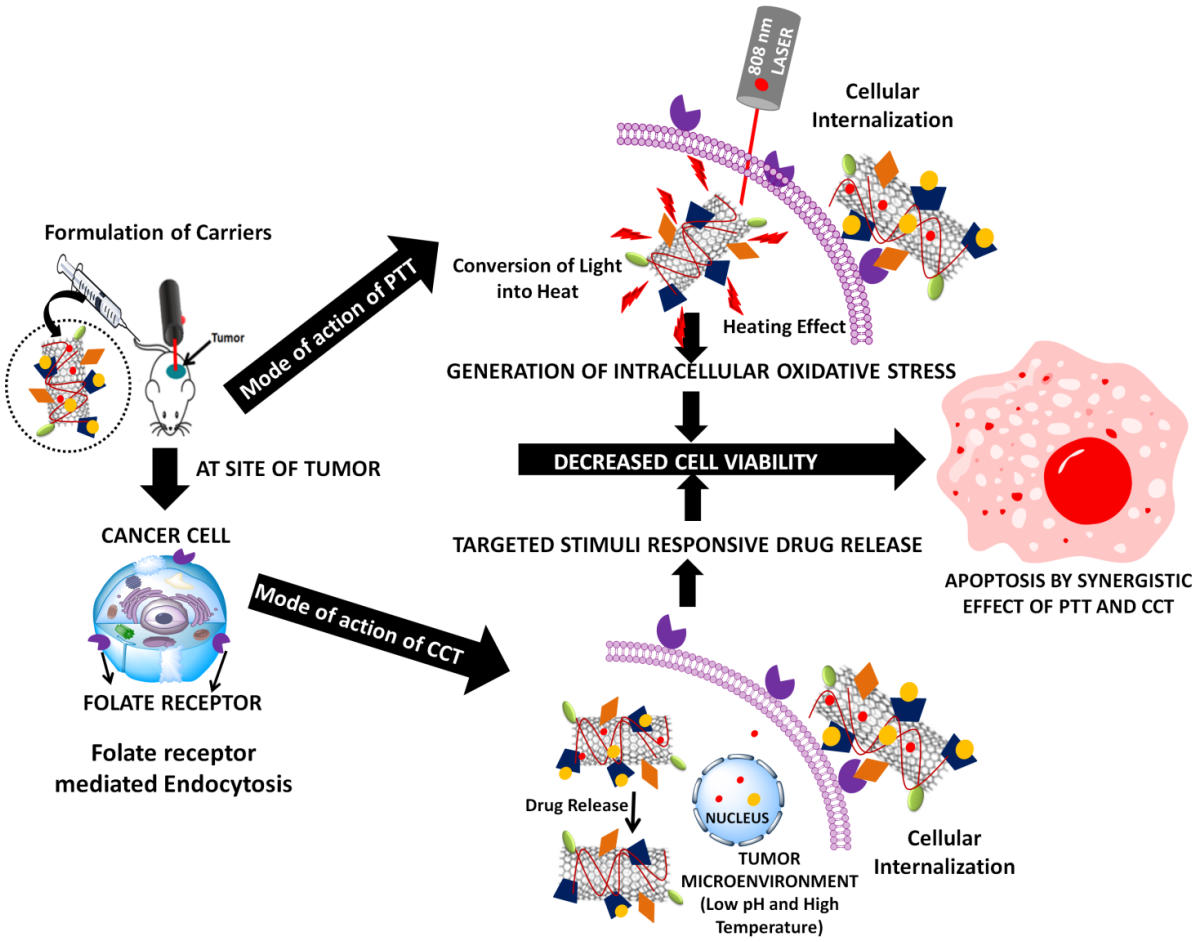


Figure 8: Probable mechanism of action of the nanoconjugates in inducing cell death at the cellular level.

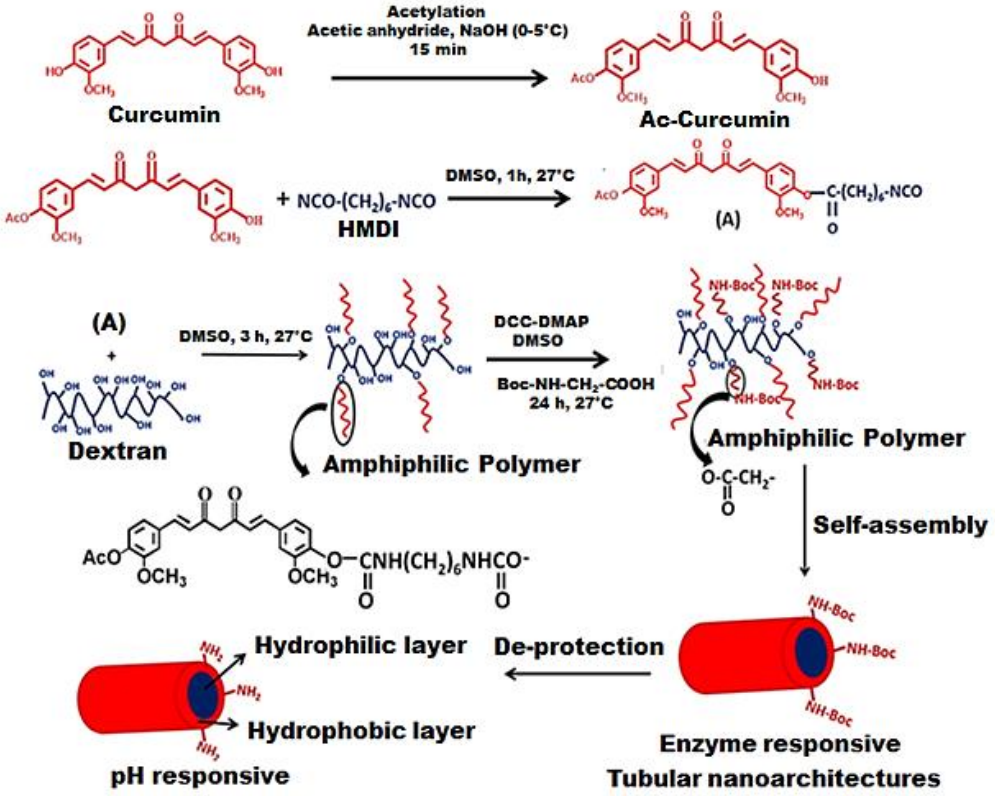
Chapter 4: Tumor homing dextran derived amphiphilic self-assembling polymer nanoarchitectures for stimuli responsive drug delivery.

The pharmaceutical industries associated with designing new drug molecules for cancer treatment are constantly facing the issue of poor therapeutic performance. Even after

investing huge amount of time (nearly 20 years) and capital (approximated cost of 500 million USD) for development of new drug molecules the issues of poor drug solubility, bioavailability and adverse side effects still remain a matter of concern to researchers. Nanotechnology derived formulations for delivering existing drugs thus emerged as a promising strategy to improvise their efficacy. Despite of various advancements in the design of synthesis, such materials bear certain disadvantages like residual toxicity. These challenges led the researchers towards the pursuit of developing biomaterial derived "smart polymeric nanocarriers". Owing to their wholesome non-toxicity, these bio-macromolecular carriers have emerged as much efficient drug carriers. This argument is supported by the fact that out of various nanomedicines approved by regulatory bodies for cancer management, approximately 40% are either protein polymer conjugates or liposomes (Eg. Doxil, which is a liposomal formulation of Doxorubicin). Thus the development of such bio-macromolecular smart polymers with improvised efficacy and their effective clinical translation are the key steps in repositioning various anticancer drugs for more patient compliant treatment.

Towards this goal, we have strategized the synthesis of dextran derived random amphiphilic co-polymers **(figure 7)**. Amphiphilic polymers possess stimuli responsiveness due to the presence of both hydrophilic and hydrophobic monomers in their skeleton. Additionally, they can self-assemble in into various nanosized morphologies by the virtue of various functionalities associated with the respective monomers. Interestingly such architectures are promising candidates as drug carriers owing to their long term stability, enhanced drug loading and excellent permeability.

Two different polymers were synthesized having dextran as the hydrophilic component, one of the polymer had octylamine as the hydrophobic unit whereas in the other curcumin was selected as the hydrophobic unit. Moreover both these polymer variants were conjugated with amino group protected glycine that rendered dual (pH and enzyme) responsivity to the carriers. The variation of the hydrophobic component demonstrated interesting diversity in size which in turn affected both the drug loading and release behaviours. The octylamine system exhibited spherical vesicles and curcumin system produced tubustecan vesicles **(figure 11).**



Scheme 9: Schematic for synthesis of pH and enzyme responsive DEX-CUR co-polymer and representation of their subsequent self-assembly into tubular nanoarchitectures

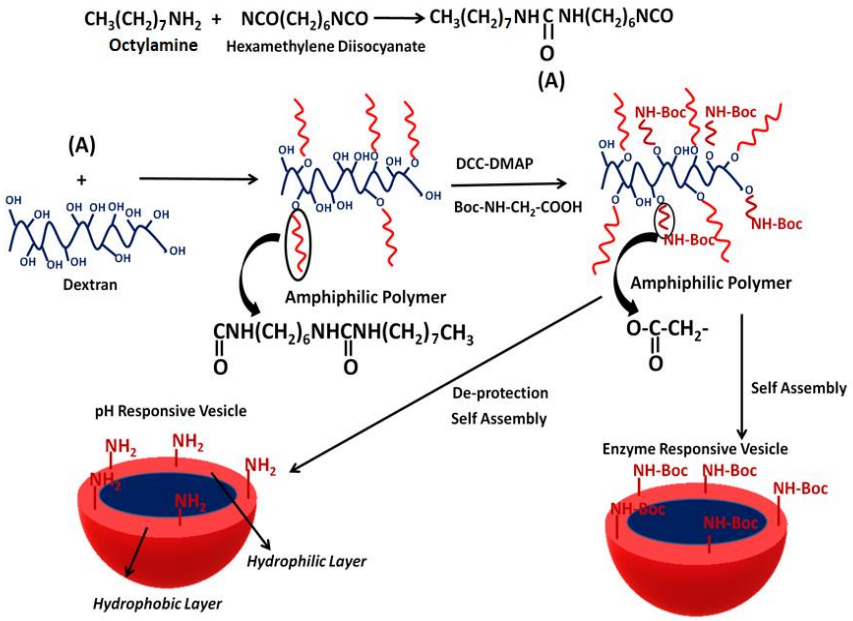


Figure 10: Schematic for the synthesis of amphiphilic polymer and their subsequent self assembly into spherical vesicles

The polymers were characterized by various spectroscopic and microscopic techniques as shown in **table 3**.

Table 3: Various properties of the carriers assessed via different techniques

Properties	**Technique/Instrument**
Structural Elucidation	NMR & FTIR Spectroscopy
Morphology and Size	FESEM, HRTEM, DLS, AFM
Self-assembly and vesicle formation	FTIR, self-quenching, fluorescence spectroscopy & microscopy
Drug Loading and Release	UV-vis Spectroscopy
Preclinical Evaluation	*In-vitro* and *In-vivo* studies

Due to tubular morphology the curcumin derived vesicle was capable of encapsulating more cargo of drug as compared to spherical ones **(Table 4)**. Further they also demonstrated a "needle like cellular internalization" thus accounting for a more pronounced cell death in vitro and enhanced tumor regression in vivo **figure 12**.

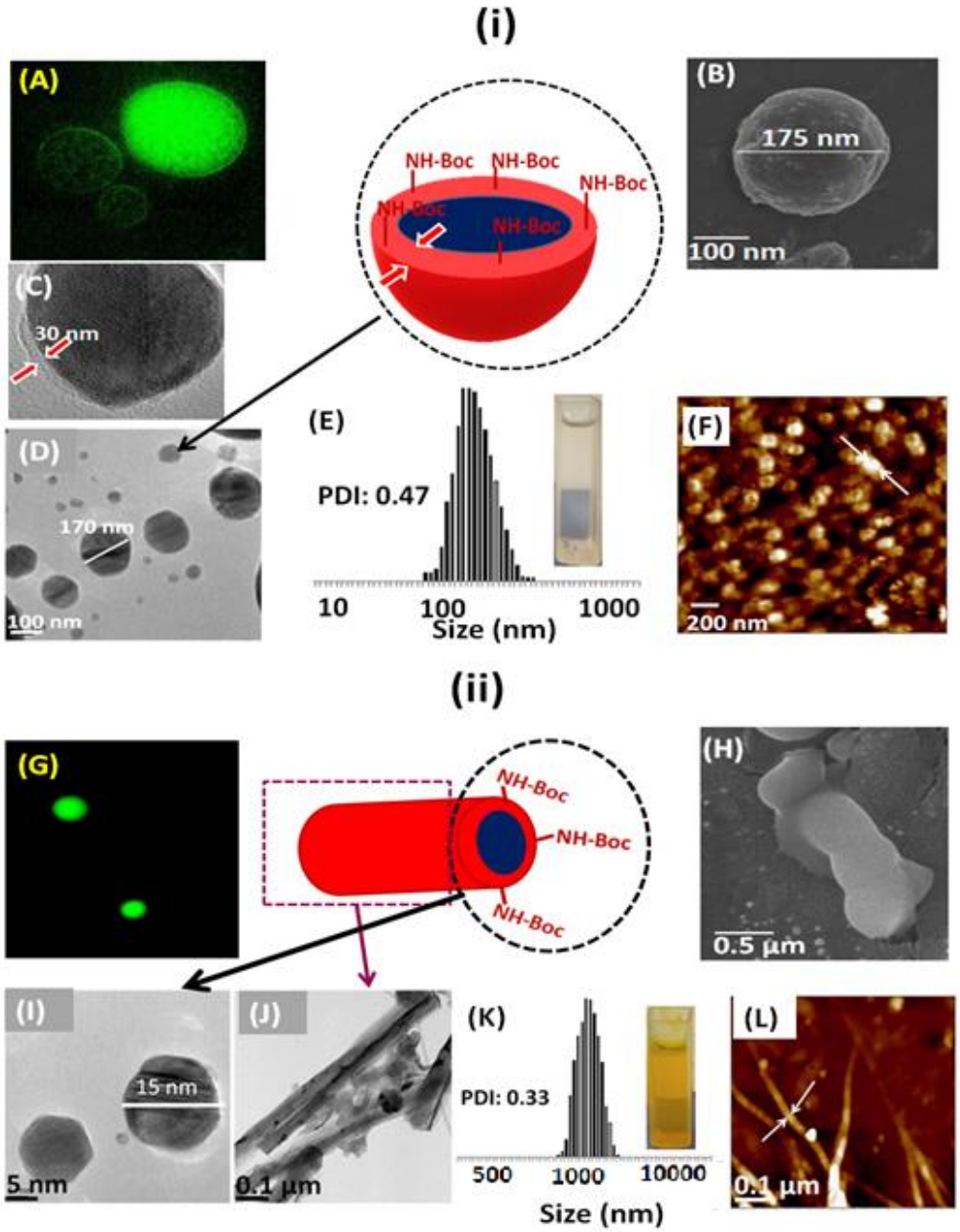


Figure 11: (i) Characterization of DEX-OA_{ER} (A) fluorescence microscopic (B) FE-SEM (C) & (D) HR-TEM images (E) DLS histogram with (inset: digital image of a vial containing the vesicular solution and (F) AFM image; (ii) characterization of DEX-CUR_{ER} (G) fluorescence microscopic (H) FE-SEM (I) & (J) HR-TEM images (K) DLS histogram with (inset: digital image of a vial containing the vesicular solution) and (L) AFM image. (The samples were maintained at 0.1 mg/ml for DLS and 0.05 mg/ml for rest of the imaging)

Table 4: Comparison of the drug entrapment efficiencies (DEE) and Drug loading capacities (DLC) of the synthesized nanocarrier

Carrier	Drug	DEE (%w/w)	DLC (%w/w)
DEX-CUR$_{ER}$	DOX.HCl	55	5.5
DEX-CUR$_{PR}$	DOX.HCl	53	5.2
DEX-OA$_{ER}$	DOX.HCl	50	5.1
	Curcumin	23	2.2
DEX-OA$_{PR}$	DOX.HCl	49	4.9
	Curcumin	25	2.6

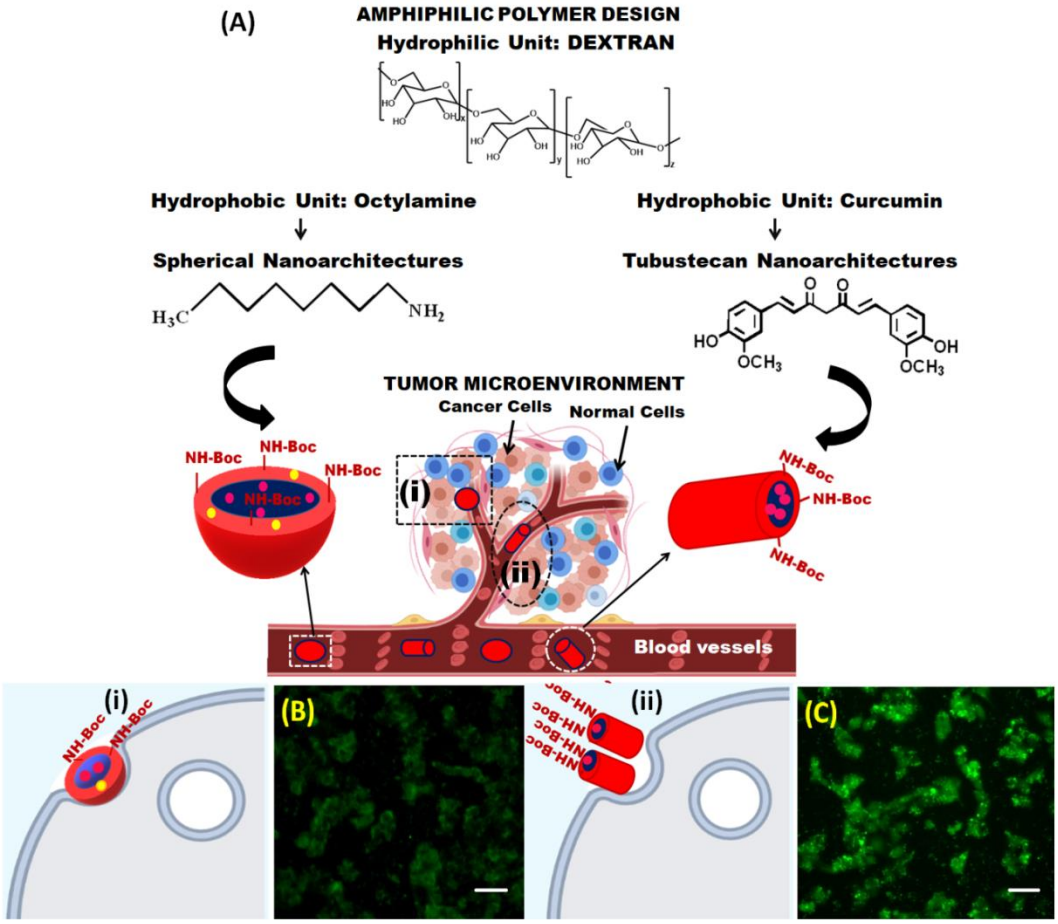


Figure 12: Schematic representation depicting (A) difference in morphologies of the nanoarchitectures upon change in the hydrophobic unit and (i), (ii) their cellular internalization; (B), (C) fluorescence microscopy images of calcein encapsulated nanoarchitectures internalized in MCF-7 cell lines (Scale 100 µm).

Chapter 5: Targeting the tumor microenvironment via hierarchical disassembly of curcumin micelles: The pro-drug strategy

The efficacy of drug delivery gets drastically affected in the body as the carrier has a tendency to face several barriers. Certain barriers include rapid drug leakage in-vivo, insufficient circulation of blood, limitations encountered by the carrier in tumor penetration etc. Thus it becomes imperative to rationally develop sophisticated delivery systems that can transform via adaptation towards physiological conditions and in response to tumor related stimuli (pH, temperature, enzymes etc.). These types of delivery agents ensure a targeted drug release and with appropriate designing strategy they can also be made to carry multiple drugs thus eliminating the issue of multidrug resistance.

Hence a rational pH sensitive and hierarchically degrading vesicle containing labile groups was synthesized. The presence of pH gradient inside various cell organelles of the cancer cells was employed to attain a desirable degradation and subsequent drug release. A pro-drug strategy was employed for further enhancing the pharmacological response of curcumin by incorporating it in the polymeric backbone via appropriate chemical modifications.

The hydrophilic end of the copolymer was functionalised with active cancer targeting ligand biotin which binds strongly to the biotin receptors widely over-expressed on cancer cell surfaces **(figure 13)**.

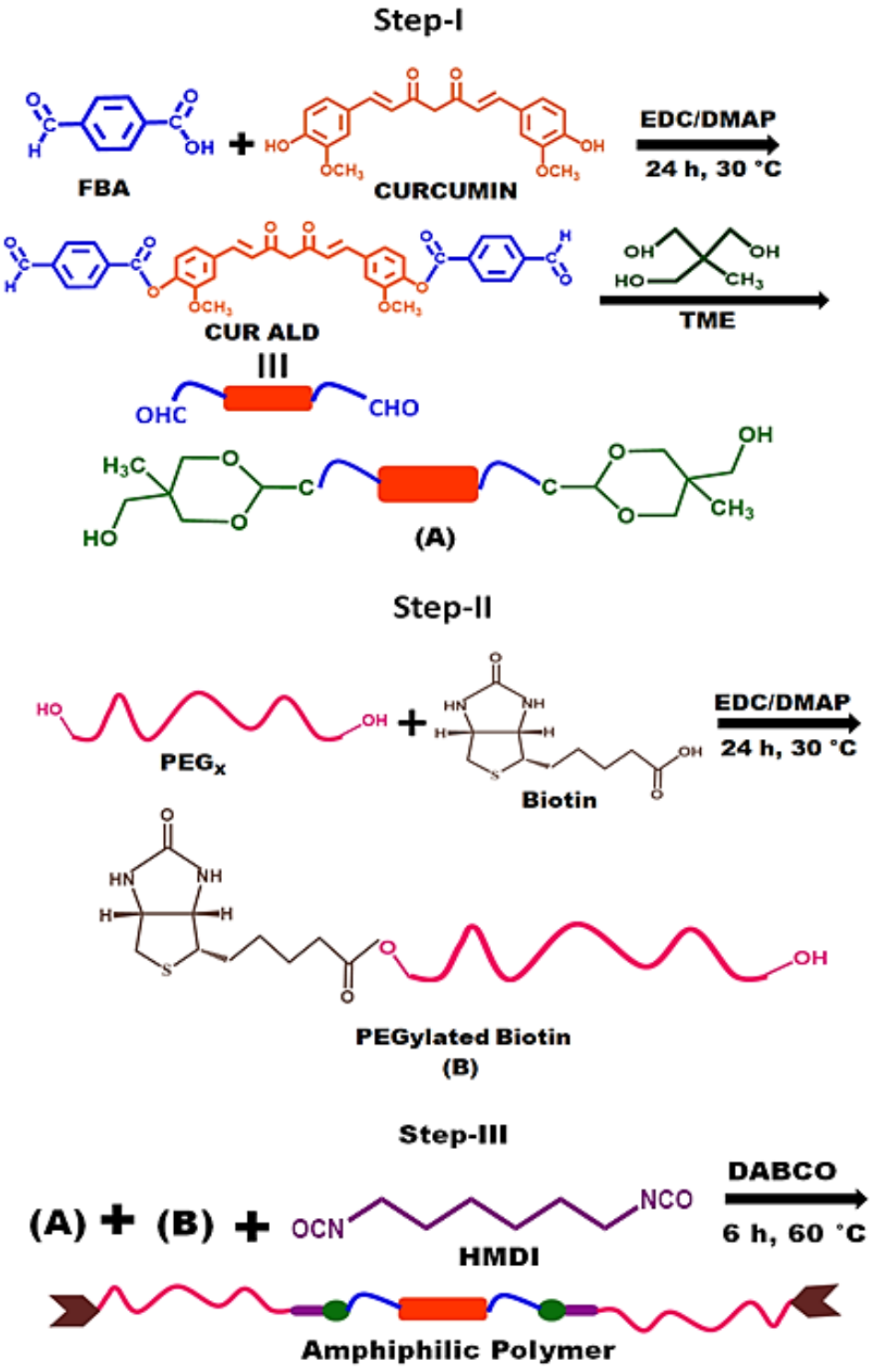


Figure 13: Synthetic route for preparing biotin tagged, acetal and ester linkage containing; pH, enzyme dual responsive amphiphilic polymer capable of self-assembling into prodrug micelle.

The resulting amphiphilic copolymer can self-assemble into a stable vesicle in an aqueous environment (as determined by the techniques mentioned in **table 5**), acting as an efficient nanocarrier for other cancer chemo-therapeutic drugs (e.g. Doxorubicin Hydrochloride). As a result, both the chemotherapeutic agent (DOX.HCl) and the chemo sensitizer (Curcumin) can be efficiently delivered into the target cancer cell at the same time.

Table 5: Various properties of the carriers assessed via different techniques

Properties	**Technique/Instrument**
Structural Elucidation	NMR & FTIR Spectroscopy, GPC
Morphology and Size	FESEM, HRTEM, DLS & AFM
Self-assembly and micelle formation	CMC determination by pyrene encapsulation
Drug Loading and Release	UV-vis Spectroscopy
Preclinical Evaluation	*In-vitro* and *In-vivo* studies

Conclusion

To summarize, this work aims towards addressing various challenges encountered in cancer therapy viz. non targeted delivery of drugs, development of multi-drug resistance by the cancer cells, toxicity caused by the carriers and poor bioavailability of certain potent drugs. Several stimuli responses such as pH, temperature, light and enzyme alone and in combination were taken into account for combinatorial therapy. Targeting ligands and pro-drug strategy were employed for delivery of solitary/multiple drugs to address MDR issue. The nanocarriers exhibited good to excellent drug entrapment efficiencies. Although the multifunctional nanoconjugates exhibited higher drug entrapment efficiency, they have the disadvantages due to inherent toxicity of the core nanomaterial. The polymeric nanoassemblies are promising due to resemblance with cell structures.

Moreover, the hierarchical disassembly of the nanoassemblies causes more selective cancer cell death due to sequential drug release thereof. All the nanocarriers showed a sustained release of the loaded drugs. The nanoassemblies demonstrated more sustained release upto 30-35 h as compared to the nanoconjugates (24 h). Efforts were dedicated to enhance the bioavailability of the natural anticancer agent curcumin by entrapment as well as by pro-drug strategy. All the results were evaluated in vitro on various anticancer cell lines (MCF-7 breast cancer & Hela cervical cancer cell lines) as well as in vivo on hepatocellular carcinoma

induced nude mice model. Thus the anticancer potential of the nanocarriers was demonstrated and validated as desired.

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List of Publications

Research Papers:

1. **Das, M**.; Solanki, A.; Joshi, A.; Devkar, R.; Seshadri, S.; Thakore, S. β-Cyclodextrin based dual-responsive multifunctional nanotheranostics for cancer cell targeting and dual drug delivery. ***Carbohydrate Polymers*****, 2019,** *206***,** 694-705.
2. **Das, M**.; Nariya, P.; Joshi, A.; Vohra, A.A.; Devkar,R.; Seshadri, S.; Thakore**, S.** Carbon nanotube embedded cyclodextrin polymer derived injectable nanocarrier: A multiple faceted platform for stimulation of multi drug resistance reversal. ***Carbohydrate Polymers*****, 2020,** *247*, 116751-116763
3. **Das, M.**; Joshi, A.; Devkar, R.; Seshadri, S.; Thakore, S. Tumor homing dextran and curcumin derived amphiphilic dextran derived functional polymer self-assembling to tubustecan nanoarchitectures: A strategy for adorning the golden spice (curcumin) for taming the Red Devil (DOX). **(2021-Under review)**
4. **Das, M.**; Joshi, A.; Devkar, R.; Seshadri, S.; Thakore, S. Vitamin-H channeled self-therapeutic P-gp inhibitor nanomicelles for targeting the tumor milieu by pH and enzyme triggered hierarchical disassembly. **(2021-Under review)**

Review Articles:

1. Solanki, A.; **Das**, **M.**; Thakore, S. A review on carbohydrate embedded polyurethanes: An emerging area in the scope of biomedical applications. ***Carbohydrate Polymers*****, 2018** *181***,** 1003-1016

2. **Das, M**.; Solanki, A.; Ganesh, A.; Thakore, S. Emerging hybrid biomaterials for oxidative stress induced photodynamic therapy. ***Photodiagnosis and Photodynamic Therapy*****, 2021,** *34***,** 102259-102268

Book Chapters:

1. Title of the Chapter: **"Exploring the potential of polymers in cancer management"**

Authors: Sonal Thakore**,** Archana Solanki, **Manita Das**

Details: **Chapter 4, p 113-133**

Name of the Book: **Materials for Biomedical Engineering: Organic Nano and Micro structures**

ISBN: **978-0-12-919433-2,** Publisher: **Elsevier**

2. Title of the Chapter: **"Carbohydrate derived functional nanomaterials for drug delivery and environmental remediation"**

Authors: **Manita Das,** Falguni Shukla, Sonal Thakore

Details: **Chapter 16**

Name of the Book: Handbook of Functionalized Nanomaterials: Environmental Health and Safety

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Status: **In Press**

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