

TAILOR-MADE POLYSACCHARIDE VESICLES FOR DRUG DELIVERY TO CANCER CELLS

A THESIS

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OF THE DEGREE OF

DOCTOR OF PHILOSOPHY

BY

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March 2015

Dedicated to
My Teachers



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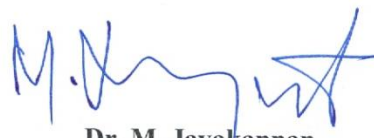
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CERTIFICATE

Certified that the work incorporated in the thesis entitled “*Tailor-made Polysaccharide Vesicles For Drug Delivery to Cancer Cells*” Submitted by **Mr. Pramod P.S.** was carried out by the candidate under my supervision. The work presented here or any part of it has not been included in any other thesis submitted previously for the award of any degree or diploma from any other University or Institution.

Date: 24th March 2015
Pune (MH) India


Dr. M. Jayakannan
(Thesis Supervisor)
24/3/2015

DECLARATION

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Pramod

SYNOPSIS

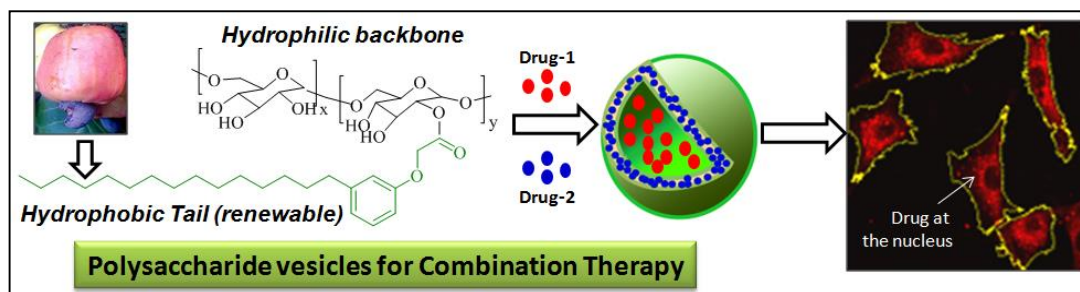
Design and development of polymer based controlled drug delivery systems has been emerging as important area of research for cancer treatment. Among the various challenges, delivering multiple anticancer drugs based on single polymer nano-carrier is particularly emerging as challenging task to enhance the tumor resistance to individual drug molecules and their therapeutic efficacies. Polymer vesicles (or polymersomes) are important classes of nano-carriers for delivering multiple drug loads such as hydrophilic and hydrophobic drugs together in a single dose. Polysaccharides are one of the most promising systems owing to their natural abundance, superior biodegradability and biocompatibility and so on so forth. However, structural optimizations in the hydrophilic polysaccharide with appropriate hydrophobic units are challenging task to produce polysaccharide vesicles. Thus, the polysaccharide vesicles for delivering multiple anticancer drugs (both hydrophilic and hydrophobic together) is less explored area in drug delivery research. The thesis is aimed to develop new polysaccharide vesicles that are stable for encapsulation and preservation of drugs to achieve better cancer treatment.

This thesis has been divided in to five major chapters:

1. Introduction Chapter: First chapter provides literature survey on polymer based drug delivery and the need for the polysaccharide vesicles for delivering anticancer drugs.
2. Development of Dextran Vesicular Carriers: Dextran vesicular nano-scaffolds were developed based on polysaccharide and renewable resource hydrophobic unit. The roles of the hydrophobic segments on the molecular self-organization of dextran backbone as vesicles were investigated in detail. Dextran vesicles were encapsulated with drugs and their intracellular administration was investigated in detail.
3. Polysaccharide Multidrug Carrier for Synergistic Killing: Combination therapy based on single polymer scaffold has been studied. Simultaneous delivery of hydrophobic drug camptothecin (CPT) and hydrophilic drug doxorubicin hydrochloride (DOX) was accomplished in breast and colon cancer cells.

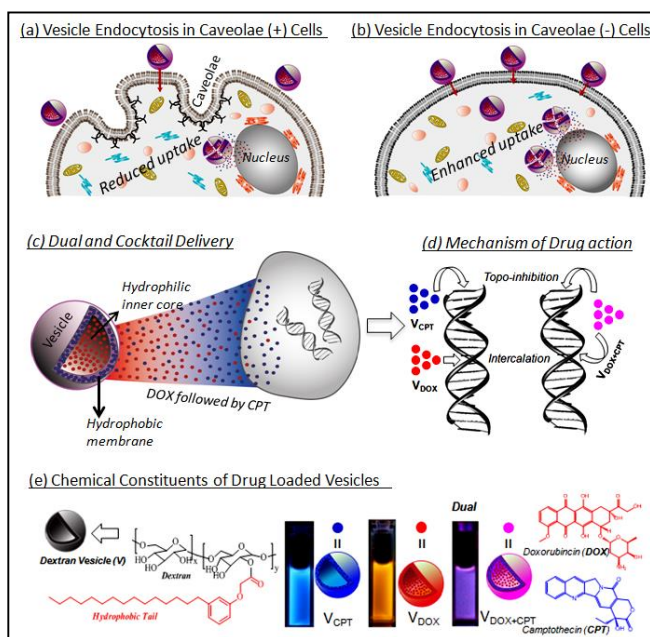
4. pH Responsive Polysaccharide Vesicles for Conjugated and Physically loaded DOX Delivery: pH and enzyme dual responsive polysaccharide vesicular nano-scaffolds were developed for the administration of doxorubicin via physical loading and chemical drug conjugation to the breast cancer cells.
5. Real-time Drug Release Analysis of Polysaccharide Vesicles: Time resolved fluorescence spectroscopic approach was developed to resolve real-time enzymatic cleavage of cell penetrable polysaccharide vesicles. Binding energy data were generated using AUTODOCK programme and the role of esterase enzyme on the cleavage of polysaccharide based vesicle was established.

This thesis work has been organized in five chapters. Chapter-1 starts with a brief introduction on the concept of drug delivery and role of polymers in drug delivery applications. This chapter provides examples of polymers used for drug delivery and the concept of passive selectivity to cancer site via enhanced permeability and retention (EPR) effect. The packing parameters governing molecular self organization of small molecules and polymers were discussed. The vesicular self organization, its structure, properties and examples of polymers were also explained. Towards the end, examples of polysaccharides as drug delivery vectors along with structure, functionalization methods and examples of self organized structures have been discussed.



In the **second chapter**, a new hydrophobic segments based on *renewable resource* was anchored to make dextran (a polysaccharide analogue) based polymer vesicles. In this approach, all three components like hydrophilic polymer backbone (dextran), hydrophobic tail (from pentadecylphenol, from cashew nut fruit) and drug molecules (anticancer drug CPT from plant) were chosen from natural resources. The dextran vesicular scaffolds were capable of loading water soluble guest (Rhodamine-B, RhB,

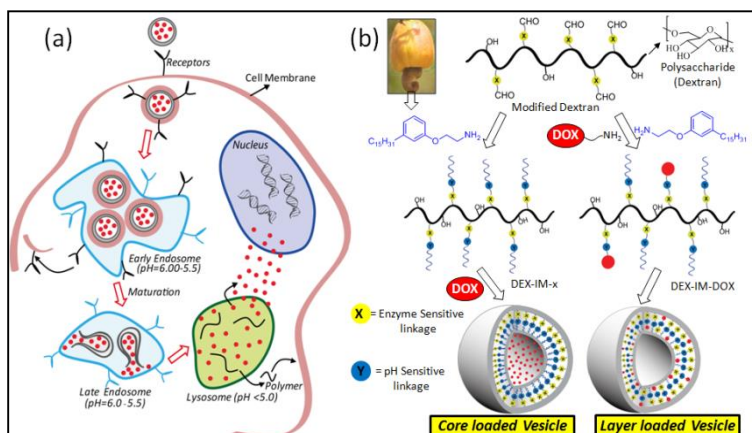
water soluble dye) in its interior hydrophilic cavity and water insoluble polyaromatic anticancer drug (Camptothecin, CPT) in its unilamellar hydrophobic membrane. It was also able to protect the plasma sensitive CPT lactone pharmacophore against the hydrolysis by ten times better than the CPT alone in PBS. The aliphatic ester linkage connecting the hydrophobic tail with dextran was found to be ruptured by esterase under physiological conditions for fast releasing of CPT or RhB. Cytotoxicity of the dextran vesicle and its drug conjugate were tested on mouse embryonic fibroblasts cells (MEFs) using MTT assay. The dextran vesicular scaffold was found to be non-toxic to living cells. CPT loaded vesicles were found to be 2.5 fold more effective in killing fibroblasts compared to that of CPT alone in PBS. Confocal microscopic images confirmed that both RhB and CPT-loaded vesicles to be taken up by fibroblasts compared to drug/dye alone, showing a distinctly perinuclear localization in cell.



The **third chapter** illustrates the development of multiple drug loading and sequential drug delivering polysaccharide nano-vesicle for administering anticancer drugs DOX (topoisomerases II inhibitor) and CPT (topoisomerases I inhibitor) to achieve synergistic killing of cancer cells. This nanovesicle facilitated the investigation of dual drug loaded single-vesicle versus the cocktail of

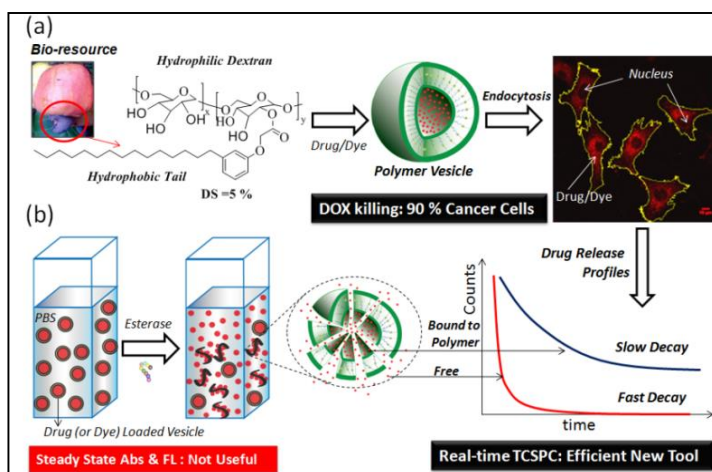
individual drug loaded nano-vesicles. Further, evaluation of the role of caveolae in endocytosis of these polymer scaffolds was also made. Endocytosis of this nano-vesicle in caveolae lacking (Cav-1^{-/-}) MEFs was seen to be better. This differential uptake is also seen in caveolin-1 (and hence caveolae lacking) breast cancer (MCF7) and colon cancer (DLD1) cells. The dual loaded polysaccharide nano-vesicles containing DOX and CPT act synergistically to promote killing of cancer cells. When administered as a cocktail having more CPT than DOX, synergistic killing of cancer cells was enhanced the

most suggesting that a cocktail of these drugs could potentially be the best candidate for delivering multiple anticancer drugs with the best efficacy. The results provide significant new insights into polysaccharide loaded dual drug action of DOX: CPT drug combination in the breast and colon cancer cells..



The **fourth chapter** describes the development of pH and enzyme dual responsive polysaccharide vesicular nano-scaffolds for the delivery of doxorubicin both physical loading and chemical drug conjugation

to the breast cancer cells. Dextran was suitably modified with renewable resource 3-pentadecyl phenol via imine and aliphatic ester linkages that act as pH and esterase enzyme stimuli for delivering drugs. These dual responsive polysaccharide derivatives self-organized as nano-vesicles in water. Water soluble anticancer drug doxorubicin DOX.HCl, highly potent DNA intercalating agent, was chosen for loading in the vesicular hydrophilic core and its amine derivative (hydrophobic form) was anchored through imine chemical linkage. The current design provided two vesicular scaffolds: (i) DOX loaded at the core of the nascent polysaccharide vesicles and (ii) DOX-conjugated at the hydrophobic layer of the polysaccharide vesicles having identical morphology. The acid labile benzylic imine linkages were cleaved under acidic pH (≤ 6.0) conditions which lead to disassemble of the dextran vesicles. *In-vitro* studies revealed that about 70-80 % of drug was well retained under circulatory conditions under pH = 7.4 at 37 °C. At low pH=6.0 to 5.0, both imine and ester linkages cleaved instantaneously to release 100 % of loaded drugs. In MCF7 cells, DOX loaded vesicles exhibited better cell killing efficiency than free drug and drug conjugated vesicles and the confocal microscopic images confirmed that DOX in nano-vesicles were taken up better by cells compared to free DOX molecules.



The *fifth chapter* deals with a facile method based on time resolved fluorescence technique to estimate real time drug release parameters from enzyme responsive and cell penetrable polysaccharide vesicles. The vesicles loaded with RhB and DOX are chosen

for the drug release studies (described in Chapter 2 and 3). Remarkably, a significant difference was observed in the TCSPC life time of free RhB and RhB loaded in the vesicles. The limitations of normal absorbance, emission and dynamic dialysis method to distinguish the species such as drug/dye bound to polymer (loaded) and released into PBS was successfully overcome using time resolved fluorescent (TCSPC) technique. The fractional contribution of the dye loaded in the vesicles and released into solution (PBS) after esterase enzyme treatment was meticulously estimated using TCSPC method, without any additional treatment such as dialysis, centrifugation etc. Moreover, the ability of enzyme to rupture the polysaccharide vesicles was investigated using docking studies. The peculiar positioning of the molecules in active site of enzyme and the binding energy data generated using AUTODOCK programme supported its capability to attack the ester linkage on polysaccharide based vesicles. The study bestowed an entirely new method to obtain authentic information about drug release parameters from polysaccharide vesicles. The results also point towards the potential applications of the newly developed dextran vesicles for the multiple drug delivery to cancer cells. They also challenge some of our existing understanding of dual delivery of drugs and the role of caveolae in nano-vesicle uptake.

The last chapter summarizes the outcome and future direction of research work carried out in the Ph.D. thesis.

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Chapter 1

Introduction

1.1. Introduction to Drug Delivery

The process of drug development comprises two major sections: (i) designing a new drug candidate from a lead molecule and (ii) developing a drug delivery method for a defined target site or pathway (see Figure 1.1).¹ Drug designing or drug discovery process involves identification of target, validation of target, lead discovery, preclinical development and clinical development.² On the other hand, drug delivery strategies encompasses engineering new technologies as a formulation or device that enables the administration of a therapeutically active molecule in the body and enhances its efficacy and safety by regulating the rate, period and site of release in physiological systems.³



Figure 1.1. *Drug development strategies*

Drug discovery is an essential practice to be initiated when there is a disease or a clinical condition devoid of proper therapeutics available in the market.⁴ Probability of a lead molecule designed in the laboratory during preliminary stage of drug discovery programme and making it to market is 1 in 10,000.⁵ Moreover, factors such as poor pharmacokinetic properties, lack of efficacy and high toxic profiles are some of the challenges needed to be addressed prior to clinical development. In addition to synthetic and scientific challenges, other aspects such as stringent regulatory obligations make drug discovery a complex and year lasting process. One way to combat the above mentioned huge risk of drug invention process is to develop good delivery strategies for existing drug molecules.

The landscape of drug delivery method/vehicle development is extremely competitive and fast evolving. Conventional delivery methods such as injection, infusion, ingestion (oral delivery: tablet, capsules, liquid orals etc.), and inhalation (Nebulizer, dry powder inhaler etc.) are still popular in medical practice for the drug

administration.⁶ However, challenges still exist in the area of improving stability, efficacy and safety profiles of administered drug molecules. Some drug molecules especially biological drug candidates undergo destabilization process in blood plasma by various proteolytic enzymes leads loss of activity and precipitation of unwanted immunogenic reactions.⁷ Moreover, the conventional delivery systems may cause dose dumping and the excess of drug molecules reached in the systemic circulation and body tissues other than target site may cause toxicity.⁸ For example, the narrow biological differences between healthy cells and cancer cells prevent chemotherapeutics (drugs used for treatment of cancers) from its differentiation and lead to severe side effects. Other problems like defective pharmacokinetic profile (ADME) of drug molecules, patient's noncompliance, environmental impacts etc. also have to be addressed.⁸

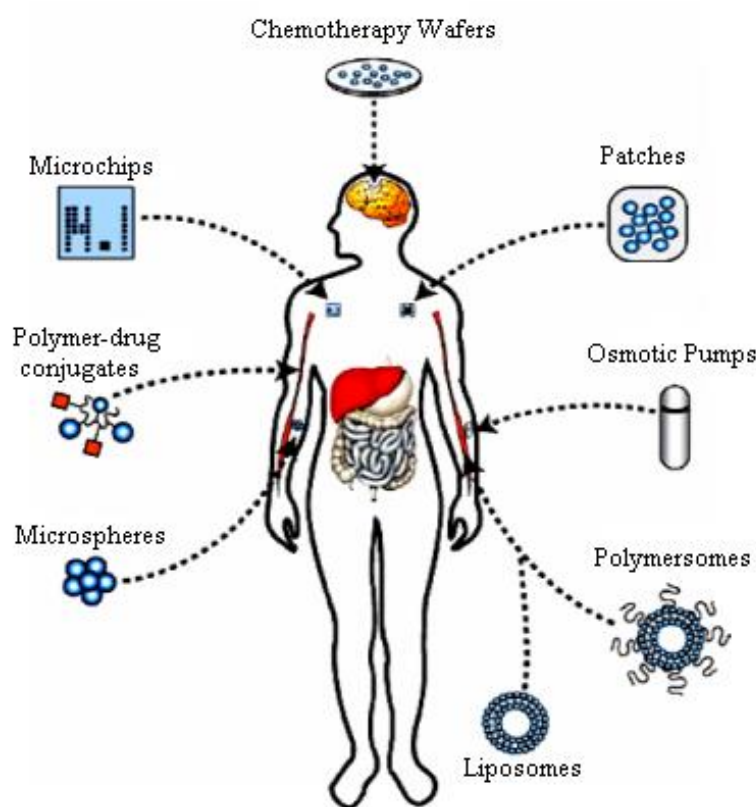


Figure 1.2. Advanced Drug delivery approaches (Adopted from Moses et al. *Cancer cell*. 2003, 65, 347-3515).

Efforts were focussed in the development of new drug delivery devices/method (See Figure 1.2) for achieving targeted delivery in a controlled or sustained manner.⁹ Wafer implants for chemotherapy to brain tumors¹⁰, transdermal patches for medication delivery to blood stream via skin¹¹, wireless microchip based implants for controlled delivery¹², osmotically controlled drug delivery pumps are

some of the newest trend in this field.¹³ The wide spread use of these devices are still limited due to various reasons: invasiveness and infections associated with implanted devices, low bioavailability, bulkiness and expensiveness of transdermal patches, occasional pain, bruising and inconsistent delivery from other devices. Recent emerging technology in this field is drug loaded or conjugated self organized structures that can act as long circulating controlled delivery system for better treatment.¹⁴

1.2. Self organized Structures for Drug Delivery

Self assembled nanostructures have attracted rapidly growing interest due to its ability to act as drug carrier to target site which can enhance the drug efficacy while lowering the toxicity and provide protection to plasma labile drugs. Wide range of such structures made up of a variety of materials including polymers, lipids and inorganic materials are shown in figure 1.3.¹⁵

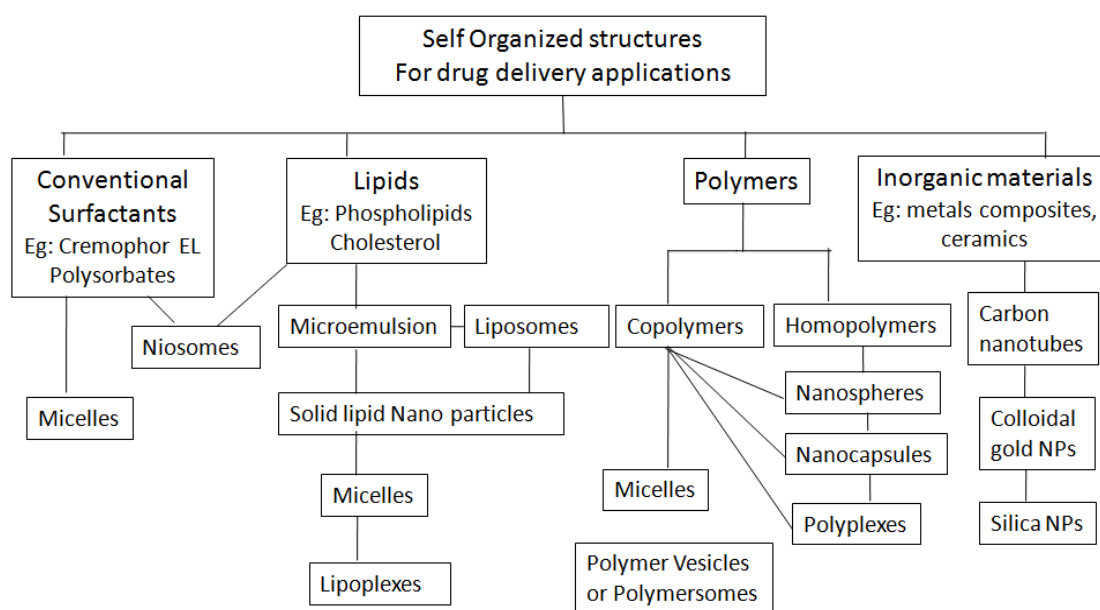


Figure 1.3. Classification of Self organized structures and materials used for drug delivery applications (Adopted from Letchford et al. *Eur.J.Pharm. Biopharm.* **2007**, 65, 259-265).

The amphiphilic small molecules and polymeric molecules can self-assembled in solution to form range of structures including liposomes, micelles, vesicles, nanorods, nanospheres, disc like micelles and inverted structures etc. It is rather difficult to predict the kind of morphologies formed from each amphiphilic structures. A dimensionless critical packing parameter “p” was introduced by

Israelachvili and coworkers and it can be used to predict the geometry of self organized structures from small molecule based surfactant molecules.¹⁶ Here, $p = v/a_0l$, where l and v are the optimal length (normal to the interface) and volume of the hydrophobic chain, respectively, and a_0 is the optimal surface area per molecule composing the aggregate. This packing parameter can be calculated using following table for a conventional surfactant molecule¹⁷

Table 1.1. Comparison of geometric formula and packing parameter for small molecule surfactant (adopted from ref. 17)

	Sphere	Cylinder	Bilayer
$V = gv_0$	$4\pi R^3/3$	πR^2	$2R$
A	$ga = 4\pi R^2$	$ga = 2\pi R$	$ga = 2$
A	$3v_0/a$	$2v_0/R$	v_0/R
$P = v_0/al_0$	$v_0/al_0 \leq 1/3$	$v_0/al_0 \leq 1/2$	$v_0/al_0 \leq 1$

Based on the above calculation, one can predict the morphology of self assembled structures as follows. $p < 1/3$ for spherical assemblies: $1/3 \leq p \leq 1/2$ for cylinders: $1/2 \leq p \leq 1$ for vesicles and $p \sim 1$ for bilayer.¹⁷ Therefore, the morphology of self-assembled structure changes in relation with the packing parameter. The above three factors, v , a_0 , and l , depend on environment in which the molecule present such as temperature, pH, solvent properties such as type of organic solvents, ratio of water to organic solvent, concentration of amphiphilic molecule and salinity and so on so forth. Moreover, the molecular structure of amphiphile (hydrophilic to hydrophobic ratio) also affects the packing parameter and thereby the self assembly. For example single hydrophobic tailed molecules have $p < 1/2$, on the contrary, molecules with two hydrophobic tail and one hydrophilic head groups have $p > 1/2$. The former forms spherical or cylindrical assemblies and latter forms vesicles or bilayers.¹⁷

Based on this prediction and various optimization processes, a plethora of organic (small molecule like anionic, cationic and non-ionic surfactants, lipids, etc.)¹⁸ and inorganic (metal, metal oxides, magnetic, semi conductor etc.)¹⁹ delivery carriers were developed for various therapeutic applications. Among inorganic delivery vectors, gold nanoparticles²⁰ and silica nanoparticles²¹ were widely studied for drug delivery, gene delivery and diagnostic imaging purposes. For example, gold nanoparticles can be easily functionalized through renowned gold-thiol synthetic strategy (thiol, multiple thiols, cyclic disulfides, mixing with free amines, see figure 1.4a).²⁰ Gold particles thus synthesized to a variety of shapes including spheres, shells

rods, etc and were shown to bind with various nucleic acid materials, anticancer drugs (eg: doxorubicin, cisplatin, paclitaxel, tamoxifen, 5-FU etc.) and antibacterial agents (eg: vancomycins, ampicillins, streptomycins etc.) (see figure 1.4b and c).²⁰ Similarly, mesoporous silica nanoparticles were also extensively studied for various pharmaceutical and therapeutic applications.²¹ The toxicity of metal particles or capping ligand and change of pharmacokinetic properties of loaded/conjugated drug molecules are some of the challenges yet to be addressed in the case of inorganic nanoparticles.²⁰

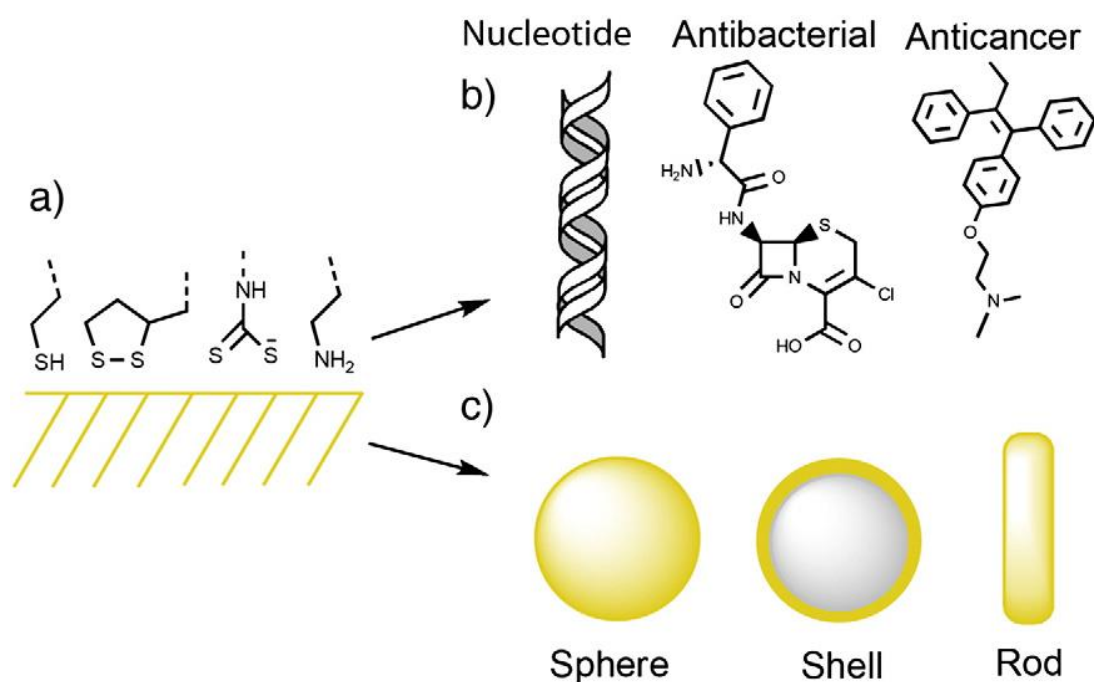


Figure 1.4. Gold nanoparticles-drug conjugate synthetic strategy (a), types of drug used (b) and various shape of NPs (c) (Adopted from Vigderman et al. *Adv. Drug Deliv. Rev.* **2013**, 65, 663-676).

The prediction model proposed for small molecule surfactants is not applicable for bigger polymeric structures. Eisenberg and coworkers have proposed model for polymeric structures. According to this model, the copolymers having hydrophilic to hydrophobic ratio greater than 1:1 forms spherical micelles.^{22,23} On the other hand, vesicles or inverted nanostructures were formed for 1:2 and 1:3 ratios respectively. However, studies on various polymeric amphiphiles showed numerous exceptions from the above mentioned guidelines. Thus, it can be concluded that the balance among the hydrophilic/hydrophobic ratio is significant factor but not the only conclusive parameter to decide self assembled morphologies. Other factors such as

kinetic factors and free energy contribution also affect the final self organized structures.^{22, 23}

1.3. Polymers Based Drug Delivery

Tremendous progress has been made in the advancement of modern drug delivery with the use of polymer based carriers to bring out spatiotemporal drug release.²⁴ Rational model proposed by Ringsdorf in 1975, forms the basis for most of the development in this area (See Figure 1.5).²⁵ As seen in this model, polymeric structures allows attachment of solubilizer unit for improved aqueous solubility, targeting group for enhanced cellular uptake and binding to target, and drug conjugation via sensitive linker for controlled delivery. Compared to small molecule based amphiphiles, availability of numerous functional groups on the polymer backbone permits such chemical modification.

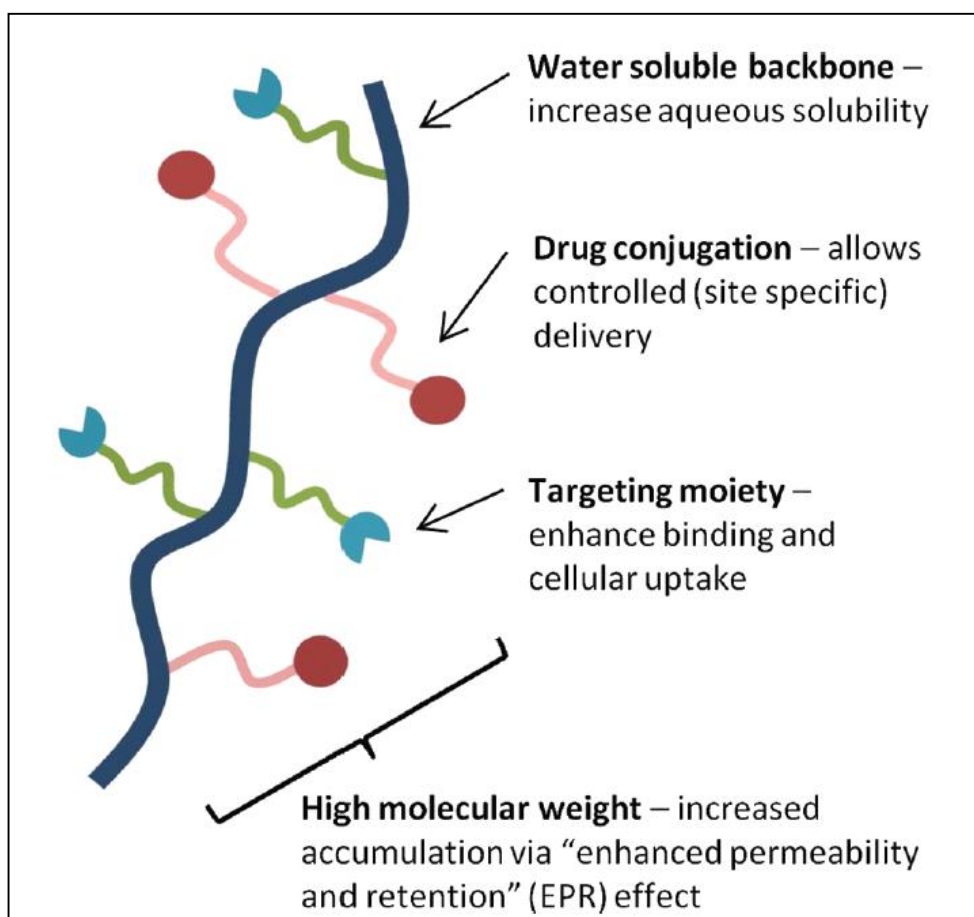


Figure 1.5. Ringsdorf model of polymer drug conjugate for delivery applications (Adopted from Larson et al. *Chem.Mater.* **2012**, 24, 840-853).

The lower critical association concentrations (CACs or minimum concentration required for the amphiphiles to form self organized structure in solution) of polymeric carrier compared to small molecule amphiphiles provides superior stability against larger dilution in physiological conditions. Thus polymeric carrier allows change of drug pharmacokinetic properties by providing protection from rapid metabolism and faster clearance.²⁵ Moreover, it guards sensitive drug molecules from harsh conditions in the body and enable them to reach therapeutic effective concentration at the target site. It also avoids dose fluctuation associated with frequent drug administrations. The aforementioned advantages of polymeric carriers inspired the researchers worldwide to develop anticancer polymer drug conjugate in order to combat the high toxicity, side effects, low therapeutic index, poor water solubility and metabolic instability of chemotherapeutic agents.²⁵ The guideline principle for polymer based anticancer drug conjugate is passive targeting achieved via enhanced permeability and retention (EPR) effect.²⁶

Neoangiogenesis (new blood vessel formation) was induced from even 2 to 3 mm clusters of tumors for catering escalated need of oxygen and other macro and micro nutrients.²⁷ The detailed investigations by Matsumura and Maeda in 1986 showed that the newly formed blood vessels to tumors possess a unique anatomical and pathophysiological architecture.²⁸ Typical features of these blood vessels includes irregularity in shape, leaky or defective nature, poorly aligned or disorganized endothelial cells having large fenestrations, deficiency of basement membrane and perivascular cells etc. Moreover, tumor tissues are characterized by deprived lymphatic drainage channels and slow venous returns. The faulty nature of blood vessels allows leakage of blood plasma components including macromolecules, lipid and nanoparticles into tumor tissues. On the other hand, poor lymphatic clearance allows the leaked macromolecules to retain in the tumor tissue and subsequent extravasation to tumor interstitium (See Figure 1.6). EPR effect assist the localisation of about 10 to 50 fold higher than normal tissues within 1-2 days.²⁶⁻²⁸ The same process is also responsible for the passive selective administration of polymer based macromolecules conjugated/loaded drug molecules in to cancer tissues.

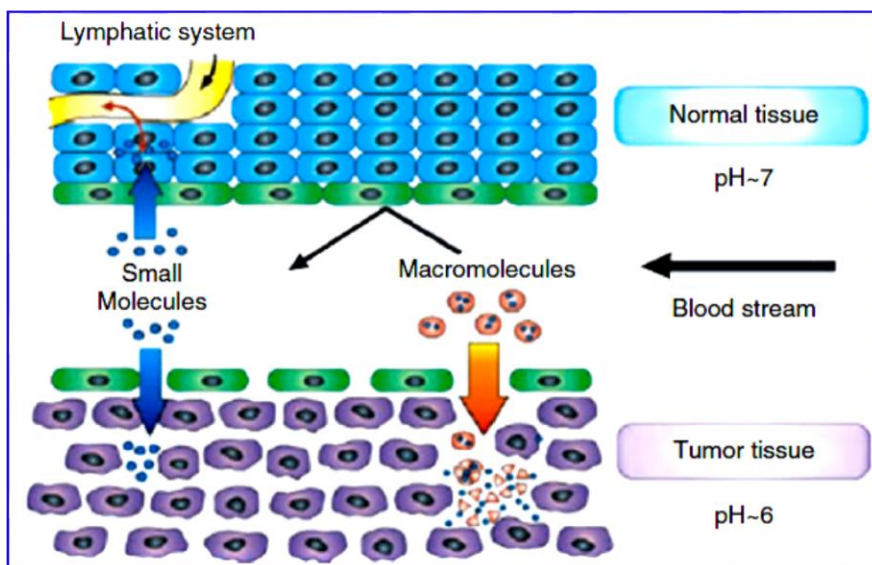


Figure 1.6. Passive targeting of Polymer Drug conjugate by EPR Effect (Adopted from Fanget al. *Adv. Drug Deliv. Rev.* **2011**, 63, 136-151).

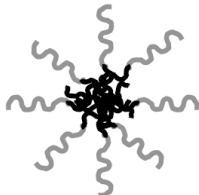
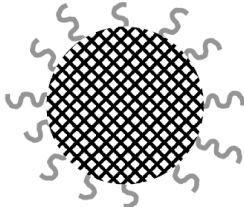
The size and molecular weight of the drug carriers play a major role on the EPR-effect.²⁶⁻²⁸ Particle sizes of 100 to 250 nm with polymer molecular weights less than 40,000 showed enhanced EPR effect for drug delivery.²⁶ However, this effect is not valid for low molecular weight drugs due to its rapid diffusion into circulating blood, and succeeding renal clearance. So the small molecules or nano-particles of < 10 nm based pro-drugs have been identified to show lack of passive selectivity for majority of tumor cells.²⁶⁻²⁸ Hence, EPR effect is now considered to be the main driving force for macromolecule based passive targeting to cancer tissues. The high molecular weight polymer chains possess preferential binding in solid tumor tissue due to the leaky character of tumor blood vessels. Additionally, the binding of hydrophilic segments on the hydrophobic polymeric backbone and vice versa enhances the water solubility of resulting amphiphile and also lowers its toxicity till the loaded drug is delivered to the target site. The combination of these factors makes polymer based controlled drug delivery approaches more attractive for improved delivery and efficacy.²⁶⁻²⁸

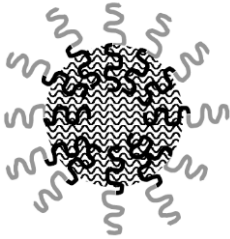
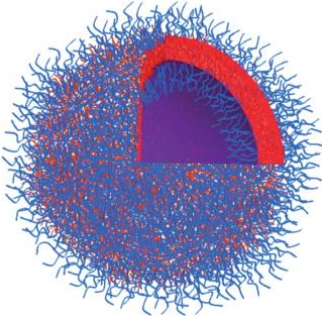
As described earlier, polymer amphiphiles, (block copolymer, random copolymer or graft copolymer) self organize in solution to form various morphologies such as micelles, nanoparticles, nanocapsules, polymeric vesicles or polymersomes and so on (See Table 1.2 for details).¹⁵ Such nanometer sized (1 to 500 nm) polymeric structures offer several advantages: Circulatory longevity with slow rate of renal

clearance, high loading capacity, allowance of direct intravenous injection without blocking blood vessels, controlled delivery by diffusion or polymer degradation mechanism, long exposure of drug at target site via EPR effect, enhanced aqueous solubility of hydrophobic drugs etc. As a result, there have been growing interests in developing new polymeric drug carriers for drug delivery applications.

Polymeric micelles usually have diameters in the range of 10 to 100 nm.²⁹ Structurally, it possesses a core-shell architecture in which the hydrophobic segment of the polymer amphiphiles excluded from the aqueous environment (see entry 1 in table 1.2). This inner core is surrounded by corona composed of the hydrophilic blocks. The hydrophobic inner core provides a space for encapsulation of lipophilic drugs. Low critical micellar concentrations (CMCs) of polymeric micelles make it as an attractive strategy for drug delivery purposes. Nanoparticles are colloidal solid particles in which hydrophobic drug can encapsulate or entrap or chemically conjugate to inner polymeric matrix.^{30, 31}

Table 1.2. Table shows polymeric self organized structures and its characteristics features (Adopted from Letchford et al. *Eur.J.Pharm. Biopharm.* **2007**, 65, 259-265).

Type of Self Assembly	Schematic representation of morphologies	Characteristics
1. Micelle		Size: 10-100 nm Mobile fluid like hydrophobic core and stabilizes hydrophobic drugs. Low CMC (critical micellar concentration)
2. Nanospheres/ Nanoparticle		Size: 10-200 nm Solid colloidal particle Hydrophobic drug entrapped, encapsulated or chemically bound to the polymer matrix.

3. Nanocapsules		Size: 100-300 nm Single layer polymer membrane surrounding an oily core or drug reservoir. Encapsulate hydrophobic drug in the core.
4. Polymersome/ Polymervesicle		Size: 5 nm-500 nm Bilayer polymer membrane surrounds an aqueous reservoir. Encapsulate hydrophilic drugs in core and hydrophobic drugs in membrane. Dual drug loading carriers

Polymeric micelles, nanoparticles and nanocapsules offer platform for delivering hydrophobic drug molecules.²⁹⁻³¹ However, hydrophilic therapeutics such drug, gene (DNA) and proteins were not possible to load in the above carriers. Vesicles with an aqueous reservoir and hydrophobic bilayer membrane serve the purpose of delivery of not only the hydrophilic molecules but also hydrophobic molecules.³²

Vesicles are ubiquitous higher order self organized system exist in all living organisms from archaebacteria to human cells.³³ These specialized assemblies are formed by the self directed association of hydrophobic chains of amphiphilic molecules with the expression of hydrophilic segments on both side of the membrane. The typical structures of vesicles are shown in the table 1.2 (entry 4). Structurally, it has a hydrophilic hollow cavity along with a hydrophobic membrane as seen in the figure. Naturally occurring viral capsids possess vesicular geometry formed by the self organization of viral polypeptides. Vesicles found in cells are composed of amphiphilic lipid molecules (phospholipids) and are termed as lipid vesicles.³⁴

Inspired from the endocytic and exocytic vesicles, researchers have developed synthetic lipid vesicles, which were termed as liposomes (see figure 1.7).³⁴ Since their first reported use in 1965, the importance and reasonableness of liposomes has been identified and persistently improved upon. A large number of drugs, proteins, enzymes and genetic materials (DNA-lipoplexes) have been encapsulated in aqueous or lipid phases of liposomes. Lipid materials used for the preparation of liposomes can be anionic, cationic, neutral or mixture thereof and have a hydrophilic head group and hydrophobic tail. Under aqueous conditions, these molecules form entropically favorable bilayer leaflets, having hydrophobic tails facing each other and hydrophilic head groups facing externally. The close contact of hydrophobic tail with water makes bilayer less stable, which leads to closing its edges to form vesicular structures. The abilities to load wide range of therapeutic materials, availability of various compositions, morphologies, differential release characteristics, lack of immunogenic response and low cost, are the main advantage liposomes.³⁴

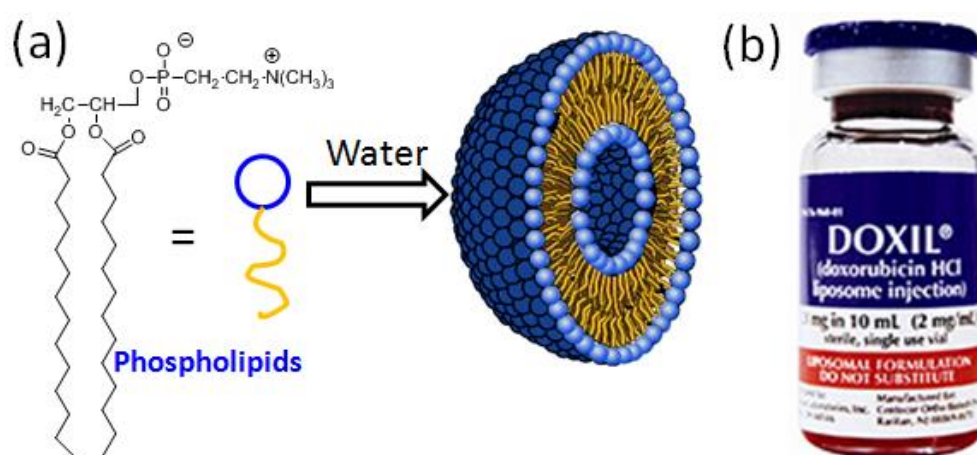


Figure 1.7. (a) Schematic representation of vesicle formation from phospholipid molecules in water (b) Clinically approved liposomal dox-DOXIL.

Table 1.3.. Table depicts the liposomal formulations and type of drug

Drug	Product
Doxorubicin	Doxil, LipoDOX, Myocet
Daunomycin	DaunoXome
Vincristine	Marqibo
Cytarabine	Depo-cyt
Estrogen	Estrasorb
Amphotericin B	Amphotec, Abelcet

Both liposomes and cellular vesicles are made up of lipid molecules having molecular weight less than 1 kDa. These low molecular weight phospholipids impart with lateral fluidity and softness to biomembrane of vesicles that appear beneficial to various cellular processes and detrimental to loading and delivering of therapeutic materials. Compromised mechanical and thermodynamic stability divulged by low molecular weight phospholipids trigger leakage of cargoes under *in-vivo* conditions.³⁵ Consequently therapeutic materials seep out from liposomes (short blood circulation half lives) prior to target site. To overcome these shortcomings of liposomes, PEG lated lipids (stealth liposomes) were developed and it provided not only the polymeric properties but also protection from the clearance by reticulo endothelial system (RES).³⁶ DOX loaded stealth liposomal formulations (Doxil, Myocet) are widely used for the treatment of various cancers (see figure 1.7b for image of Doxil available in market). However, serious adverse effects such as neutropenia, thrombocytopenia, peripheral neuropathy, hand-foot syndrome (redness, tenderness and peeling of the palms of the hand and soles of the feet) etc, limit its long term clinical use. Some of the marketed liposomal formulations enlisted in the table 1.3.³⁶

In retrospect, nature selected polypeptides (made of 100 to 200 amino acids, about 10 kDa) constituted viral capsids for protecting viral genetic materials. Motivated from viral capsids, researchers have developed polymer based vesicular structures, known as polymersomes.³⁷ Compared with liposomes, polymersomes have several distinctive advantages. Elasticity, permeability, mechanical stability and thickness of molecularly thin enveloping membrane of polymersomes can be controlled by selecting hydrophobic block of the copolymer having suitable molecular weight.³⁵ The high molecular weight of polymers compared to lipids offer thicker, stronger and tougher membrane to polymersomes and makes it inherently more stable than liposomes. Furthermore; the greater chemical diversity bestowed by the polymeric building blocks assist the development of a variety specialized polymersomes including stimuli responsive and targeted drug delivery vehicles.^{38,39} Moreover, like liposomes, the interior hydrophilic hollow cavities of polymeric vesicles stabilize water soluble molecules. Molecularly thin hydrophobic membrane of polymersomes can solubilize adequate quantity of water insoluble molecules. Owing to these dual encapsulation capabilities and higher mechanical stability, polymersomes become most promising self assembled system and found wide range of applications in nano medicine, material science, and biology.^{32, 33}

1.4. Polymer Vesicles

Pioneering work in the area of polymersomes was extensively carried out by Discher and co-workers using block copolymer poly (ethylene oxide)-b-poly-(ethyl ethylene) (PEO-b-PEE).^{22, 23} Later, numerous polymers have been explored in the literature for the preparation of polymeric vesicles. Block copolymers investigated for the purpose of vesicle formation have identical amphiphilic character as that of lipids, but be composed of covalently connected two or more distinct polymer segments. The ultimate form of copolymer can be managed by their synthesis to obtain block (diblock, triblock, multiblock), random block and graft copolymers (see figure 1.8a)⁴⁰

Polymer vesicles are believed to form in two steps: Initially bilayer type membranes formed from polymer sheets. Later the change in interfacial curvature and corresponding change in packing parameters support the closure of the membrane to form hollow structures (see figure 1.8b).⁴¹ Recent detailed simulation studies proved that the formation vesicles go through more complex steps.⁴¹ Based on these studies, two different mechanisms were proposed. In mechanism I, block copolymers self organize to form small micelles which evolve into large micelles and cylindrical flat shaped micelles. These large discs like micelles later close to form polymersomes.⁴¹

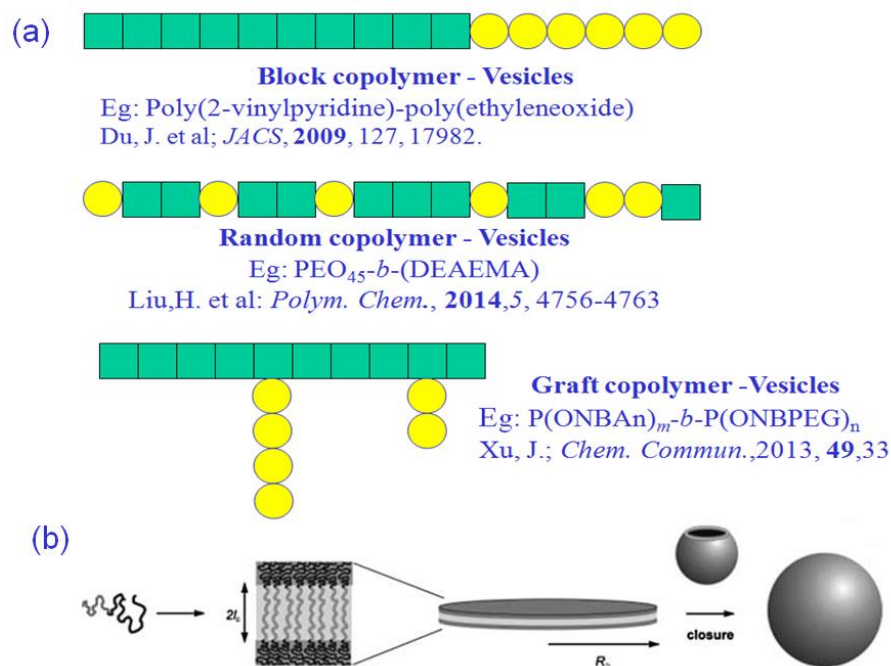


Figure 1.8. (a) Diagrammatic representation of various copolymers and (b) mechanism of vesicle formation (Adopted from Antonietti, et al. *Adv. Mater.* **2003**, 15, 1323-1333)

According to mechanism II, small micelles initially formed grow up to form larger micelles, which subsequently take solvent to reduce energy via evaporation condensation like processes to form vesicles. The amphiphilic block copolymers, amphiphilic alternating copolymers, hydrophilically modified copolymers etc. were reported to form vesicles.⁴² Poly (N-isopropylacrylamide)-b-poly (ethylene glycol) (PNIPAAm-PEG),⁴³ Poly(styrene)-b-poly(acrylic acid) (PS-PAA),⁴⁴ Poly (vinyl pyridine)-b- poly (ethylene glycol),⁴⁵ Poly (butadiene)-b-poly(glutamic acid) (PBD-PGA)⁴⁶ Poly (lactic acid)-b- poly (ethylene glycol) (PLA-PEG),^{47,48} polyethers like poly(butylene oxide)-poly(ethylene oxide) (selectively permeable vesicle)⁴⁹ or poly(ethylene oxide)-poly(lactic acid), poly(ethylene oxide)-polycaprolactone and poly(ethylene oxide)- poly(trimethylene carbonate) (biodegradable vesicles)⁵⁰ are the some of the examples of diblock copolymers which self organize to form polymersomes. These copolymers have diverse conformation depending on its architectures.

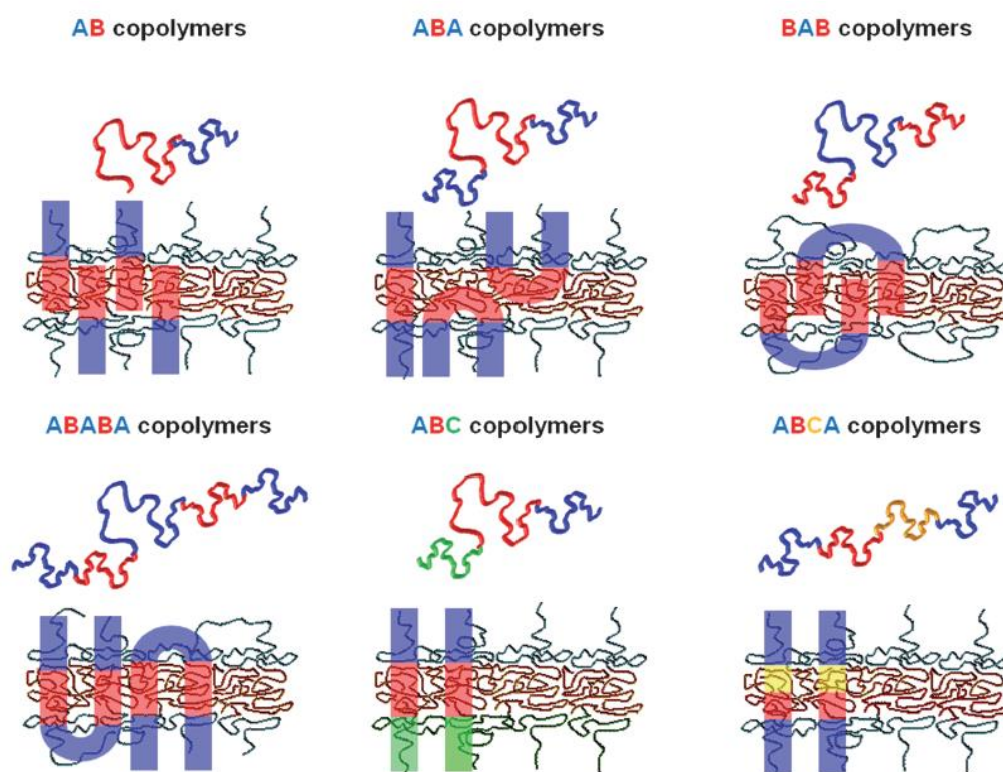


Figure 1.9. (a) Membrane conformation of polymersomes formed by diblock, triblock and multiblock copolymers (Adopted from Lopresti et al. *J.Mater.Chem.* **2009**, 19, 3576-3590).

Tri-block copolymers may be in two forms: hydrophobic-hydrophilic-hydrophobic or hydrophilic-hydrophobic-hydrophilic copolymers.⁴⁰ The former

attains one molecular conformation that can direct membrane formation and the chain end of hydrophobic segments assembles into membrane. In contrast, hydrophilic-hydrophobic-hydrophilic type of triblock accomplish two probable conformations: Hydrophilic chains express on the same side of the membrane and hydrophobic block forms a loop or they can stretch forming a monolayer with the two hydrophilic blocks at the two sides of the monolayer membrane.⁵¹⁻⁵³ Analogous conformations can be produced by other higher copolymers as shown in the figure 1.9.

There is rising appeal in cellular targeting and delivery of therapeutically and biologically pertinent molecules such as protein, anticancer drugs and genes.³⁵⁻⁴⁰ The bioavailability of these molecules can be enhanced by loading into nanocarriers. Polymersomes play a significant role for this purpose owing to its large hydrophilic compartment and hydrophobic membrane to load both water soluble and insoluble molecules (see figure 1.10). Additionally, low critical vesicular concentration (CVCs) and slow chain exchange dynamics of amphiphilic block copolymers impart slow dissociation to polymersomes.

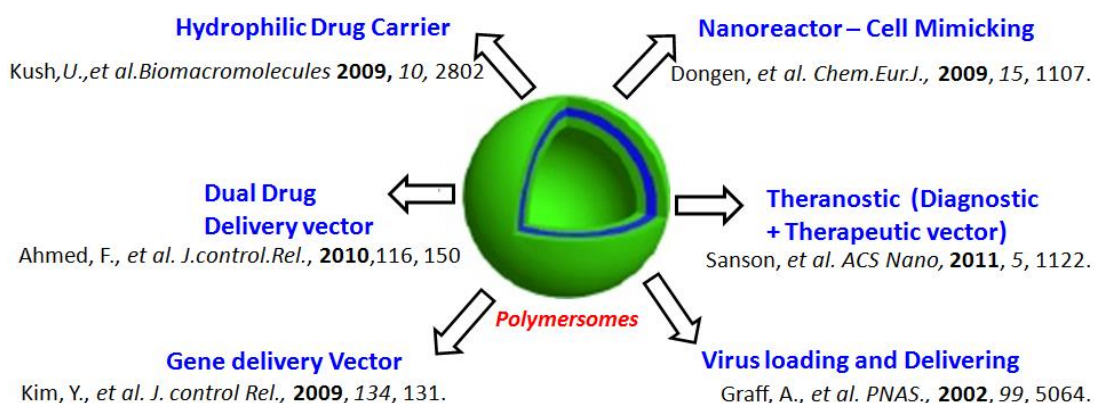


Figure 1.10. Therapeutic applications of polymersomes (adopted from ref 54-60).

Water soluble anticancer drug molecule like DOX.HCl has successfully encapsulated into vesicles. For instance, Lecommandoux and coworkers reported poly(g-benzyl L-glutamate)-block-hyaluronan (PBLG-b-HYA) based polymersomes for loading and delivering DOX.HCl as shown in the Figure 1.11a.⁵⁴⁻⁵⁷ Several investigators showed dual encapsulation capabilities of polymersomes. Chen, *et al.* demonstrated polymersomes self organized from diblock copolymer of PEG and polycarbonate containing trimethoxybenzylidene acetals (PTMBPEC) loaded with both water soluble DOX.HCl and water insoluble Paclitaxel (PTX) anticancer drug molecules (See Figure 1.11b).⁵⁸

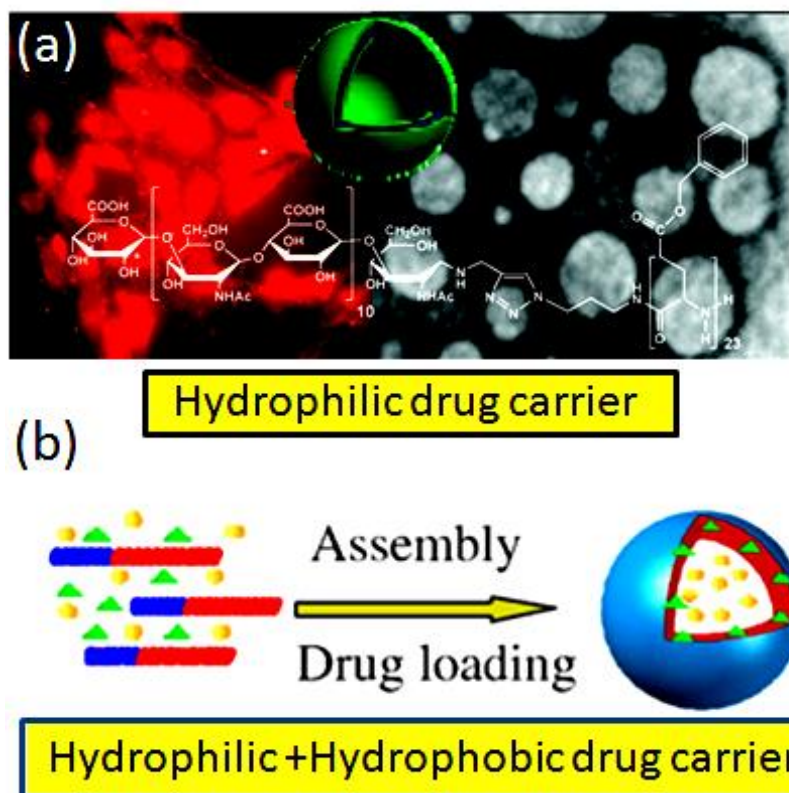


Figure 1.11. Applications of polymersomes in (a) hydrophilic drug delivery and (Adopted from Upadhyay *et al.* *Biomacromolecules*, **2009**, 10, 2808- 2808) (b) dual drug delivery DOX (interior) and PTX (on the walls) (Adopted from Ahmed *et al.* *J.control.Rel.*, **2010**,116, 150).

The delivery of genetic materials (eg. siRNA, DNA) is extremely challenging due to presence of degrading endolysosomal enzymes and clearance by the organs such as liver and spleen. So permeation of these oligonucleotides to target site is most strenuous objective to be achieved. Encapsulation of water soluble genetic materials in the inner aqueous cavity of polymersomes protects from oligo-degradation and clearance from the body. For example, Lomas *et al.* explored polymersomes formed from PMPC-PDPA diblock copolymer for DNA delivery applications.⁵⁹ The study proved that the polymersomes ensured protection of GFP encoded plasmid. The expression of GFP proteins within the cytosol established the delivery capabilities of vesicles. Kim *et al.* showed RNAi or antisense oligonucleotide (AON) loading abilities of polymersome prepared from the blend of fluorescent labelled PEG-polycaprolactone and PEG-polybutadiene (1:4 ratio).⁶⁰ The *in vitro* cellular studies illustrated endolysosomal escape of loaded polymersomes and delivery to cytosol or

nucleus of cells. Mouse model studies using AON loaded polymersomes displayed intracellular delivery and expected protein expression.

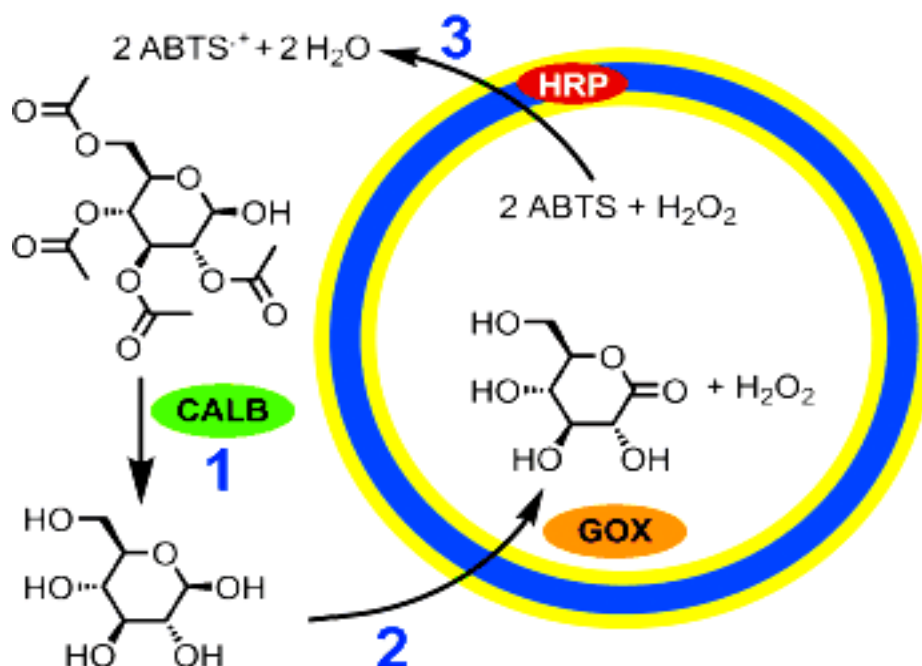


Figure 1.12. Applications of polymersomes as a nanoreactor to mimic cellular enzymatic reactions (Adopted from Dongen *et al.* *Chem.Eur.J.*, **2009**, 15, 1107).

The intracellular environment is extremely complex medium, in which numerous multistep reactions take place. These enzyme catalyzed multistep reactions are achieved by compartmentalization of intracellular environment. Polymersomes with hydrophobic membrane and inner hydrophilic cavity can mimic such compartments and act as nanoreactor to provide protective environment to enzymes, substrates and products. The controlled permeability allows diffusion of materials in and out of the vesicles. Dongen *et al.* illustrated a three enzyme cascade reaction as shown in the figure 1.12. In this approach one enzyme trapped in the hydrophilic cavity, and second enzyme was loaded in the hydrophobic membrane. The third enzyme was anchored on the surface of the polymersome via clickable linkages.⁶¹

Sanson *et al.* reported polymersome formed from poly(trimethylene carbonate)-b-poly(L-glutamic acid) (PTMC-b-PGA) blockcopolymer loaded with magnetic iron oxide nanoparticles (maghemite or $\gamma\text{-Fe}_2\text{O}_3$) in the hydrophobic membrane and DOX in the inner aqueous reservoir (See Figure 1.13).⁶² These superparamagnetic hybrid polymersomes serves as a theranostic (diagnostic + therapeutic) via magnetic resonance imaging (MRI) with the help of embedded iron

oxide particle and chemotherapy by controlled delivery of loaded DOX molecules. However, membrane permeability polymersomes explained in the above example is not tuneable. Graff *et al.* reported an elegant methodology in the direction of the creation of semi-permeable polymersomes by reconstitution of LamB protein in PDMS-b-PMOXA polymersomes. LamB protein was easily identified by bacteriophage lambda and utilized to dock the phage.⁶³ Subsequently, the phage injected its DNA into the polymersome and was well captured by HR-TEM imaging method.

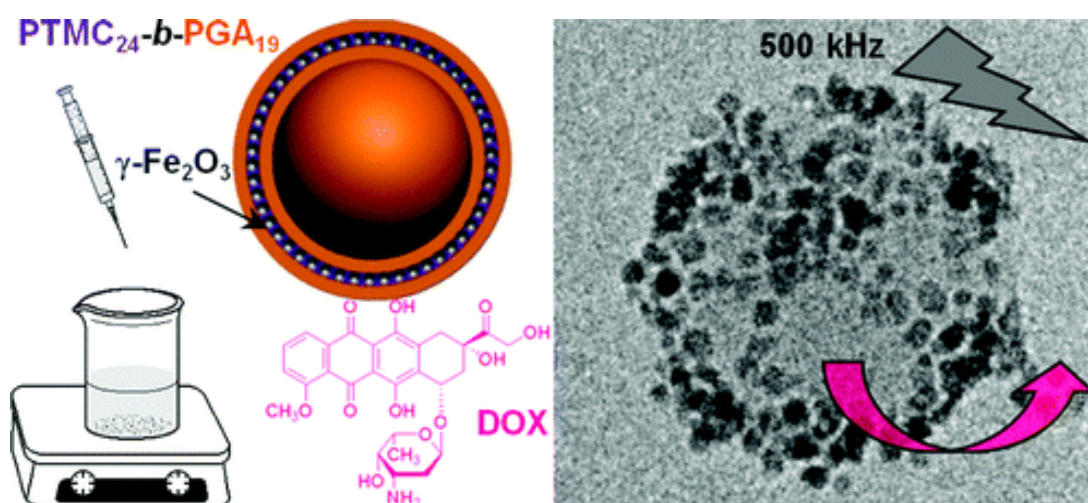


Figure 1.13. Applications of polymersomes as diagnostic (Fe_2O_3 nanoparticle on the walls for MRI imaging) + therapeutic DOX (theranostic) (Adopted from Sanson *et al.* ACS Nano, **2011**, 5, 1122).

The above discussion proved that an assortment of properties can be incorporated in the molecular design of polymersomes. Other than encapsulation of hydrophilic and hydrophobic molecules and conjugation of drug molecules, programmable disassembly (stimuli responsive polymeric materials), attachment of recognition antibodies, proteins, glycolipids, and carbohydrates for targeting to specific site, surface functionalization to achieve altering circulation half lives, biocompatibility etc. can be integrated during molecular design processes of polymersomes.^{32, 33} Recently, many efforts have been aimed at the development of internal or external stimuli responsive polymersomes for programmable delivery.

1.5 Stimuli Responsive Polymer Vesicles

Stimuli responsiveness of polymersomes means its capability to accept, convey the stimulus, and retort with a useful effect. The responsive delivery is highly desirable since fast and slow delivery lead to systemic side effects and cellular resistances to drug molecules respectively. In the past few years, various endeavours were focussed to the preparation of intelligent polymeric vesicles that respond to internal stimuli such as pH, enzymes, redox potential, temperature and external stimuli such as light, ultrasound and magnetic field.³⁵ The internal stimuli sensitive vesicles are more attractive due to their prospective cellular targeting applications, industrial scale up and cost considerations. Examples of the internal stimuli sensitive polymer vesicles are described below.

1.5.1. pH Sensitive Polymer Vesicles:

Existence of wide range of pH gradients in microenvironment of organ, tissue, cell organelles, and extra cellular environment in physiological system inspired the development of pH responsive block copolymer vesicles.⁶⁴ Du *et al.* developed diblock copolymer vesicles from biocompatible monomer, 2-(methacryloyloxy)ethyl phosphorylcholine (MPC), and a second monomer, 2-(diisopropylamino)ethyl methacrylate (DPA).⁶⁵ The tertiary amine group gives pH sensitivity to the membrane wall. Upon changing the solution pH from 2 to above 6, PMPC-PDPA formed vesicles spontaneously which loaded DOX and release under acidic condition (see figure 1.14).⁶⁵

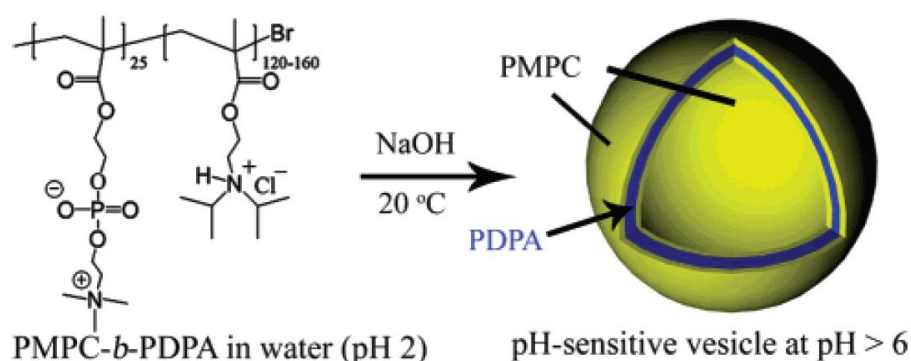


Figure: 1.14. Preparation of PMPC-PDPA block copolymer vesicles (Adopted from Du *et al.* *J.Am.Chem.Soc.* **2005**,127, 17982-17983).

Same group reported PMPC-*b*-PDMA-*b*-PDPA and PMPC-*b*-PDPA-*b*-PDMA triblock copolymers as well as PMPC-*b*-P(DMA-stat-DPA) block-statistical

copolymer with variable pH trigger point from 6.2 to 6.8.⁶⁶ They also reported pH-responsive degradable chimaeric polymersomes poly(ethylene glycol)-b-poly(2,4,6-trimethoxybenzylidene-1,1,1-tris(hydroxymethyl)ethane methacrylate)-b-poly(acrylic acid) (PEG-PTTMA-PAA) triblock copolymers (see figure 1.15).⁶⁷ These polymers were prepared by reversible addition-fragmentation chain transfer copolymerization (RAFT) method. The polymersome loaded with DOX.HCl underwent disassembly between pH 4.0 and pH 5.0. MTT assays in HeLa cells showed that DOX.HCl-loaded PEG-PTTMA-PAA polymersomes produced high anti-tumor activity with IC₅₀ of 1.48-1.67 µg/mL, close to that of free DOX.HCl.

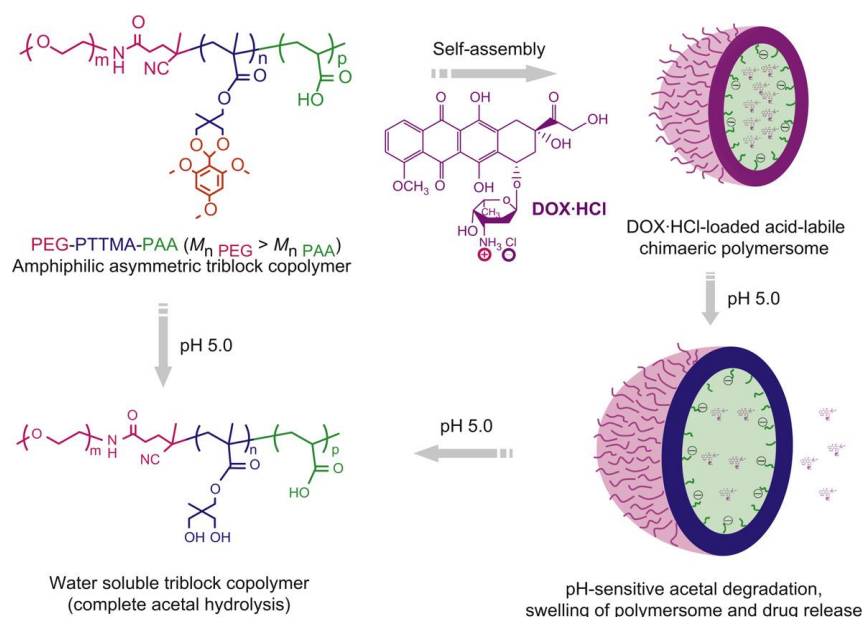


Figure: 1.15. Schematic representation of pH-responsive chimaeric polymersomes prepared from PEG-PTTMA-PAA triblock copolymers (PEG block longer than PAA block) for loading as well as endosomal pH-triggered elution of anticancer doxorubicin hydrochloride (Adopted from Du et al. *Biomaterials*. **2012**,33, 7291-7299).

Yang et al. reported preparation of pH responsive vesicle from folate (FA) or methoxy)-poly(ethylene glycol)(Mw:5000)- poly(glutamate hydrozone doxorubicin)-poly(ethylene glycol)(Mw:2000)-acrylate.⁶⁸ The inner hydrophilic PEG layers were cross linked using acrylate group to increase the *in-vivo* stability while folic acid (FA) serves active tumor targeting. DOX chemically conjugated using pH sensitive hydrazone linkage to polyglutamate segment of triblock copolymer and it formed the hydrophobic membrane of polymersomes. Super paramagnetic iron oxide (SPIO) nanoparticle was loaded into the aqueous cavity of polymersome to perform magnetic

resonance imaging (MRI). The whole assembly was schematically shown in Figure 1.16.

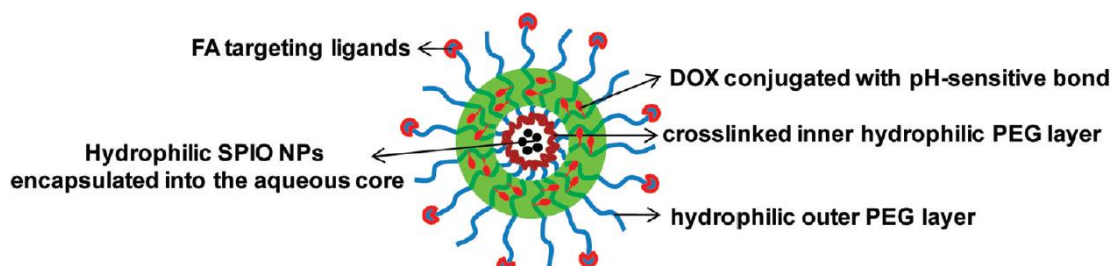


Figure: 1.16. Schematic representation of pH-responsive SPIO/DOX loaded vesicles prepared from amphiphilic triblock copolymer having PEG cross linked inner layer (Adopted from Yang et al. ACS Nano. 2010,4, 6805-6817).

DOX chemically conjugated was shown to get released under acidic condition through destabilization of hydrazone linkage. MTT assay in HeLa cells proved the cytotoxic potential of SPIO/DOX-loaded vesicles.

1.5.2. Temperature Sensitive Polymer vesicles

Thermo-sensitive polymers display a volume phase transition at defined temperatures, are used for the preparation of temperature-responsive polymersomes.⁶⁹ Polymers soluble in an aqueous environment at room temperature or under cold condition, while on heating precipitates, have a lower critical solution temperature (LCST). Polymers that become soluble on heating in an aqueous condition have an upper critical solution temperature (UCST). Temperature sensitive polymersomes are useful for treating tumors having slightly higher temperature (2 to 5° C) compared to normal cells.⁷⁰

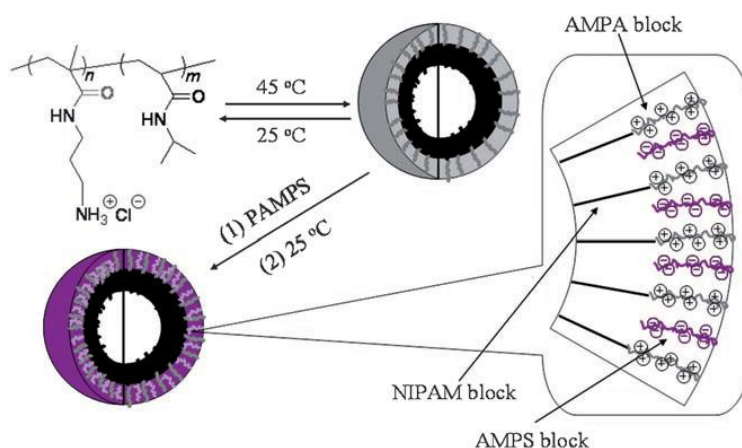


Figure: 1.17. Structure and schematic representation of temperature-responsive PAMPA-b-PNIPAM forming vesicles and subsequent cross linking using PAMPS (Adopted from Li et al. Angew.Chem.Int. Ed.. 2005, 45, 5792-5795).

Li *et al.* reported an elegant temperature responsive diblock copolymer poly(N-(3-aminopropyl)methacrylamidehydrochloride)-block-(N-isopropylacrylamide) (PAMPA-b-PNIPAM) synthesised using RAFT polymerization method. The polymer exists as unimers in water at room temperature, but when the solution temperature increased above LCST of PINIPAM polymer, formation of polymersomes was observed (See Figure 1.17).⁷¹ The vesicles were further locked by adding oppositely charged PAMPS: poly(N-(3-aminopropyl) methacrylamide hydrochloride) polyelectrolyte.

1.5.3. Enzyme Sensitive Polymer Vesicles

Expression levels of enzymes vary in pathological conditions such as cancer and other inflammations. This altered expression profiles can be exploited as an elegant biocompatible strategy to achieve targeted delivery of therapeutics. For instance, Guo *et al.* reported supramolecular binary vesicle composed of p-sulfonatocalix[4]arene as macrocyclic host and natural enzyme cleavable myristoylcholine guest molecules. The vesicles were proved to be dissociated specifically in the presence choline esterase enzyme. (See Figure 1.18).⁷² Choline esterase enzyme is overexpressed in Alzheimer's disease and hence, present system was proposed to be an ideal drug carrier for Alzheimers disease.

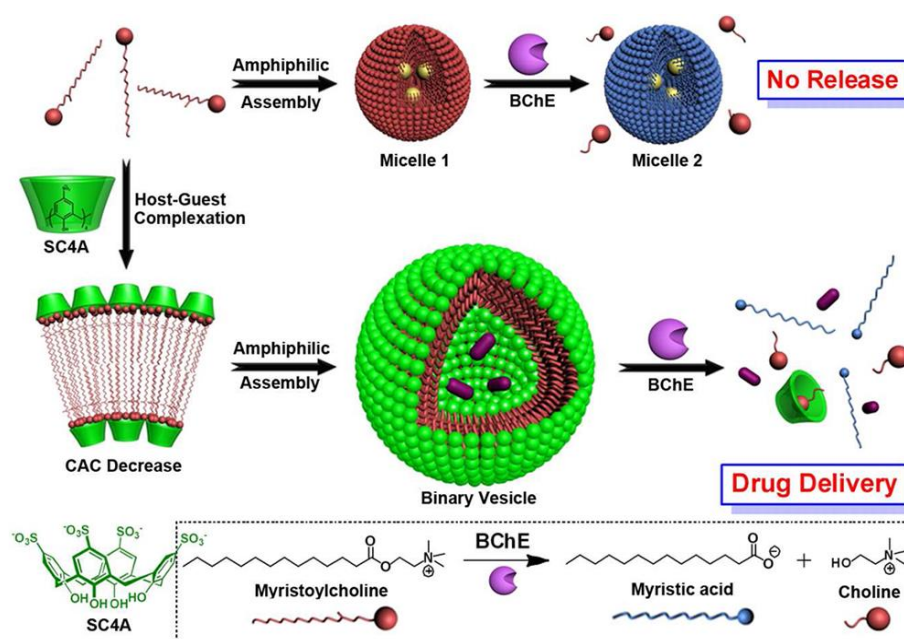


Figure: 1.18. Structure and schematic representation of choline esterase-responsive supramolecular binary vesicles formed from p-sulfonatocalix[4]arene and myristyl choline (Adopted from Guo *et al.* *J.Am.Chem.Soc.* **2012**,134, 10244-10250).

Some cancer cells are over expressed with thiols including glutathione (GSH, 10 μ M in human plasma, milli molar concentration in cancer cells) offer large disparity in redox potential between the extracellular and intracellular environments. Therefore molecule with disulfide bonds undergoes rapid cleavage in the cancer intracellular conditions. Ren *et al.* exploited the above concept for the development of polymersomes from disulfide-bond-linked triblock copolymer of poly(ethylene glycol) (PEG) and poly(ϵ -benzyloxycarbonyl-L-lysine) (PzLL).⁷³ Confocal studies using DOX loaded vesicles incubated Hela cells proved the rapid degradation of vesicles and subsequent accumulation of DOX in nucleus.

Even though much advancement has been happened in the past decade, most polymer vesicular systems reported so far suffer from serious limitations of being not biodegradable, non biocompatible, slow responsiveness to internal and external stimuli, or a lack of target capability. Moreover, most of the synthetic polymers have debatable biodegradability and biocompatibility, limited water solubility, and lack of innate bioactivity or bioadhesion property, which restrict their application for drug delivery.³³ Naturally available polysaccharide system which combat most of the above disadvantages of synthetic polymers could be considered as alternative platform for drug delivery application.⁷⁴

1.6. Polysaccharide as Building Block for Drug Delivery Systems:

Polysaccharides composed of many molecules of monosaccharide units joined together by glycosidic bonds. These carbohydrate polymers are obtained from various natural resources such as animal origin (chitosan, heparin, hyaluronan and chondroitin), algal origin (e.g: Alginate and carrageenan), plant origin (e.g: starch [mixture of amylose and amylopectin], cellulose, pectin, guar gum) and microbial origin (e.g.: dextran and xanthan gum).⁷⁵ Polysaccharides can have linear or branched design depending upon the nature of constituent monosaccharide units. The chemical structures of some of the important polysaccharides are given in the figure 1.19. The superior biocompatibility, biodegradability, stability, availability in range of molecular weight, hydrophilicity, non toxicity and low cost makes this natural material most attractive scaffold for drug delivery applications.^{74,75} Under physiological conditions, most of the polysaccharides undergo enzymatic degradation to form monosaccharide or oligosaccharide units which are recycled for cell signaling and structural support or storage functions.⁷⁶ For example, enzyme glycosidase

participates in the breakage of glycosidic linkages of many polysaccharides.⁷⁷ Moreover, other lysosomal degradative enzymes such as esterase and proteases also rupture lengthy polysaccharide chain subsequent to endocytosis.⁷⁸ On the other hand, dextranase and hyaluronidase are example for polysaccharide specific enzymes which hydrolyze precisely dextran and hyaluronic acid, respectively.⁷⁷

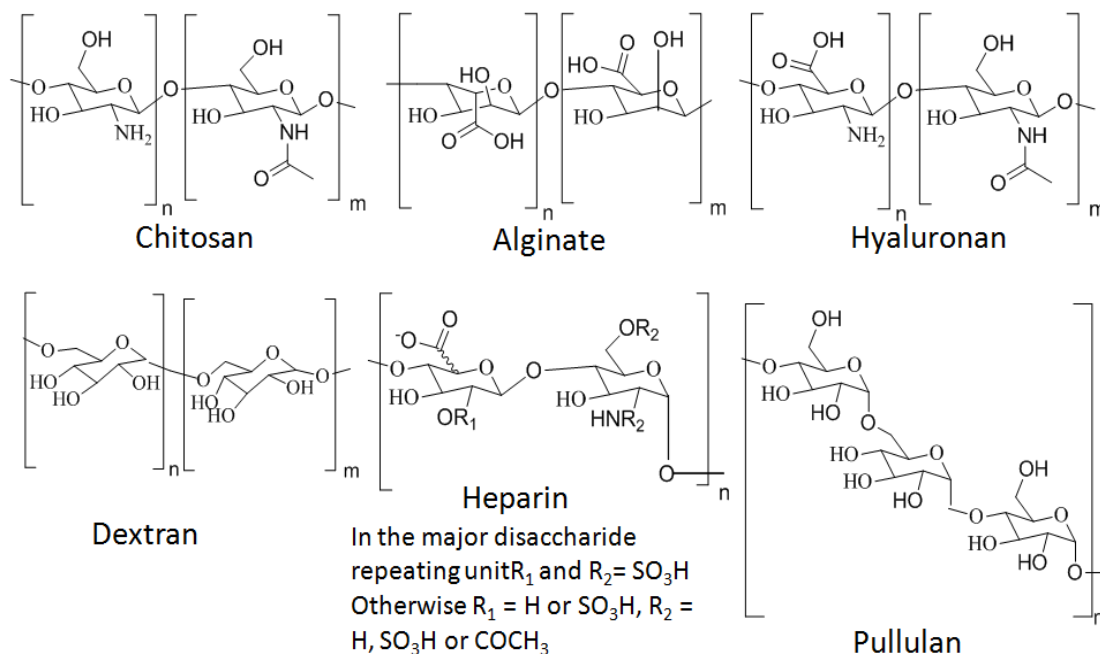


Figure: 1.19. Chemical Structures commonly used polysaccharides for drug delivery applications (Adopted from Mizrahy et al. *Chem. Soc. Rev.* **2011**, 41, 2623-2640).

Presence of large number of functional group on the molecular chain assists effortless chemical as well as biochemical modification of polysaccharides. Starch, dextrin, dextran and cellulose have abundance of hydroxyl group for modification.^{75,79} The carboxyl group in hyaluronic acid, alginate, heparin⁸⁰ and amine group in deacetylated chitosan⁸¹⁻⁸³ can be readily derivatized with tailored/determined properties. Additionally, the occurrence of hydroxyl group and amine group on polysaccharide backbone enhances its aqueous solubility. The solubility, hydrophobicity, biological and physiochemical properties of polysaccharide can be tuned by monomer modification via oxidation, esterification, amidation, sulfation and grafting techniques. The solubility of chitosan under acidic condition can be enhanced by increasing deacetylation process.⁸¹⁻⁸³ Another advantage of polysaccharide is its inherent bioactivity especially bioadhesive properties on mucosal layers. Positively charged polysaccharide (chitosan) can bind to negatively charged mucosal surfaces.⁸⁴

⁸⁵ The innate mucoadhesive property of polysaccharide helps in targeting of specific organs and to improve drug residence time. All the above advantages provide credibility to polysaccharide for its potential in drug delivery applications.

For instance, cationic polysaccharide chitosan based systems were extensively studied as carriers for drugs, proteins, DNA, Si RNA, peptones etc. Zhou *et al.* reported chitosan modified using hydrophobic deoxycholic acid hydrophilic glycidol molecules (see figure 1.20).⁸⁶ The resultant amphiphilic molecules were shown to form nanoparticles of sizes between 160 to 200 nm in water. Doxorubicin was encapsulated in nanoparticles and showed significant antitumor efficacy against MCF 7 breast cancer cell lines.

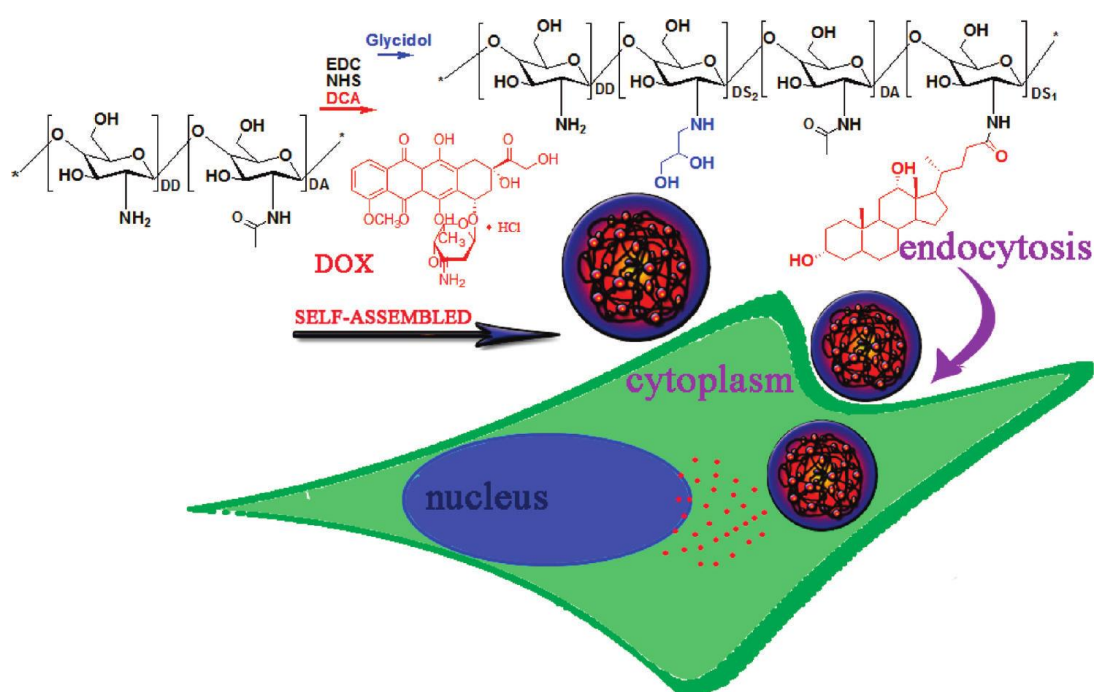


Figure 1.20. Synthetic route of Glycidol-Chitosan-Deoxycholic acid amphiphilic molecule and its doxorubicin loading for intracellular drug release (Adopted from Zhou *et al. Biomacromolecules*, **2010**, 11, 3480-3486).

Hyaluronic acid is an important polysaccharide widely employed for drug delivery due to its high water solubility, biodegradability, non immunogenicity and CD44 binding capacity.⁷⁴ Moreover the availability of large number of functional groups on its backbones allows easy conjugation of drug molecules. Recently, El-dakdouk *et al.*, demonstrated doxorubicin conjugated polysaccharide hyaluronic acid coated with super paramagnetic iron oxide nanoparticles (SPION) for treatment against drug resistant CD 44 over expressed ovarian carcinoma (See figure 1.21).⁸⁷

The *in-vivo* studies using ovarian cancer bearing mice showed preferential accumulation of DOX molecules and reduction of tumor. Additionally, the presence of SPION nanoparticles allowed to monitor nanoparticles accumulation using MRI.⁸⁷

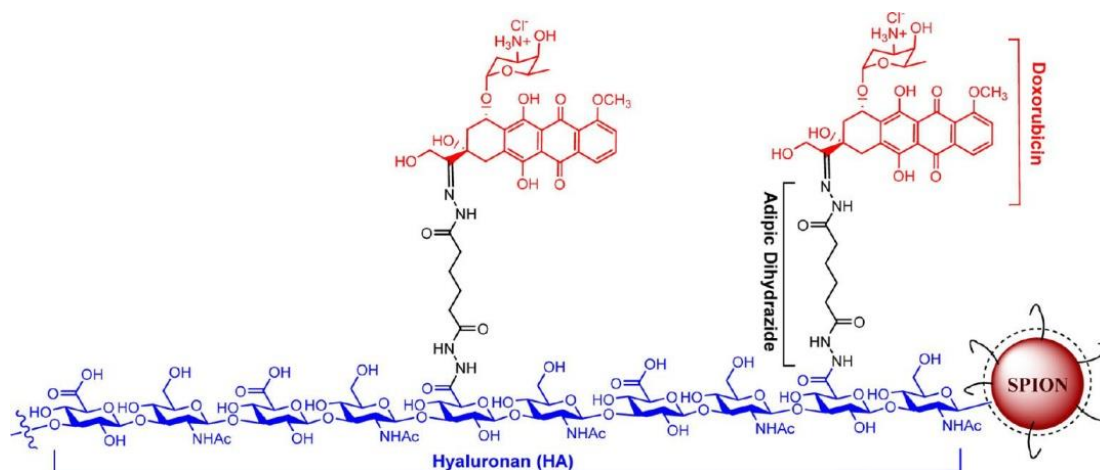


Figure 1.21. The chemical structure of Hyaluronan conjugated with doxorubicin anticancer drug molecules and SPION nanoparticles. (Adopted from El-dakdouk *et al.* ACS Appl. Mater. Interfaces, **2014**, 6, 697-705).

Alginate is an anionic polysaccharide widely used for developing drug delivery carrier due to its high biocompatibility, water solubility and mucoadhesive properties.⁷⁴ For example, Zhang *et al.* developed glycerhethinic acid modified alginate loaded with doxorubicin drug molecule for delivery to liver cancer cells (see figure 1.22).⁸⁸ *In-vivo* studies in mice proved the selective accumulation of nanoparticles in liver and subsequent reduction of tumor.

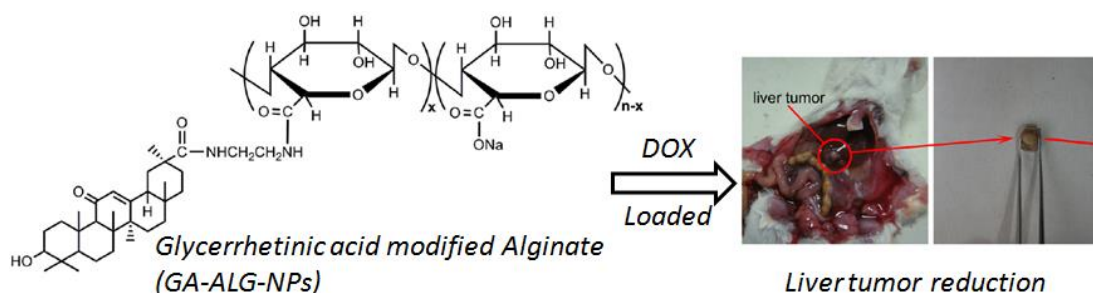


Figure 1.22. The chemical structure of glycerhethinic acid modified alginate and doxorubicin delivery to liver cancer cells (Adopted from Zhang *et al.*, Biomaterials, **2012**, 33, 2187-2196).

Pullulan is a neutral homopolysaccharide obtained produced from starch by the micro organism *Aureobasidium pullulans*.⁷⁴ Hydrophilicity, adhesive properties and low viscosity upon water dissolution makes Pullulan an attractive starting material for drug delivery vector development. Akiyoshi *et al.* reported hydrophobic cholesterol modified pullulan nanoparticles. These nanoparticles were later showed for its ability to carry various protein molecules including insulin hormone.⁸⁹

Compared to various polysaccharide described above, micro-organism derived dextran has been widely used in clinics for more than five decades as antithrombolytic agents, MRI contrast components and, plasma volume expander etc.^{90,91} Hence, suitable chemical modification of this clinically valuable polysaccharide-dextran into amphiphilic structures and self-assembling them as nanometer sized morphologies for drug delivery application is very much in demand.⁹²⁻⁹⁴ The structural properties, functionalization techniques and examples of self organized strictures from dextrans are described below.

1.7. Polysaccharide: Dextrans

Dextrans are family of neutral polysaccharides obtained from the cultures of gram positive anaerobic cocci microorganism such as *Leuconostoc mesenteroids* and *Streptobacterium dextranicum*.⁹¹ Clinically, dextrans are used for more than six decades as plasma volume expander, antithrombolytic agent and peripheral blood flow promoter etc.⁹¹ More than 2000 tonnes of dextran are produced annually for various medical and industrial applications. Structurally, it consists of anhydro-D-glucopyranose ring predominantly linked by α -(1 $\rightarrow$ 6) glycosidic linkages with some degree of branching at α -(1 $\rightarrow$ 4) positions.^{91, 95} Dextrans with branching at α -(1 $\rightarrow$ 3) and α -(1 $\rightarrow$ 2) positions are also obtained from various microbial cultures (see figure 1.23). The degree of branching depends on the type cultures used for the production and is vary from 0.5 to 60 %.⁹⁵ In addition to degree of branching, molecular weight (MW), relative amount of particular type of glycosidic linkages, solubility, optical activity, and physiological action of dextran can be tuned by selecting appropriate strains of microorganism.⁹⁵

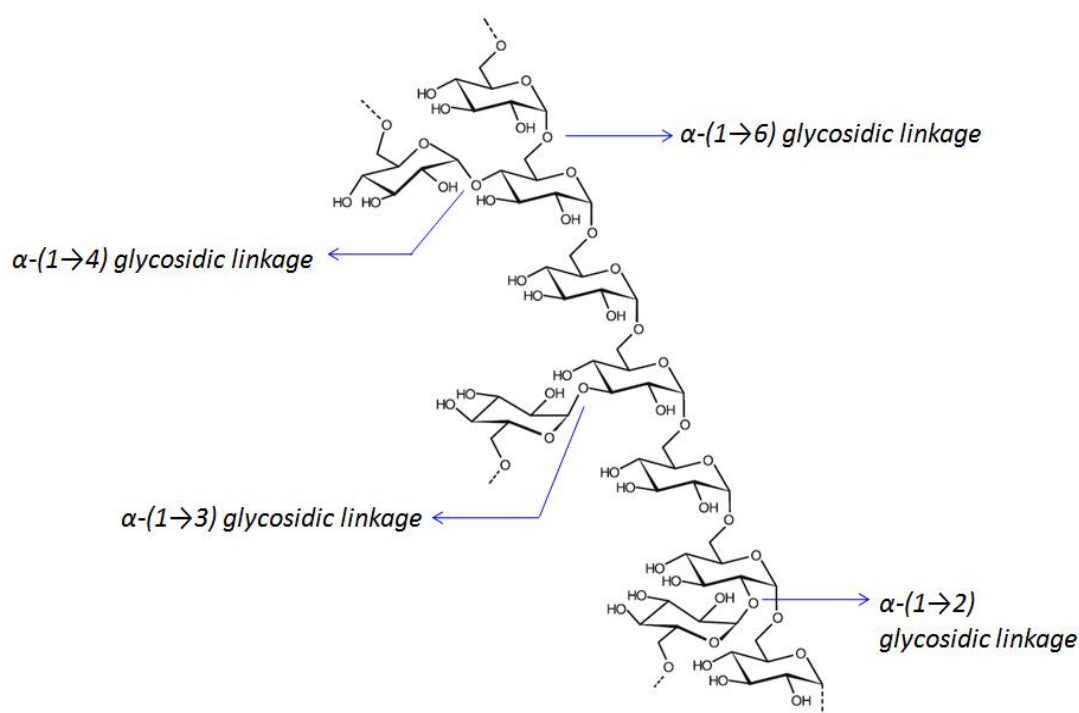


Figure 1.23. Chemical structure of dextran showing α -(1 $\rightarrow$ 6) glucose units and having branching at α -(1 $\rightarrow$ 4) or α -(1 $\rightarrow$ 3) or α -(1 $\rightarrow$ 2) positions depending upon type of bacterial strains used for the production. (Adopted from Heinze et al. *Adv. Polym. Sci.* **2006**, 205, 199-291).

The high molecular weight (9×10^6 to 5×10^8 g/mol) and polydispersity of native dextrans are reduced partial depolymerisation methods (e.g. acid hydrolysis) followed by fractionation techniques.⁹¹ Dextrans with narrow molecular weight range from 4-200 kDa and polydispersity < 1.5 are available commercially for chemical modification. The presence of large number of hydroxyl group aid easy chemical conjugation, and enhances the water solubility of dextran polymer. The aqueous solubility of dextran is decreased with increase in degree of branching. Dextrans with higher branching ($> 45\%$, α -(1 $\rightarrow$ 3)) have been determined to be practically insoluble in water. Most of the commercially available and clinical dextrans (40 kDa or 70 kDa) have low degree of branching (0.5 %). The presence of α -(1 $\rightarrow$ 6) glycosidic linkages improves chain mobility, which assist the solubility of dextrans in DMSO, DMF, DMA/LiCl, ethylene glycol, glycerol and 2M aqueous glycine. Dextrans attain rod like shape at low concentration and achieve more compact coil geometry at higher concentrations.⁹⁵

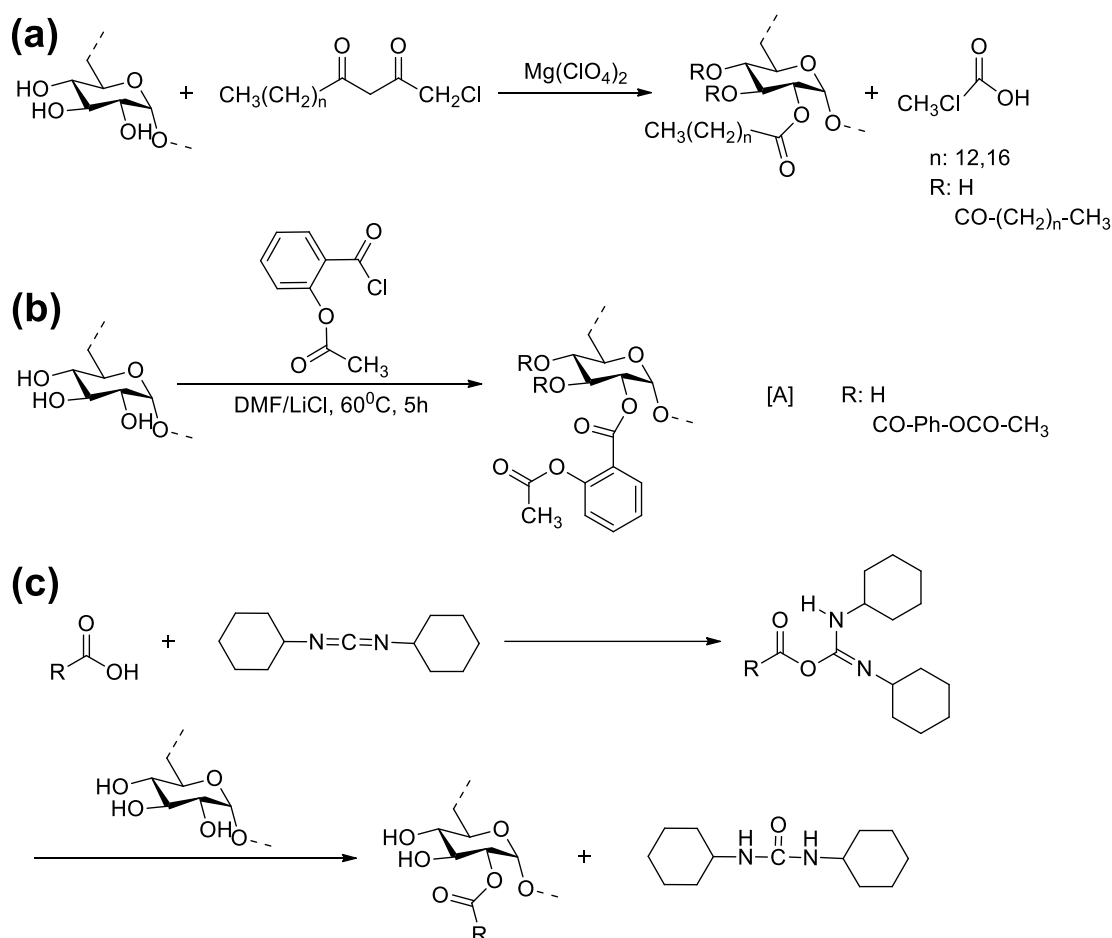
Dextrans have negligible bioavailability after oral administration.⁹⁶ This is due to the prevention of bigger sized dextran (1.5 nm) through aqueous pores (< 0.75 nm) of gastrointestinal tract. The higher molecular weight fraction of dextran persist

longer time in plasma after direct systemic administration. On the other hand dextrans having $M_w < 20$ kDa eliminated within 3h from circulation.⁹⁷ Metabolism of dextrans is catalyzed by enzyme dextranase (α -1-glucosidase)⁹⁸ and takes place in liver, kidney, spleen and lower GIT. The rate of metabolism is increased with increase in molecular weight. On the other hand, studies showed that the rate of degradation by dextranase enzyme is very slow for chemically modified dextrans.⁹⁹ Similar to metabolism, renal excretion of dextrans are also exhibited M_w dependency. Studies revealed that the renal elimination of dextran having MW less than 10 kDa occurred exclusive of any molecular limitations.⁹⁹ However, renal clearance of dextrans is reduced with increase in MW and approaches to almost zero at MW equal to 40 kDa. The greater hydrodynamic radii of higher MW dextrans limited its passage through glomerular capillary wall and thus lessen renal clearance.

Functionalization reactions of dextrans were extensively investigated for designing materials of interest. The relatively higher acidity of hydroxyl group at second position (C_2) provides easy access for nucleophilic substitution reactions. The ^1H NMR spectral analysis of derivatized dextran exhibited diminished intensity of OH protons (at second position), which proves the position of substitution on polymer back bone.⁹⁵ The reactivity of dextrans OH group is declined in the order $C_2 > C_3 > C_4$. Among various dextran derivatives, esters and ethers are most commonly employed material for various delivery applications.

Esterification of Dextrans

Inorganic esters of dextran such as dextran phosphate, dextran sulphates are less commonly employed in clinics. On the other hand, organic esters found wide range of pharmaceutical applications.⁹² One of the most commonly employed methods for the synthesis of organic dextran esters is heterogeneous reactions of carboxylic acid anhydrides and polymer suspended in pyridine. The above method can also be used to introduce longer aliphatic chains on dextran back bone, where corresponding chloroacetic anhydride and dextran have to be reacted in the presence of magnesium perchlorate as shown below in Scheme 1.1a.¹⁰⁰

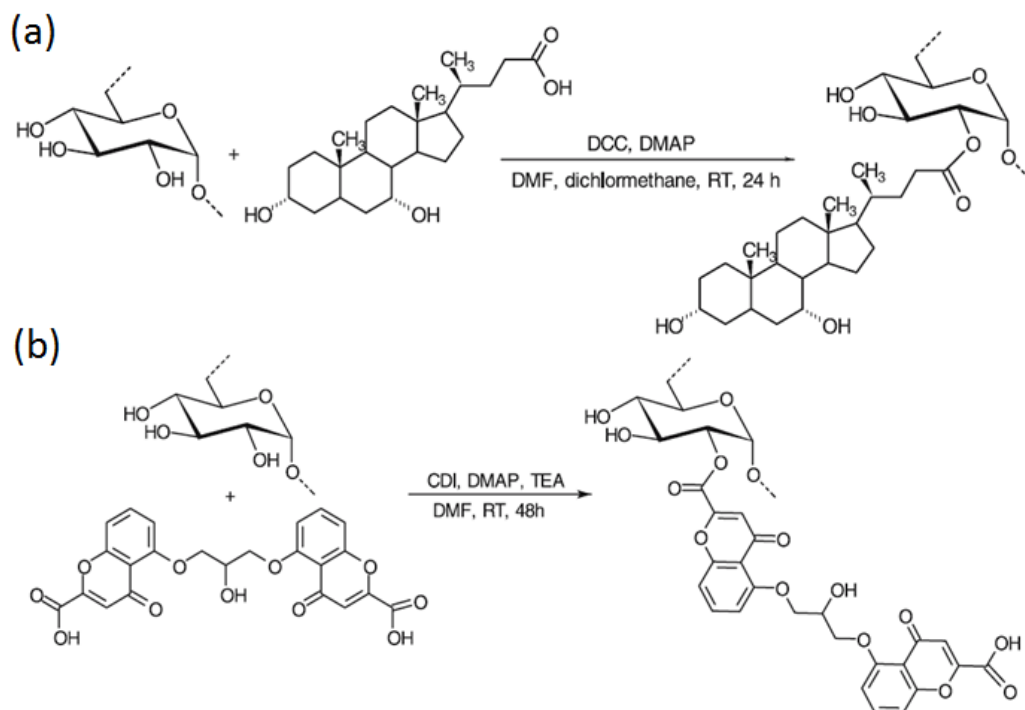


Scheme 1.1. (a) Dextran esterification reaction with anhydrides in the presence of magnesium perchlorate catalyst. (b) Dextran esterification reaction via acid chloride coupling reaction (c) Dextran esterification using carboxylic acid in-situ activated with dicyclohexyl carbodiimide (DCC) (Adopted from ref. 100-102).

The acylation reaction of dextran yield derivative with determined hydrophobic properties. The chain length of substituent and degree of substitution (DS) on dextran backbone affects the water solubility of the synthesized derivative. The aqueous solubility of dextran derivatives remained unchanged for $DS \leq 0.26$ (Scheme 1.1b).¹⁰¹ Dextran completely dissolved in DMSO or DMF/LiCl was homogeneously reacted with acid chlorides to yield corresponding esters. Esterification reaction of more complex carboxylic acid with polysaccharide hydroxyl group can be performed by using dialkyl carbodiimide type of coupling reagent. N,N'-dicyclohexyl carbodiimide (DCC) is most frequently employed for such coupling reactions.¹⁰²

Activation of carboxyl group with DCC followed by the condensation reaction with dextran hydroxyl group deferred esters. The bulky hydrophobic group were established on dextran surface by DCC/DMAP reaction, where DMAP act as catalyst

of acyl transformation reaction. For example, bile acid was attached on dextran via DCC/DMAP coupling reaction as shown below in scheme 1.2a.¹⁰³



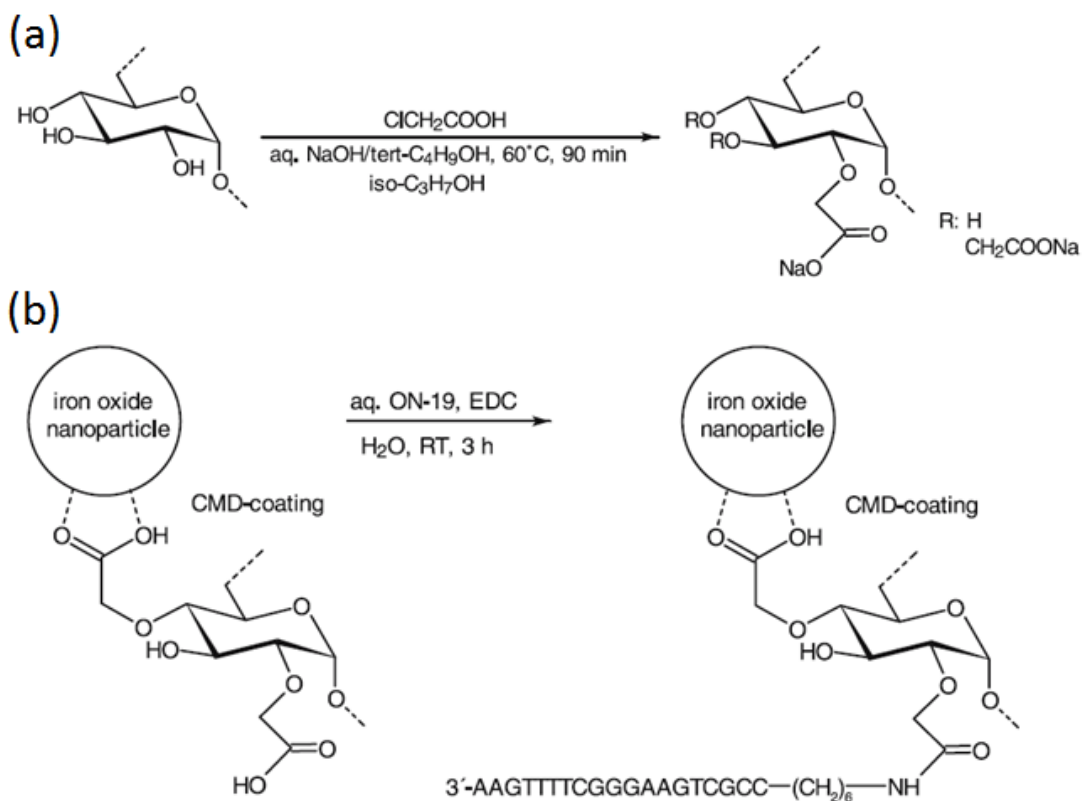
Scheme 1.2. (a) Dextran esterification reaction with bile acid in the presence of DCC/DMAP condition, (b) Dextran esterification reaction with cromoglycic acid in the presence of CDI coupling agent (adopted from ref. 103-104)

N,N'-carbonyldiimidazole (CDI) is non toxic and more efficient coupling reagent than DCC. The carboxylic acid got first activated with CDI to form imidazolide, which upon reaction with dextrans to form esters. For example, dextran was covalently bonded with cromoglycic acid in the presence of CDI, TEA and DMAP as shown above scheme 1.2b.¹⁰⁴

Dextran Ethers

Dextran ethers are widely exploited to introduce spacer group for further drug conjugation. Etherification of dextrans bestowed more stable polymer derivatives with entirely different physicochemical properties. The establishment of ether moieties on the dextran backbone slows down the hydrolytic or enzymatic degradation process. Enormous efforts have devoted to synthesise various dextran ethers, starting from simple methylation to attachment of long alkyl chains and hydrophilic chains like ethyleneglycols. Carboxymethylation of dextran (CMD) is most renowned etherification reaction due to its wide range of applications for making further drug

conjugates or coating materials. CMD can be easily prepared by the reaction of dextran and chloroacetic acid in water/ organic solvent mixtures contains strong alkali, as shown below in scheme 1.3a.¹⁰⁵

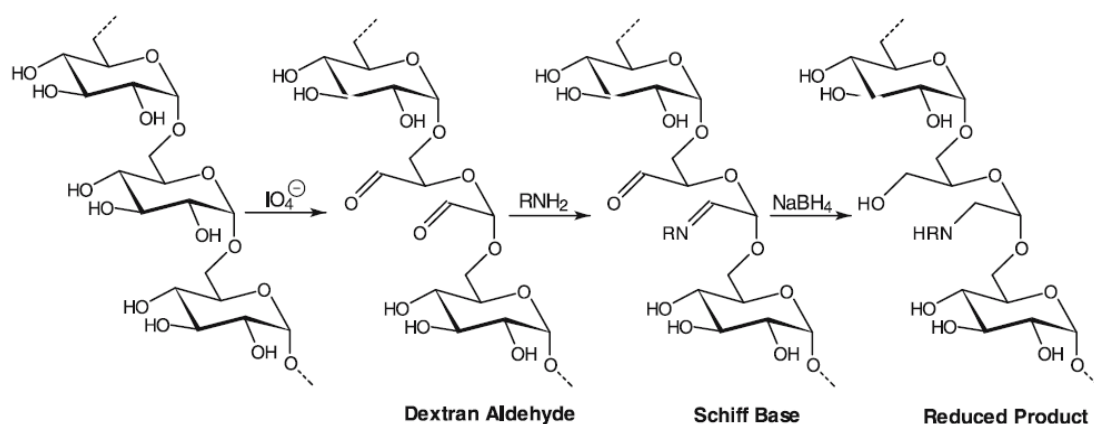


Scheme1.3 (a) Synthesis of carboxy methylated dextran (CMD) using chloroacetic acid under alkaline condition, (b) Carboxy methyl dextran coated iron oxide nanoparticles coupled with oligonucleotide ON-19.(Adopted from ref. 105-108)

Carboxyl functionalized dextran was studied for its ability to conjugate with bioactive proteins, drugs and Iron oxide nanoparticles etc. For instance, Wagner *et al.* demonstrated iron oxide nanoparticle coupled with a few of the carboxyl (-COOH) group of carboxy methyl dextran and its subsequent conjugation with cell penetrating peptides as shown above in scheme 1.3b. The faster endocytosis of the dextran coated iron oxide NP was shown to aid tumor diagnosis and therapy.¹⁰⁶

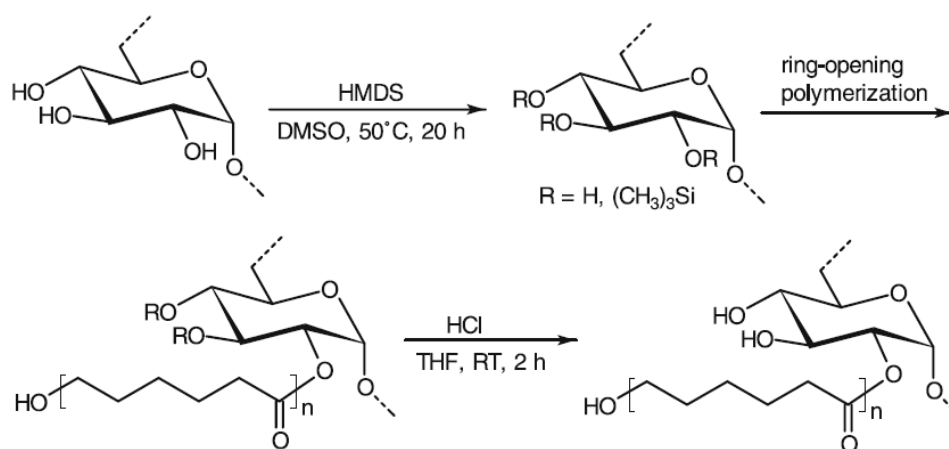
Dextran Schiff bases

Schiff bases formation reactions are considered to be most versatile method to synthesise various dextran conjugates. Dextrans have to be oxidised to obtain reactive aldehyde derivative, which upon reaction with -NH₂ terminated bioactive molecules such as proteins, enzymes, drugs or fluorescent dyes offer corresponding Schiff bases. The final step involves the reduction of Schiff bases to yield stable conjugate.¹⁰⁷



Scheme 1.4. Bioactive molecules covalently conjugated to terminal amino group of dextran generated using oxidation, Schiff base formation and reduction processes¹⁰⁷

Heinze *et al.* recently reviewed other methods of dextran derivatives such as cyanogen bromide addition and subsequent coupling amine containing drugs, preparation of carbonates of dextran, p-touene sulphonic acid esters of dextran, thiolated dextran, silyl dextran, ring opening polymerization using PLA (PLA grafted dextran copolymer) and ϵ -caprolactone (PCL grafted dextran copolymer) etc. to obtain necessitated structures and/or conjugates.^{95, 108}



Scheme 1.5. Partial silylation of dextran and subsequent ring opening polymerization with caprolactone to obtain hydrophobic poly(ϵ -caprolactone) grafted dextran.¹⁰⁸

1.8. Dextran based self Nanoscaffolds

The high biodegradability, biocompatibility, hydrophilicity and ease of chemical functionalization put together dextran a versatile polysaccharide for drug/gene delivery applications. A wide variety of dextran based graft copolymers, block copolymers and random copolymers have been explored in the literature to make diverse self organized structures for diagnosis, imaging and treatment of

cancers. Among this dextran based nanoparticles, micelles and hydrogel were most commonly employed for drug/gene delivery and tissue engineering applications.⁹⁶

Frechet and coworkers have reported pH responsive acetalated dextran derivative soluble in organic solvents such as dichloromethane ethyl acetate or acetone.^{109,110} The material thus formed was self assemble to nano particles (see figure 1.24), which was shown for its ability to load hydrophobic and hydrophilic molecules.

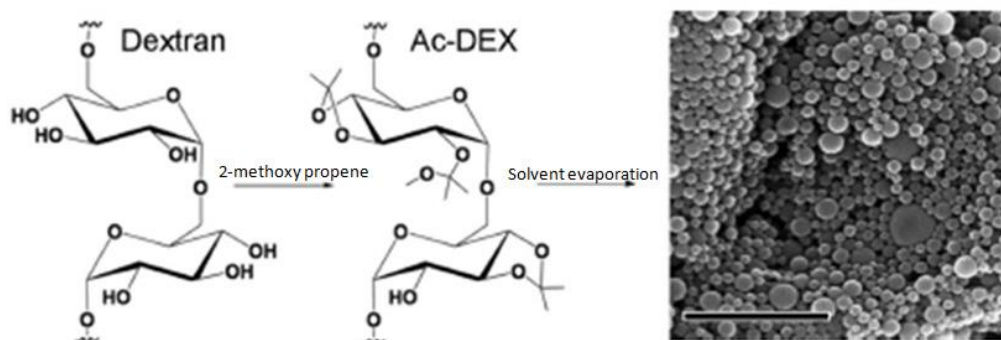


Figure 1.24.: Synthetic scheme for acetalated dextran derivative preparation and nanoparticles formed using solvent evaporation method (Adopted from Broaders et al. *J. Am. Chem. Soc.* **2011**, 133, 756-758).

Li et al. reported nanoparticles based non toxic lipoic acid (LA) conjugated dextran derivatives (Dex-LA) for Doxorubicin (DOX) delivery into cancer cells.¹¹¹ The number of lipoyl group per 100 anhydro glucose unit (AHG) or degree of substitution (DS) was regulated by carefully selecting appropriate LA/AHG feed ratio.

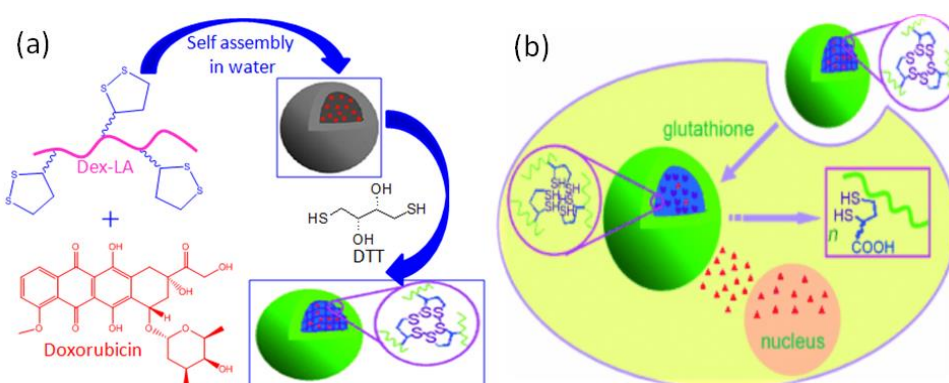


Figure 1.25. (a) Reversibly stabilized multifunctional dextran nanoparticles loaded with doxorubicin anticancer drug obtained by the reaction between dextran and lipoic acid and crosslinked using catalytic amount of DTT. (b) Schematic representation of decrosslinking and release of doxorubicin from dextran nanoparticles in the presence of glutathione abundant in cancer cells (Adopted from Schatz et al. *Angew. Chem.Int. Ed.* **2009**, 48, 2572-2575).

DEX-LA with high DS (80%) was selected for crosslinking with catalytic amount of dithiotheritol (DTT) to get reversibly stabilized multifunctional dextran nanoparticles. DOX loaded in the crosslinked particles were shown to get released upon the action of glutathione (GSH) enzyme abundant in the cancer cells. The approach is schematically shown in the figure 1.25.¹¹¹

Liebert *et al.* demonstrated two step esterification reaction of dextran with propionic anhydride and subsequently with polyglutamic acid.¹¹² The second esterification was performed using CDI as coupling agent and thus avoided DCC/DMAP method (see figure 1.26). The reaction yielded an amphiphilic molecule named as dextran propionate polyglutamate, where both propionate and polyglutamate moieties were entirely biocompatible. The degree of substitution on dextran back bone was calculated to be 70 %, (almost 50 % for propionate and close to 20 % for polyglutamate) which may completely affect basic dextran properties. Self assembly was induced into new dextran derivative by dialysis method, which resulted into the formation of uniform nanoparticles as shown in the figure 1.26. The authors proposed this new biocompatible material devoid of all kinds of harmful chemical impurities for future biological and medical applications.¹¹²

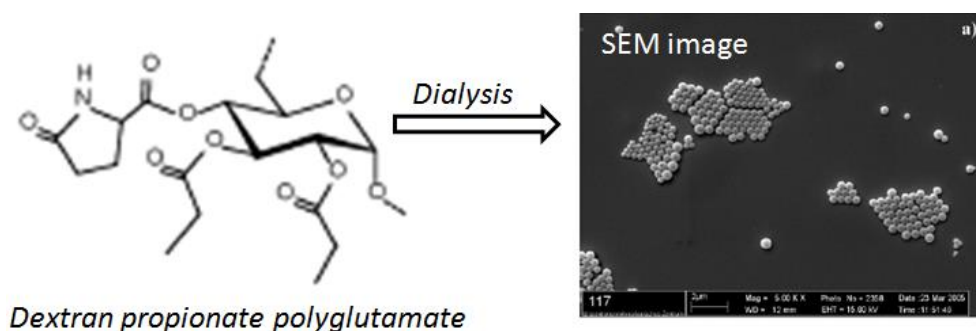


Figure 1.26. SEM image of spherical nanoparticle prepared from dextran having biocompatible propionate and polyglutamate moieties (Adopted from Liebert *et al.* *J. Am. Chem. Soc.* **2005**, 127, 10484-10485).

Du *et al.* reported stearic acid (SA) grafted dextran amphiphiles for loading and delivering hydrophobic DOX anticancer drug molecules.¹¹³ The carboxylic acid group of stearic acid was esterified with hydroxyl groups of dextran polymer via DCC/DMAP reaction. DS on dextran backbone was carried from 6 % to 84 % by changing feed ratio of stearic acid in the reaction. The synthesised DEX-SA derivatives were self assembled in PBS to form micelles as shown in the figure. 1.27.

DOX loaded into micelles and was demonstrated to suppress the growth of drug resistant MCF 7 (breast cancer) cells. *In-vivo* studies using cancer induced mice (A 549 cells transplanted) models proved the superior cancer reduction capacity of DOX loaded dextran micelles compared to free doxorubicin hydrochloride molecules (see figure 1.27).

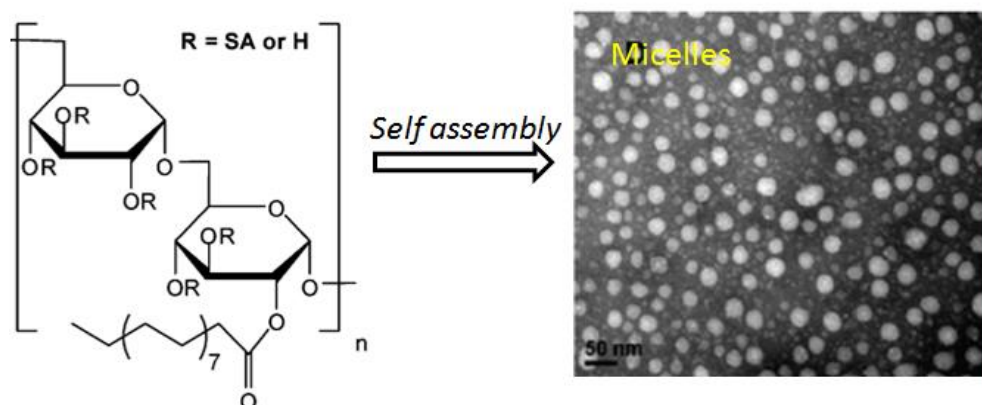


Figure 1.27. Stearic acid grafted dextran amphiphiles self assembled to form micelles in the aqueous conditions shown in SEM image (Adopted from Du *et al.* ACS Nano, 2010, 4, 6894-6902).

Disulphide linked dextran-b-poly (ϵ -caprolactone) diblock copolymer (DEX-SS-PCL) was developed by Sun *et al.* for reduction responsive delivery of doxorubicin in cancer cells (see figure 1.28).¹¹⁴ The thiol disulphide exchange reaction between PCL-SH and dextran orthopyridyl disulphide bestowed DEX-SS-PCL which self assembled to form micelles of about 60 nm size. The approach is schematically shown in the figure 1.28. DOX loaded in the micelles displayed faster release in the presence of DTT (Dithiothreitol, molecule equivalent to GSH). The reduction environment in cancer cells due to over expressed GSH (glutathione) cleaved the micelles and rapidly released DOX molecules into cytoplasm and nucleus. The MTT assay proved cytotoxic potential of drug loaded scaffold.

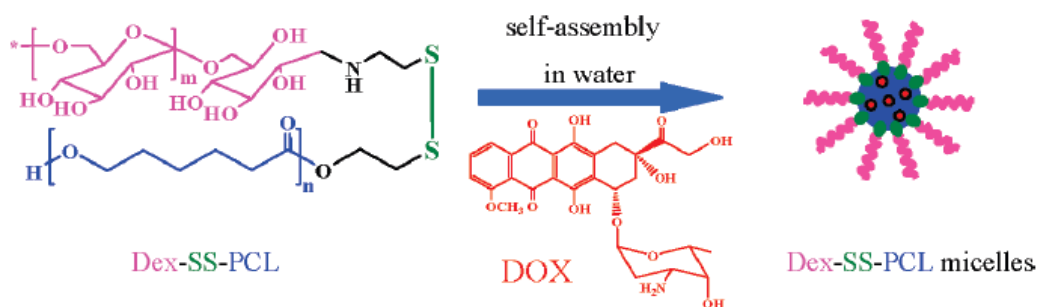


Figure 1.28. Representation of DOX loaded micelles formed from the self assembly of dextran-SS-PCL diblock copolymer in water (Adopted from Sun *et al.* Biomacromolecules, 2010, 11, 848-854).

Houga *et al.* investigated self assembly of dextran-*b*-polystyrene (DEX-*b*-PS) in organic solvents and water (See Figure 1.29).¹¹⁵ Polystyrene (PS) is a toxic non biodegradable material and its block length (hydrophobic content) in the copolymer was varied from 7 % to 92 % w/w, while the hydrophilic dextran molar mass was kept constant at 6600 g/mol. The study revealed that the block copolymer with low hydrophobic weigh fraction (7 %) self assembled to form core shelled micelles and nanoparticles. High degree of substitution yielded vesicles as shown in the figure 1.29.

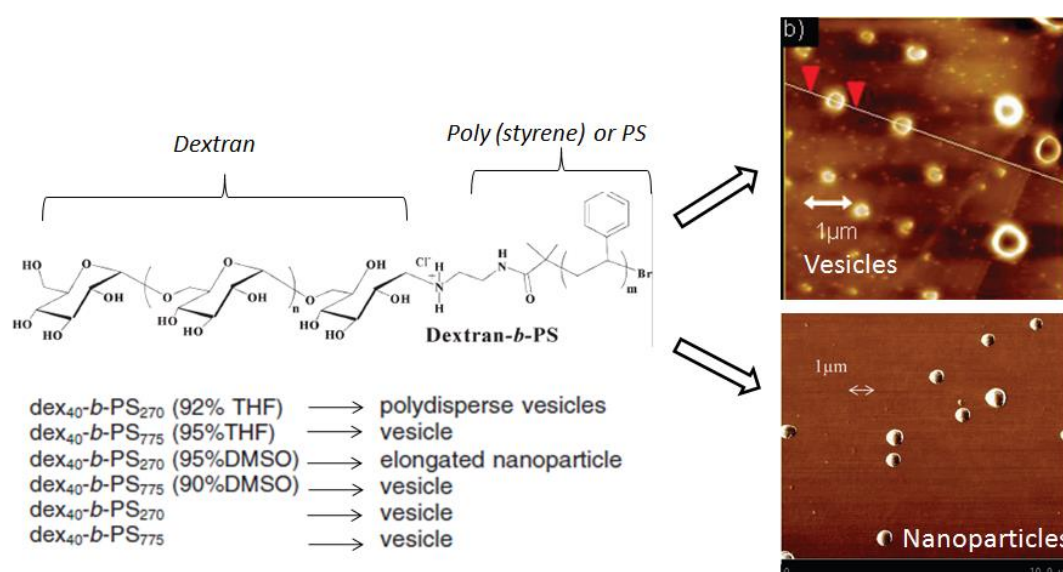


Figure 1.29. The chemical structure of Dextran-*b*-Polystyrene (DEX-*b*-PS) and AFM image of vesicles and nanoparticles formed by varying block length of hydrophobic (PS) content. Table depicts kind of self assembly formed from various DEX-*b*-PS in different solvent compositions. (Adopted from Houga *et al.* *Biomacromolecules*, **2009**, 10, 32-40.)

The above study emphasizes the need for the development of clinically useful polymer based drug delivery vehicles for cancer therapy. Polymer vesicle or polymersome prepared from polysaccharides or polypeptides based block copolymer are considered to be one step on the way of mimicking natural vesicles formed from the self assembly of amphiphilic lipid, protein or carbohydrate molecules. The overall composition, presence of interdigitating hydrophobic chain and type of conformation exhibited by polysaccharide chain highly influence the formation of vesicular geometry. Therefore, only a few copolymers based on dextrans were reported to form vesicles.

1.9. Aim of the thesis:

From the previous discussions, it is understood that the polymer vesicles provide several technological advantages such as dual drug encapsulation ability, efficient cell penetrating capacity due to its ability to mimic cell membrane and resistance against plasma proteins, etc, which are not possible with other self-organized structures. Thus, developing new generation of nano-scaffolds for drug delivery from clinically useful polysaccharides such as dextran is a very important and promising area of research for cancer therapeutics. Polysaccharide based vesicles are one of the least explored polymer vectors for drug delivery research till date due to the difficulty in identifying appropriate hydrophobic units and lack of knowledge of degree substitution required for getting vesicular geometry etc. The above discussions emphasized the need for new structural modifications on polysaccharide to make vesicles for drug delivery applications.

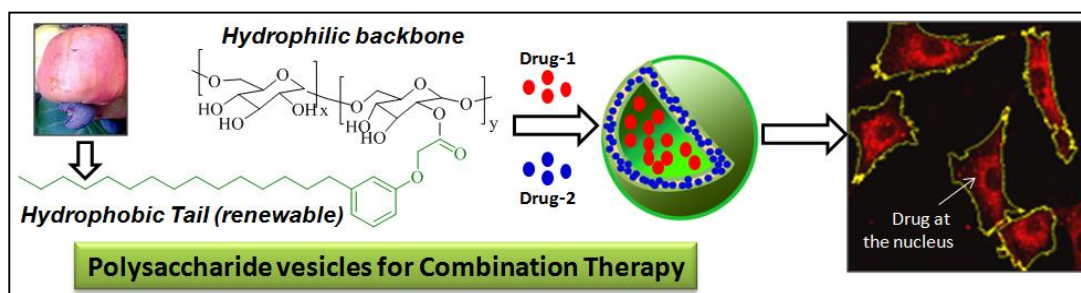


Figure 1.30. Polysaccharide design for vesicular geometry and its dual drug loading capabilities. Confocal microscope image of DOX loaded vesicles at the perinuclear site in the breast cancer cells

This thesis work is focused on design and development of new dextran vesicles for the delivery of anticancer drugs such as camptothecin (CPT topoisomerase inhibitor) and doxorubicin (DOX, DNA intercalating agent) (see figure 1.30). Multiple drug loading and sequential drug delivering abilities of dextran vesicles were also evaluated to achieve synergistic killing effects in cancer cells. Efforts were also put to develop dual stimuli responsive polysaccharide vesicles for DOX physically loading and chemically conjugating on dextran scaffolds to obtain better killing effects in cancer cells. A new technique based on time resolved fluorescence technique was also identified to conquer the drawbacks of dynamic dialysis method of *in-vitro* drug release studies.

First chapter provides complete literature survey on polymer based drug delivery and the need for the polysaccharide vesicles for delivering anticancer drugs.

In the second chapter, all three components of polymer-drug conjugate like hydrophilic polymer backbone (dextran), hydrophobic tail (Pentadecyl phenol from cashew nut shell liquid) and drug molecules (Camptothecin, plant derived anticancer drug) were chosen from naturally available resources. The drug's pharmacophore stabilizing capabilities of dextran vesicles and the killing effect of drug loaded vesicles compared to free drug were assessed in detail. Third chapter deals with the application of above polysaccharide vesicles in dual drug loading and its comparison with drug cocktails (at comparable molar ratios). The role of the caveolar endocytic pathway and targeting to cancer cells were also studied using caveolae containing (Cav-1 ^{+/+} MEFs) and lacking (Cav-1 ^{-/-} MEFs, MCF7 and DLD1) cells. The fourth chapter describes the development of dual responsive polysaccharide vesicles for both physically loaded and chemically conjugated DOX drugs having identical morphology. The pH responsiveness was achieved by imine chemical linkage (stimuli-1) and the aliphatic ester bond that connects the hydrophobic segment at the dextran backbone behaved as stimuli-2 for lysosomal enzyme: esterase. In the fifth chapter, the accurate estimation of drug release kinetics from polymeric vehicles was determined using TCSPC technique. The method was employed to distinguish the drug loaded and released from vesicles and its esterase enzyme sensitivity was analysed using AUTODOCK programme. Finally, the overall conclusion of the thesis has been summarized.

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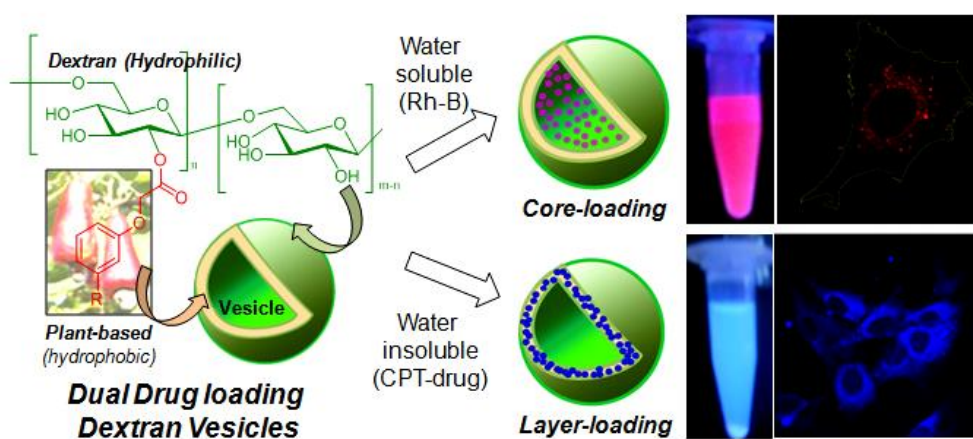
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Chapter 2

Dextran Vesicular Carriers for Dual Encapsulation of Hydrophilic and Hydrophobic Molecules and Delivery into Cells



Chapter 2

Dextran Vesicular Carriers for Dual Encapsulation of Hydrophilic and Hydrophobic Molecules and Delivery into Cells

Dextran vesicular nano-scaffolds were developed based on polysaccharide and renewable resource cashew nut liquid for dual encapsulation of hydrophilic and hydrophobic molecules (or drugs) and delivery into cells. The roles of the hydrophobic segments on the molecular self-organization of dextran backbone into vesicles or nanoparticles were investigated in detail. Dextran vesicles were found to be a unique dual carrier in which water soluble molecules (like Rhodamine-B, RhB) and polyaromatic anticancer drug (camptothecin, CPT) were selectively encapsulated in the hydrophilic core and hydrophobic layer, respectively. The dextran vesicles were capable of protecting the plasma sensitive CPT lactone pharmacophore against the hydrolysis by ten times better than the CPT alone in PBS. The aliphatic ester linkage connecting the hydrophobic tail with dextran was found to be cleaved by esterase under physiological conditions for fast releasing of CPT or RhB. Cytotoxicity of the polymer vesicle and its drug conjugate were tested on mouse embryonic fibroblasts cells (MEFs) using MTT assay. The dextran vesicular scaffold was found to be non-toxic to living cells. CPT loaded vesicles were found to be 2.5 fold more effective in killing fibroblasts compared to that of CPT alone in PBS. Confocal microscopic images confirmed that both RhB and CPT-loaded vesicles to be taken up by fibroblasts compared to CPT alone, showing a distinctly perinuclear localization in cells. The custom designed dextran vesicular provides new research opportunities for dual loading and delivering of hydrophilic and hydrophobic drug molecules.

2.1. Introduction

Mimicking biological behaviors in artificial synthetic macromolecules and understanding the role of bio-system choice for a particular structure requirement is emerging as an important area of research in chemistry-biology interface.¹ Among the various interesting properties of the biological systems, selection of suitable hydrophilic and hydrophobic units in the cell membranes through which ions, molecules or nutrients transportation occurs from extracellular to cytoplasm is one of the most interesting aspects to be understood.² Biological systems cleverly chose saturated and unsaturated chains in the cell membranes in such a way to create well defined rigid walls and flexible channels, respectively. Both in plants and sea-based living organisms such as fishes are enriched with ω -unsaturated fatty acids which are turned to be rich sources of nutrients for mammals including human beings. Cashew nut shell liquid (CSNL) is one of the rich renewable resources and industrial waste which consisting of both saturated (pentadecyl phenol-PDP) and unsaturated (cardanol) C₁₅-chains having phenolic functionality.³ (See figure 2.1).

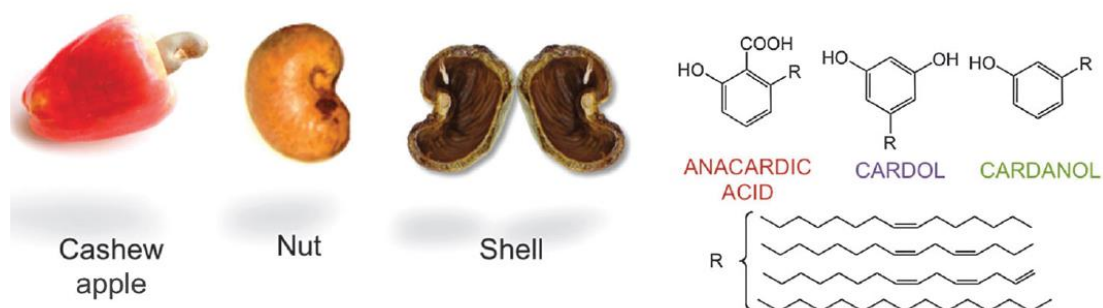


Figure 2.1. Chemical structures of Cashew nut shell liquid distillates (adopted from Balachandran *et al.* *Chem. Soc. Rev.* **2013**, 42, 427-438).

These derivatives were not edible, and therefore, extensively used by various research groups including ours as renewable resource intermediates in material science for making polymers, nanomaterials, gels, etc.⁴ From our group Anilkumar *et al.* had reported benzene sulphonic acid derivative of cardanol as a dopant to produce polyaniline microspheres, dendritic and linear nanofibres, nanotubes, etc.^{5,6} (see figure 2.2) This molecule was also used to tune the size, shape and ordering of polymeric nanomaterials.⁷ The strategy was found to be very attractive for new research activities in conducting polymer nano-materials more specifically for conducting polyaniline and related nano-materials.

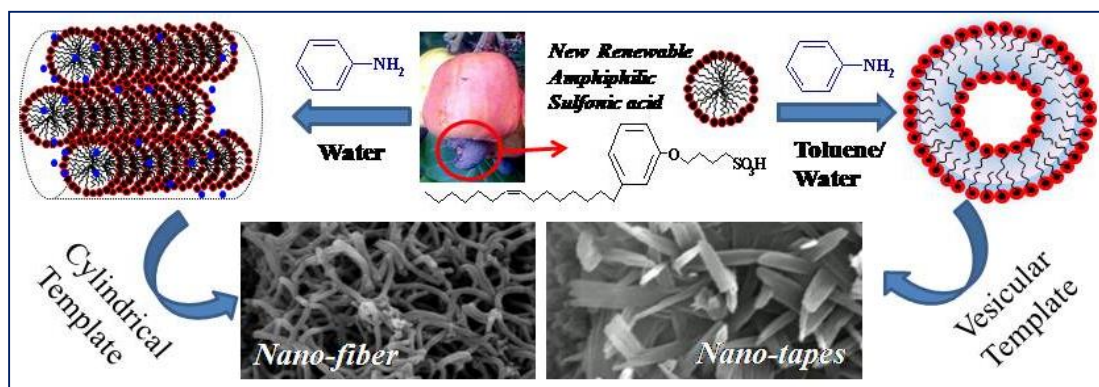
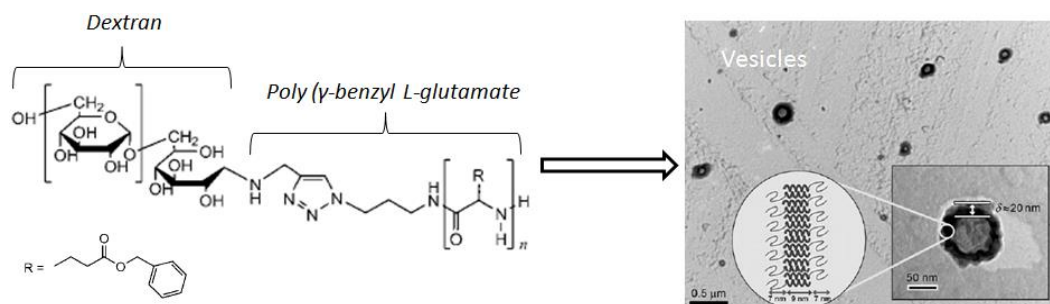


Figure 2.2. Polyaniline nanomaterials prepared using benzene sulfonic acid derivative of renewable resource based cardanol (adopted from Anilkumar et al. *Macromolecules* **2007**, 40, 7311-7319).

During the course of nanomaterial development, it was found that the PDP based surfactant molecules behave as a templating agent for producing micelles or vesicles. However, these molecules were not used as a structure directing agent in the optimization of nanoscaffolds for drug delivery research. Therefore, it is more rational to think to utilize this cashew nut based saturated and ω -unsaturated molecules for making new macromolecular derivatives, which could be useful for therapeutics such as polymer, based anticancer drug delivery. This also provides a unique opportunity to understand the role of the saturated/un-saturated units on the molecular self-organization of synthetic macromolecular nano-scaffolds and further their influence on the cellular uptake mechanism.

The synthesis of amphiphilic polymers were typically achieved by anchoring hydrophobic moieties on the water soluble polysaccharides or polyethylene glycol backbones.⁸ Both naturally occurring polymers such as cellulose, starch, chitosan and synthetic macromolecules based on polyethylene glycols (PEG)s, acrylics, ω -caprolactone and their block or random copolymers were reported as scaffolds for drug delivery.⁹ As described in chapter 1, it is known that, dextran is very unique water soluble starch based biopolymer consist of α (1-6) linked glucose monomer and is a suitable candidate for drug delivery application.¹⁰ In general, dextran is highly hydrophilic as similar to synthetic polymers like PEGs and need appropriate modification in such a way that the optimum hydrophilic-hydrophobic content for suitable self-organization. As discussed in previous chapter, a few efforts had been taken to functionalize dextran with alkyl chains or long polymers through ester or acetal linkages¹¹ for biomedical application^{12, 13}. However, most of these modified



Recently, Schatz *et al.* reported polysaccharide-peptide block copolymer vesicles as viral capsids.¹⁷ The authors described vesicle forming polysaccharide-block-polypeptide based on dextran and poly (γ -benzyl L-glutamate) (PBLG) block copolymer (See Figure 2.3). Dextran with a terminal alkyne functionality and PBLG end functionalized with azido group were coupled together in the presence of CuBr catalyst. The self assembly was induced into new dextran copolymer via nanoprecipitation method. However the drug encapsulation and delivery properties of dextran based vesicles was not studied in detail.

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A Versatile Dual Encapsulation Polymer Carrier for Hydrophilic & hydrophobic Drugs

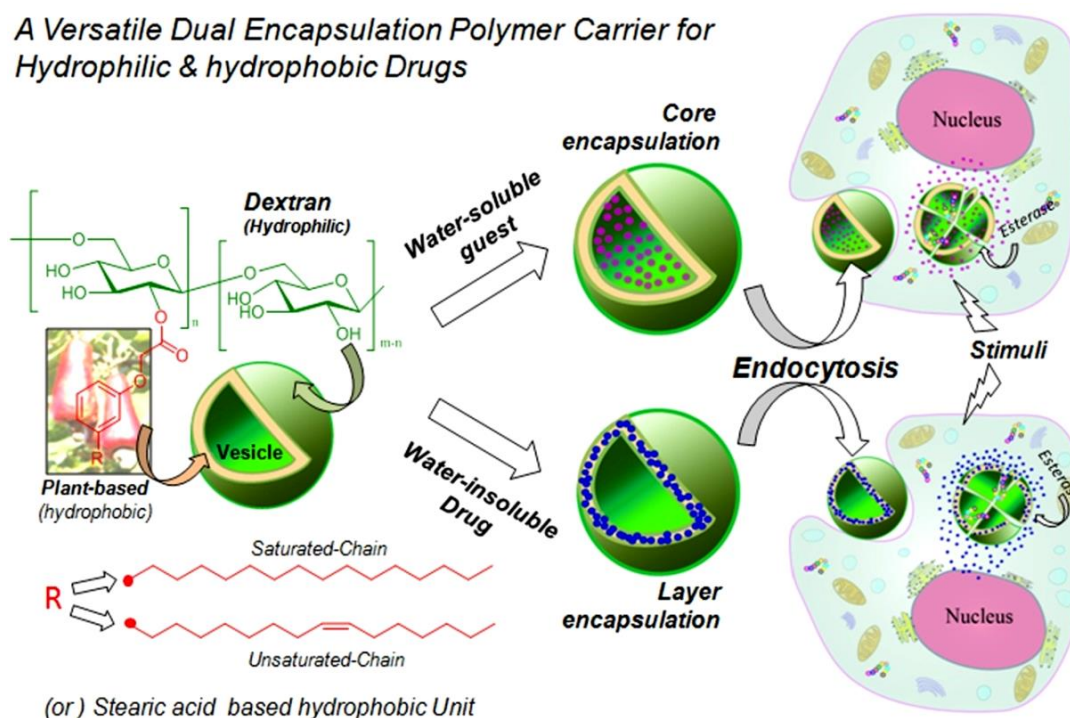


Figure 2.4 Dextran Vesicular approach for delivery of hydrophilic and hydrophobic drugs (or molecules) into cells

Further, single crystal structure was resolved to establish the role of PDP-chain hydrophobic tail interactions in the formation of uni-lamellar vesicular structures. The dextran vesicular scaffolds were capable of loading hydrophilic guest (Rhodamine-B, RhB, water soluble dye) and hydrophobic polyaromatic anticancer drug (Camptothecin, CPT) (see figure 2.4). The stability of the CPT enhanced in the vesicular structure almost ten folds compared to its isolated form. Esterase when used as stimuli to break the polymer scaffold was seen to release CPT or RhB from the vesicular scaffolds at significantly much faster rate. Cellular uptake of the both RhB and CPT loaded vesicular scaffolds were investigated in mouse embryonic fibroblasts (MEFs). The both were found to be efficiently taken up by cells with CPT drug was found to be 2.5 fold more efficient in killing the cells, when loaded in the vesicular scaffold compared to CPT alone. Confocal microscope images gave further evidence for the cellular uptake of dextran vesicles and their selective perinuclear localization in cells. The present approach opens up new drug delivery concept based on polysaccharide vesicles, more specifically in dextran vesicles, for stabilizing and delivering both hydrophobic drugs and hydrophilic molecules inside the cells.

2.2. Experimental methods:

2.2.1. Materials

Dextran ($M_w = 6,000$), 3-pentadecylphenol, Stearic acid, dicyclohexylcarbodiimide, 4-dimethylamino pyridine, pyrene, 20-(S)-camptothecin (CPT), Rhodamine B and horse liver esterase enzyme were purchased from Aldrich chemicals. Cardanol was purchased locally and purified by double distillation, followed by column chromatography. Dimethyl sulphoxide was dried over CaH_2 and distilled prior to use. Ethyl chloroacetate, K_2CO_3 , KI, KOH, and all other reagents and solvents were purchased locally and purified following the standard procedure. Wild type mouse embryonic fibroblasts were maintained in DMEM (phenol red free medium: Gibco) containing 10% (v/v) fetal bovine serum (FBS) and 1% (v/v) penicillin–streptomycin at 37° C under a 5% CO_2 humidified atmosphere. Cells were trypsinised using 0.05% trypsin (Gibco) and seeded in 96 well or 6 well (as per experiment) flat bottomed plastic plates (Costar) for all assays. Tetrazolium salt, 3-(4,5 dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide (MTT), DMSO and paraformaldehyde was obtained from Sigma. Phalloidin conjugated to Alexa 594 was obtained from Molecular Probes (Invitrogen) and fluoromount from Souther Biotech.

2.2.2. General procedures

NMR spectra were recorded using 400-MHz Jeol NMR spectrophotometer in CDCl_3 (for PDP-ester, CAR-ester) and DMSO (d_6) (for PDP-acid, CAR -acid and all DEX-derivatives) containing small amount of TMS as internal standard. FT-IR spectra of all compounds were recorded using Bruker alphaT Fourier transform infrared spectrometer. The mass of PDP and CAR derivatives were confirmed by using the Applied Biosystems 4800 PLUS MALDI TOF/TOF analyzer. High resolution mass spectra of PDP-acid cleaved from DEX-PDP were obtained from Micro Mass ESI-TOF MS spectrometer. The purity of the modified dextran were determined by gel permeation chromatography (GPC) using a Viscotek VE 1122 pump, Viscotek VE 3580 RI detector, and Viscotek VE 3210 UV/Vis detector in dimethyl formamide. The absorption and emission studies were done by a Perkin-Elmer Lambda 45 UV-Visible spectrophotometer and SPEX Fluorolog HORIBA JOBIN VYON fluorescence spectrophotometer with a double-grating 0.22 m Spex1680 monochromator and a 450W Xe lamp as the excitation source at room

temperature. The excitation spectra are collected at 375 nm and 420 nm (pyrene emission wavelength) and the emission spectra are recorded by exciting at the excitation maxima. The pyrene samples were purged with N₂ gas for at least 15-20 minutes prior to photophysical experiments. The size determination of the DEX-PDP, DEX-CAR and DEX-SA were carried out by dynamic light scattering (DLS), using a Nano ZS-90 apparatus utilizing 633 nm red laser (at 90 ° angle) from Malvern instruments. The reproducibility of the data was checked for at least three times using independent polymer solutions. The static light scattering experiment (SLS) was carried out using 3D-DLS spectrometer, from LS instruments, Switzerland. The instrument consist of a He Ne laser having wavelength of 632.8 nm, in autocorrelation mode attached to computer using Lab view interface utilizing toluene as reference. The measurement was performed from 20° to 130° by steps of 5°. Atomic force microscope (AFM) images were recorded by drop casting the samples on freshly cleaved mica surface, using Veeco Nanoscope IV instrument. The experiment was performed in tapping mode using TAP-190AL-G50 probe from Budget sensors with a nominal spring constant of 48 N/m and resonance frequency of 163.5. FE-SEM images were recorded using Zeiss Ultra Plus scanning electron microscope. For FE-SEM analysis, the samples were prepared by drop casting on silicon wafers and coated with gold. Thermal analysis of PDP-acid, CAR-acid and Stearic acid was performed using TA Q20 differential scanning calorimeter (DSC). The instrument was calibrated using indium standards. TEM images were recorded using a Technai-300 instrument by drop casting the sample on formvar coated copper grid. The fluorescent micrographs were collected using Carl Zeiss Axiovert 200 microscope. LSM710 confocal microscope was used for imaging the cells. The stability of CPT was analysed by using reverse phase HPLC system, consists of Dionex ICS3000 instrument with phenomenix c18 reverse phase column (250X 4.60mm) and UV detector, at flow rate of 1ml/min. Single crystals were subjected to data collection at 100 K on Bruker APEX duo CCD-X ray diffractometer equipped with graphite monochromated Mo Ka radiation ($\lambda=0.71073$ Å). The frames were integrated with Bruker APEX software package. The structures were solved by direct methods and refined with a full matrix least squares techniques using SHELX S v97 programs.

2.2.3. Synthesis of ethyl 2-(3-pentadecylphenoxy)acetate (PDP-ester, 1a):

3-Pentadecylphenol (10.0 g, 32.8 mmol), K_2CO_3 (13.5 g, 98.5 mmol) and a pinch of KI were taken in dry dimethylformamide (50 mL) and stirred at 80 °C under nitrogen atmosphere for 30 minutes. The content was cooled and ethyl chloroacetate (4.2 mL, 39.4 mmol) added drop wise. The reaction continued by stirring at 80 °C for 24 h under nitrogen atmosphere. The reaction mixture was poured into water (250 mL) and the product was extracted with ethylacetate. The organic layer was washed with brine, dried over anhydrous sodium sulphate and the solvent was removed to obtain the product as low melting solid. It was further purified by passing through silica gel column using 3% ethyl acetate in hexane as eluent. Yield = 7.5 g (58 %). m.p. = 38 °C. ^1H NMR (400 MHz, $CDCl_3$) δ : 7.17ppm (t, 1H, Ar-H), 6.80ppm (d, 1H, Ar-H), 6.74ppm (s, 1H, Ar-H), 6.69ppm (d, 1H, Ar-H), 4.59ppm (s, 2H, O-CH₂), 4.26ppm (q, 2H, O-CH₂-CH₃), 2.55ppm (t, 2H, Ar-CH₂), 1.48ppm (m, 2H, Ar-CH₂-CH₂), 1.28ppm (m, 27H, aliphatic-H), 0.87ppm (t, 3H, -CH₃). ^{13}C NMR ($CDCl_3$, 100 MHz) δ : 169.34 (C=O), 158.10, 142.04, 129.72, 121.776, 115.01, 112.08 (Ar-C), 65.06 (Ar-O-CH₂), 61.07 (O-CH₂-CH₃), 35.68, 31.83, 31.42, 29.57, 29.41, 29.26, 22.63 and 14.48. FT-IR (KBr), cm^{-1}), 3037, 3006 (Aromatic C-H stretch), 2925, 2854 (Aliphatic C-H stretch), 1762 (Ester C=O stretch), 1585 (ring C=C stretch), 1201 (C(=O)-O stretch) MALDI-TOF-TOF, (MW:390), m/z= 429 (M+K⁺) (see annexure for spectra).

2.2.4. Synthesis of (E)-Ethyl 2(3-pentadec-7-enyl)phenoxy)acetate (CAR ester, 1b):

Cardanol (10.0 g, 33.0 mmol), K_2CO_3 (13.7 g, 99.0 mmol), KI and ethyl chloroacetate (4.26 mL, 39.6 mmol) were reacted in dimethylformamide as described for compound **1a** and the product was obtained as colorless liquid after column purification. Yield = 8.2g (63%). ^1H NMR (400 MHz, $CDCl_3$) δ : 7.17ppm (t, 1H, Ar-H), 6.80ppm (d, 1H, Ar-H), 6.75ppm (s, 1H, Ar-H), 6.70ppm (d, 1H, Ar-H), 5.34ppm (m, 2H, vinylic protons), 4.60 ppm (s, 2H, O-CH₂), 4.26ppm (q, 2H, O-CH₂-CH₃), 2.56ppm (Ar-CH₂), 1.99ppm (CH₂-CH=CH-CH₂), 1.58ppm (m, 2H, Ar-CH₂-CH₂), 1.27ppm (m, 19H, aliphatic protons), 0.87ppm (t, 3H, aliphatic terminal CH₃). ^{13}C NMR ($CDCl_3$, 100 MHz): 169.28 (-C=O), 158.12, 144.45, 129.67, 121.68, 114.98, 112.02 (Ar-C), 130.48, 130.02 (Vinylic C), 65.05 (Ar-O-CH₂), 61.04 (O-CH₂-CH₃), 35.69, 31.70, 31.68, 31.38, 29.65, 29.16, 27.13, 22.77, 22.64, 14.52 (Aliphatic C). FT-IR (KBr), cm^{-1}), 3104, 3035 (Aromatic C-H stretch), 2925, 2854 (Aliphatic C-H stretch), 1764

(Ester C=O stretch), 1654 (C=C stretch of long tail), 1585 (ring C=C stretch), 1201 (C(=O)-O stretch) MALDI TOF-TOF, (MW:388), $m/z=427(M+K^+)$ (see annexure for spectra).

2.2.5. Synthesis of 2-(3-pentadecylphenoxy) acetic acid (PDP-Acid, 2a): PDP-ester (5.0 g, 11.6 mmol) and KOH (2.2 g, 36.5mmol) were taken in dioxane (50.0 mL) and refluxed for 24 h. After removal of dioxane, the solid content was dissolved in water (100 mL) and the un-reacted PDP-ester was removed by extracting into ethyl acetate. The aqueous solution was acidified with dilute hydrochloric acid until the content became pH = 4. The precipitate was extracted with 50 mL ethyl acetate, washed with water and dried over anhydrous sodium sulphate. The solvent was removed to obtain the product as white solid which was further purified by passing through silica gel column using 20% ethyl acetate in hexane as eluent. Yield = 2.8 g (60 %). ^1H NMR (400 MHz, d_6 DMSO) δ : 12.9ppm (s, 1H, CO-OH), 7.13ppm (t, 1H, Ar-H), 6.67ppm (m, 3H, Ar-H), 4.58ppm (s, 2H, O-CH₂), 2.49ppm (t, 2H, Ar-CH₂) (1.48ppm (m, 2H, Ar-CH₂-CH₂), 1.28ppm (m, 27H, Aliphatic protons). ^{13}C NMR (DMSO(d_6), 100 MHz) δ : 170.79 (CO-OH), 158.23, 144.46, 129.66, 121.52, 115.00, 111.90 (Ar-C), 64.83(O-CH₂), 35.68, 31.83, 31.46, 29.57, 29.26, 22.63, 14.48 (Aliphatic-C). FT-IR (KBr), cm^{-1}), 3093, 3043 (Aromatic C-H stretch), 2956, 2919, 2848 (Aliphatic C-H stretch), 1731 (Acid C=O stretch), 1577 (ring C=C stretch), 1421 (O-H bending), 1272 (C (=O)-O stretch). MALDI TOF-TOF, (MW: 362.5), $m/z= 401.5 (M+K^+)$ (see annexure for spectra).

2.2.6. Synthesis of 2(3-pentadec-7-enyl) phenoxy)acetic acid (CAR-acid, 2b): Compound CARD-ester (5g, 11.7mmol) was hydrolyzed using KOH (2.16g, 38.65 mmol) in Dioxane as described for PDP-acid. Yield = 3.1g (67%) ^1H NMR (400 MHz, d_6 DMSO) δ : 12.9ppm (s, 1H, COOH), 7.19ppm (t, 1H, Ar-H), 6.83ppm (d, 1H, Ar-H), 6.76ppm (s, 1H, Ar-H), 6.71ppm (d, 1H, Ar-H), 5.33ppm (m, 2H, vinylic protons), 4.66ppm (s, 2H, O-CH₂), 2.56ppm (Ar-CH₂), 2.00ppm (CH₂-CH=CH-CH₂), 1.58ppm (m, 2H, Ar-CH₂-CH₂), 1.27ppm (m, 19H, aliphatic protons), 0.87ppm (t, 3H, aliphatic terminal CH₃). ^{13}C NMR (CDCl₃, 100 MHz): 170.77 (-C=O), 158.25, 144.45, 129.65, 121.51, 115.01, 111.91 (Ar-C), 130.57, 130.14 (Vinylic C), 64.86 (Ar-O-CH₂), 35.68, 32.46, 31.67, 31.39, 29.63, 29.21, 27.10, 22.74, 22.62, 14.45 (Aliphatic C). FT-IR (KBr), cm^{-1}), 3380 (O-H stretch), 3093, 3043 (Aromatic C-H

stretch), 3006 (Alkene C-H stretch), 2956, 2921, 2850 (Aliphatic C-H stretch), 1733 (Acid C=O stretch), 1654 (C=C stretch of long tail), 1577 (ring C=C stretch), 1423 (O-H bending), 1272 (C (=O)-O stretch). MALDI TOF-TOF, (MW:360), $m/z=399$ ($M+K^+$) (see annexure for spectra).

2.2.7. Synthesis of DEX-PDP-x:

Dextran ($M_w=6,000$, 1.0 g, 6.2 mmol of anhydroglucose unit) and compound **1b** (2.2g, 6.2 mmol, for DEX-PDP-10) were dissolved in anhydrous DMSO (50.0 mL) and the solution was purged with dry nitrogen. 4-(Dimethylamino) pyridine (0.15 g, 1.24mmol) in anhydrous DMSO (3.0 mL) and dicyclohexylcarbodiimide (1.53 g, 7.45 mmol) in anhydrous DMSO (3.0 mL) were added. The reaction mixture was stirred for 1 hour at 25 °C and the reaction was continued at 80 °C for 24 h. The solution was cooled, filtered to remove dicyclohexyl urea and the solvent was removed under reduced pressure. The thick viscous liquid was precipitated by adding into cold methanol (100 mL). The solid was filtered and washed several times with methanol. It was dissolved again in DMSO and the purification via the precipitation technique was done at least twice. The product filtered out and dried under vacuum at 60 °C to get yellowish white solid as product. Yield = 60 %. ^1H NMR (400 MHz, d_6 DMSO): δ : 7.12ppm (s, 1H, Ar-H), 6.72ppm (m, 3H, Ar-H), 4.47, 4.82, 4.88 ppm (s, hydroxyl of dextran) 4.63ppm (s, dextran anomeric proton), 3.14-3.69ppm (dextran glucosidic protons), 2.49ppm (2H, Ar-CH₂), 1.48ppm (2H, Ar-CH₂-CH₂), 1.18-0.80ppm (Aliphatic protons). ^{13}C NMR (DMSO (d_6), 100 MHz) δ : 168.95ppm (Ester C=O), 158.2, 144.52, 129.70, 121.69, 115.48, 111.80 (Ar-C), 98.69 (dextran anomeric C), 73.81, 72.33, 70.85, 70.58, 66.52 (Dextran glucosidic Carbons), 64.95ppm (O=C-CH₂), 35.64, 31.81, 31.39, 29.55, 29.22, 25.19, 22.61, 14.49 (Aliphatic carbons). FT-IR (KBr), cm^{-1} , 3376 (O-H stretch), 2920, 2852 (Aliphatic C-H stretch), 1766 (Ester C=O stretch), 1585 (ring C=C stretch), 1453 (O-H bending), 1198 (C (=O)-O stretch).

2.2.8. Synthesis of DEX-CAR-x:

Dextran ($M_n=6,000$, 1g, 6.1mmol of anhydro glucose unit), compound **2b** (2.23g, 6.1 mmol, for DEX-CAR-10), 4-(dimethyl amino) pyridine (0.15 g, 1.24mmol), and dicyclohexylcarbodiimide (1.53 g, 7.45 mmol) were reacted in dry DMSO as described for DEX-PDP-x. Yield= 60 %. ^1H NMR (400 MHz, d_6 DMSO): δ : 7.11ppm (s, 1H, Ar-H), 6.71ppm (m, 3H, Ar-H), 5.26ppm (Vinylic Protons), 4.45, 4.80, 4.86

ppm (s, hydroxyl of dextran) 4.62ppm (s, dextran anomeric proton), 3.11-3.70 ppm (dextran glucosidic protons), 2.48ppm (2H, Ar-CH₂), 1.92ppm (CH₂-CH=CH-CH₂), 1.48ppm (2H, Ar-CH₂-CH₂), 1.20-0.79ppm (Aliphatic protons). ¹³C NMR (DMSO(d₆), 100 MHz) δ: 168.95ppm (Ester C=O), 158.2, 144.50, 129.68, 121.65, 115.50, 111.80 (Ar-C), 98.71 (dextran anomeric C), 73.82, 72.34, 70.87, 70.60, 66.54 (Dextran glucosidic Carbons), 64.95 (O=C-CH₂), 35.65, 31.83, 31.39, 29.57, 29.24, 27.11, 25.19, 22.62, 14.46 (Aliphatic carbons). FT-IR (KBr), cm⁻¹, 3380 (O-H stretch), 3045, 3003 (aromatic C-H stretch), 2922, 2854 (Aliphatic C-H stretch), 1762 (Ester C=O stretch), 1582 (ring C=C stretch), 1453 (O-H bending), 1198 (C (=O)-O stretch).

DEX-PDP-x and **DEX-CAR-x** with various degrees of substitution were obtained by changing the mole ratios of PDP-acid to dextran as 0.5, 1.0 and 3.0 in the feed and their details are given in results and discussion. **DEX-SA** with a degree of substitution of 7.0 was synthesized using similar procedure as described for DEX-PDP-x with a mole ratio of Stearic acid (SA) to dextran as 0.5 in the feed (see annexure for spectra).

2.2.9. Preparation of DEX-vesicles and critical vesicular concentration (CVC)

DEX-PDP or DEX-CAR or DEX-SA (20 mg) was dissolved in DMSO (3.0 mL) and distilled water (3.0 mL) was added drop wise into the polymer solution with stirring at 25 °C. The resulting suspension was dialyzed against distilled water using commercial cellulose ester semi-permeable membrane (SPECTRA/POR, MWCO 3500) for 24 h. The critical vesicular concentration (CVC) was determined using pyrene as a fluorescence probe. A predetermined amount (0.6 μM) of pyrene prepared in acetone was pipetted into 5 ml glass sample vials and then acetone was allowed to evaporate completely. DEX-PDP or DEX-CAR solutions at different concentration, varying from 6.2×10^{-7} M to 3.1×10^{-3} M were added under the nitrogen purge to the vial containing pyrene and equilibrate for overnight. The excitation wavelength was set at 337 nm, the excitation slit at 3 nm, and the emission slit at 5 nm. For pyrene emission spectra, the ratio of fluorescent intensity at 375 and 386 nm was calculated and plotted against logarithm concentration of polymer concentration. The CVC was estimated at a polymer concentration of onset of the increase in I_{375}/I_{386} ratio.

2.2.10. Encapsulation of hydrophilic Rhodamine-B and loading efficiency

The ability of aggregates to stabilize the water soluble molecules in the inner aqueous compartment was checked using rhodamine B, RhB. Typically, 20mg polymer conjugates and 2 mg rhodamine B were dissolved in 3ml DMSO and distilled water (3.0 mL) was added drop wise into the polymer solution with stirring at 25 °C. This solution was stirred for 12 hours and then extensively dialyzed (SPECTRA/POR, MWCO 3,500) against water. The rhodamine B stabilization in DEX-PDP-5 was checked for 7days under dialysis. But in the case of DEX-CAR and DEX-SA, rhodamine B was completely leached out within 48 hrs of dialysis. DEX-PDP-5 solution contains entrapped rhodamine B was further made upto 10ml. The absorbance of encapsulated rhodamine B was measured by diluting 100 µl of dialysed solution to 3ml using DMSO as solvent. The rhodamine B content and loading efficiency of polymer vesicles was determined from beers law using molar absorption coefficient of dye as 1,16,000. The loading efficiency of RhB was determined as 1.80 wt %.

2.2.11. Encapsulation of Camptothecin and Drug loading Efficiency

Dialysis method was used to incorporate camptothecin in vesicles. Typically, 50 mg of DEX-PDP and 5 mg of CPT was dissolved in 5ml of DMSO. Under moderate stirring distilled water (5ml) was added dropwise to the above solution. After stirring for 1h at room temperature, the solution was transferred to a dialysis bag (SPECTRA/POR, MWCO 3500) and dialysed against deionized water for 24 h. The resulting solution was filtered, to remove unencapsulated camptothecin, and lyophilized. To determine CPT loading content and encapsulation efficiency, 3 mg of freeze dried CPT loaded DEX-PDP was dissolved in 1.0 mL of DMSO. 100 µL of this solution was diluted to 3.0 mL with DMSO and the absorbance at 365 nm was measured on UV-visible spectrophotometer. The amount CPT encapsulated in the vesicles was determined using the molar extinction coefficient of CPT as 11,250. Drug loading content (DLC) and drug loading efficiency (DLE) were calculated using following equations.

$$\text{DLE (\%)} = \{ \text{Weight of drug in vesicles} / \text{Weight of drug in Feed} \} \times 100 \%$$

$$\text{DLC (\%)} = \{ \text{Weight of drug in vesicles} / \text{Weight of Drug loaded vesicles} \} \times 100 \%$$

2.2.12. Stability of CPT free drug and CPT encapsulated in DEX-PDP vesicles:

HPLC method was used to study the stability of free CPT and CPT encapsulated in DEX-PDP vesicles under physiological condition (pH 7.4 and 37 °C). The mobile phase was consisting of 23 % acetonitrile and 77 % triethylamine acetate buffer (pH 6.0). For free CPT, a stock solution of 100 µg/ml was prepared in DMSO and 1.5 ml of stock solution was mixed with 1.5 ml PBS and incubated in PBS at 37 °C. An aliquot of 100 µl was withdrawn at 0.5 h, 1 h, 2 h, 4 h, 8 h, and 24 h and mixed with 1.9 ml PBS and immediately analyzed by HPLC to determine the ratio of carboxylate to lactone. For the CPT loaded vesicles, 3 mg of freeze dried DEX-PDP-CPT samples were dispersed in 3 ml of PBS, taken in dialysis tube and incubated at 37 °C on a rotary shaker. At various time interval 100 µL of suspension was withdrawn, dissolved in DMSO and appropriately diluted with PBS (pH 7.4), then immediately analysed by HPLC.

2.2.13. In-vitro Release studies

Dialysis method was used to study the release profile of CPT and rhodamine from DEX-PDP vesicles. Briefly, 3.0 mg of DEX-PDP vesicles encapsulated with CPT or rhodamine was dispersed in 3.0 ml PBS and placed in a dialysis tube. The tube was immersed in 100 ml PBS (pH 7.4) taken in a beaker and whole solution was incubated at 37 °C. At definite time interval 3.0 ml of solution was withdrawn and replaced with equal volume of fresh PBS. Absorbance of the each aliquot has been measured and amount of the CPT or rhodamine released were calculated using Beer's law. Raw CPT release was done in a similar manner by dispersing free CPT in PBS (an amount equivalent in the encapsulated vesicles). Esterase assisted drug release studies were carried out by adding 10 U esterase enzyme into the dialysis bag.

2.2.14. Cell Viability Assay (MTT Assay)

To observe the effect of CPT and CPT loaded in vesicles, a cell viability assay was performed in wild type mouse embryonic fibroblasts using the tetrazolium salt, 3,4,5 dimethylthiazol-2,5 diphenyl tetrazolium bromide (MTT). 1×10^3 wild type mouse embryonic fibroblasts were seeded per well in a 96 well plate (Corning, USA) in 100 µl DMEM with 10 % FBS (Fetal Bovine Serum) and allowed to adhere for 24 hrs. Prior to drug treatment, medium from cells was aspirated and 0.05 µM CPT and DEX-PDP with encapsulated CPT required to achieve a final concentration 0.05 µM

CPT were prepared. These were added to 100 μ l DMEM with FBS that the cells were incubated in. A blank control, DMEM with FBS in the absence of cells and an untreated control, cells with DMEM containing FBS, were used in each experiment. All control and treated experiment wells were in triplicate. Cells were incubated for 48 hrs without a change in medium and after 48 hrs, drug containing medium was aspirated. Freshly prepared stock of MTT in sterile PBS (5 mg/ml) was diluted to 50 μ g/ml in 100 μ l DMEM with FBS and added to cells. Cells were then incubated with MTT for 4 hrs at 37 °C. Medium with MTT was then aspirated from wells and the purple formazan crystals formed as a result of reduction of MTT by mitochondrial dehydrogenase enzyme from cells were dissolved in 100 μ l of 100% DMSO (added per well). The absorbance from formazan crystals was immediately measured using microplate reader at 570 nm (Varioskan Flash) and is representative of number of viable cells per well.

Values from the triplicates for each control and treated set were noted and their means used for calculations. If one value from the triplicate had deviated substantially from the other two, it was not considered in the mean calculations. If the triplicate values were all variable the experiment was not used in the final calculations. The mean of the absorbance values for the blank control samples was subtracted from the average of the untreated control and treated samples respectively. The values thus obtained for the untreated control samples were equated to 100 % and relative percentage values for CPT and vesicles loaded CPT calculated accordingly. Percentage values thus obtained for CPT and vesicles loaded CPT treated wells were subtracted from the untreated control (100%) to determine the percentage loss in cell numbers (relative to control). Results thus obtained from 7 experiments were compiled and differences between CPT and vesicles loaded CPT treatment statistically evaluated using a paired two tail T-Test.

2.2.15. Cell Viability Assay (Crystal Violet labeling of cells)

Wild type mouse embryonic fibroblasts were seeded in 6-well plates (Corning, USA) at a density of 5×10^4 cells per well. After allowing the cells to adhere overnight, the culture medium (DMEM + 10% FBS) was removed and 2 ml of medium with 0.1 μ M drug was added to each well. Appropriate amounts of free CPT and DEX-PDP with CPT were dissolved in PBS and diluted in medium to achieve the final CPT concentration of 0.1 μ M. A blank control, DMEM with FBS in the absence

of cells, and an untreated control, cells with DMEM containing FBS, were used in each experiment. Cells were treated for 48 hours at 37 °C in a CO₂ incubator after which drug-containing medium was removed and the cells were rinsed once with PBS and fixed with 1 ml of 3.5 % paraformaldehyde in PBS (15 min at room temperature). Each well was gently rinsed twice with PBS and 2 ml of 0.05 % crystal violet in PBS was added for staining for 11 min at room temperature. The plates were then rinsed with PBS and allowed to dry inverted overnight. Crystal violet bound to the cells was dissolved in 100 % DMSO and the absorbance, which is related to the number of adherent cells in the well, was measured using a microplate reader at 590 nm (Varioskan Flash). The absorbance of the blank control well was subtracted from all control and treated wells. Values thus obtained in the control untreated wells were equated to 100 % and relative percentage values for CPT and DEX-PDP-CPT treated wells calculated accordingly. Percentage values thus obtained for CPT and DEX-PDP-CPT treated wells were subtracted from the untreated control (100 %) to determine the percentage loss in cell numbers (relative to control). Results thus obtained from four independent experiments were compiled and differences between CPT and DEX-PDP-CPT treatment statistically evaluated using a paired two tail T-Test.

2.2.16. Cellular Uptake of CPT by Confocal Microscopy

Wild type mouse embryonic fibroblasts (WT MEFs) were seeded at a density of 5×10^4 cells on flame dried cover slips placed in 6 well plates containing DMEM medium with 10 % FBS and incubated at 37 °C for 18 hours. The cells were then exposed to required concentration of CPT alone, DEX-PDP alone and CPT loaded DEX-PDP vesicles or rhodamine B loaded DEX-PDP vesicles (both dissolved in PBS) for 2 h in a CO₂ incubator at 37 °C. After incubation, drug-containing medium was aspirated from each well, cells washed twice with PBS (1mL X 2) and fixed with 3.5 % paraformaldehyde solution in PBS for 15 min at room temperature. The cells were washed twice with PBS (1 mL) and stained with phalloidin conjugated to Alexa 594 (Invitrogen) diluted 1:500 in 3 % BSA solution in PBS. After 1 hour incubation, at room temperature in the dark the excess dye was washed from the plate and cells were again gently rinsed with PBS. The cover slips were mounted on slides using fluoromount mounting medium (Southern Biotech) and dried overnight at room

temperature in the dark. The cells were imaged using a LSM710 confocal microscope using the λ 405nm (blue channel) and λ 568 nm (red channel) lasers.

Images thus obtained were opened in the Image J analysis software, and the image for each channel separated. Phalloidin stained cell images (red channel) were processed using the threshold plugin and the threshold set to fill up the entire area of the cell. A mask was then created to map the edge of the thresholded cell, which was then saved as a region of interest (ROI) in the ROI manager. This ROI was then imported into the blue channel image and used to mapping the periphery of the cell in this image. The intensity of fluorescence within this ROI (hence within the cell) was now measured, as was the area of the ROI (hence the cell). Measured intensity values were then divided by the area of the cell to get an arbitrary value representing the intensity per unit area of each cell. Similar measurements and calculation were done for CPT, empty vesicles and CPT loaded in vesicles treated cells. Values thus obtained in 10 or more cells for each treatment were analyzed and differences statistically evaluated using a paired two tail T-Test.

2.3. Results and Discussion

2.3.1 Synthesis and Structural Characterizations

The hydroxyl groups in dextran were substituted with renewable resource hydrophobic motif 3-pentadecyl phenol (PDP), cardanol (CAR) or stearic acid (SA) as shown in Figure 2.5. PDP was functionalized with carboxylic acid group and then anchored to the dextran hydroxyl group (MW = 6,000) using DCC/DMAP. The degree of substitution was varied by using different mole ratios of the PDP-acids in feed. The polymers were purified by repetitive precipitation and subsequent dialysis using semi-permeable membrane. Similarly cardanol was also modified as described for PDP and anchored in the dextran backbone. Commercial available SA was directly used for dextran modification. The structure of the PDP-attached dextran (DEX-PDP) was confirmed by NMR and FT-IR spectroscopic analysis.

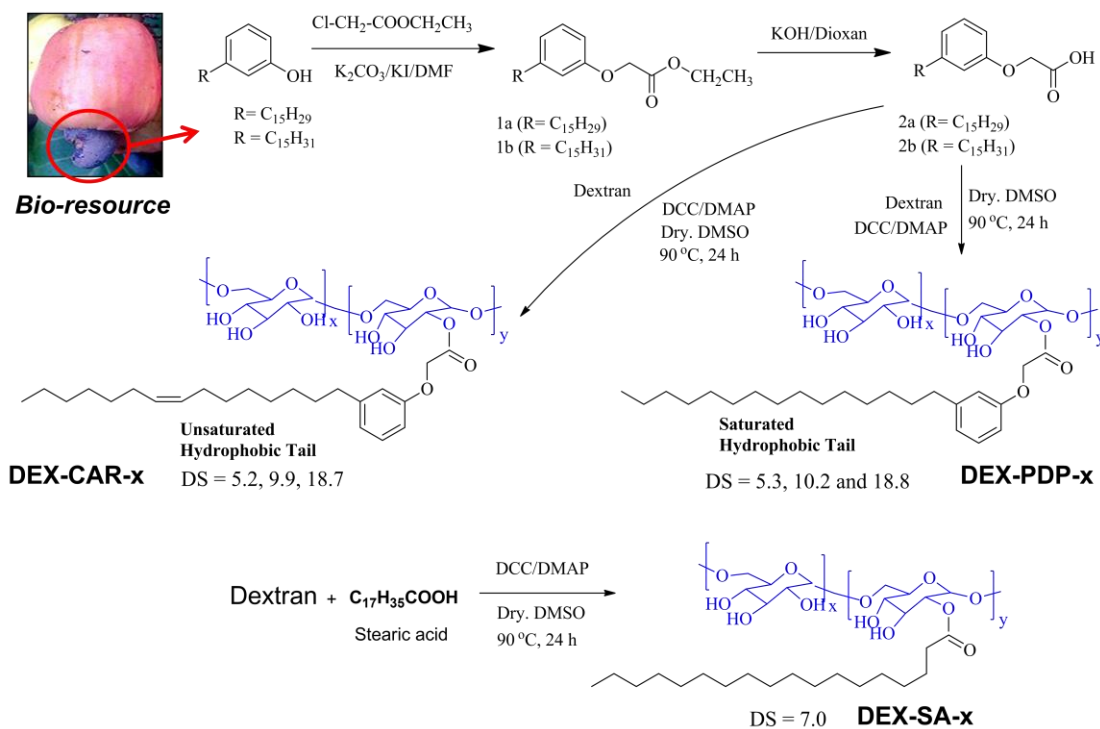
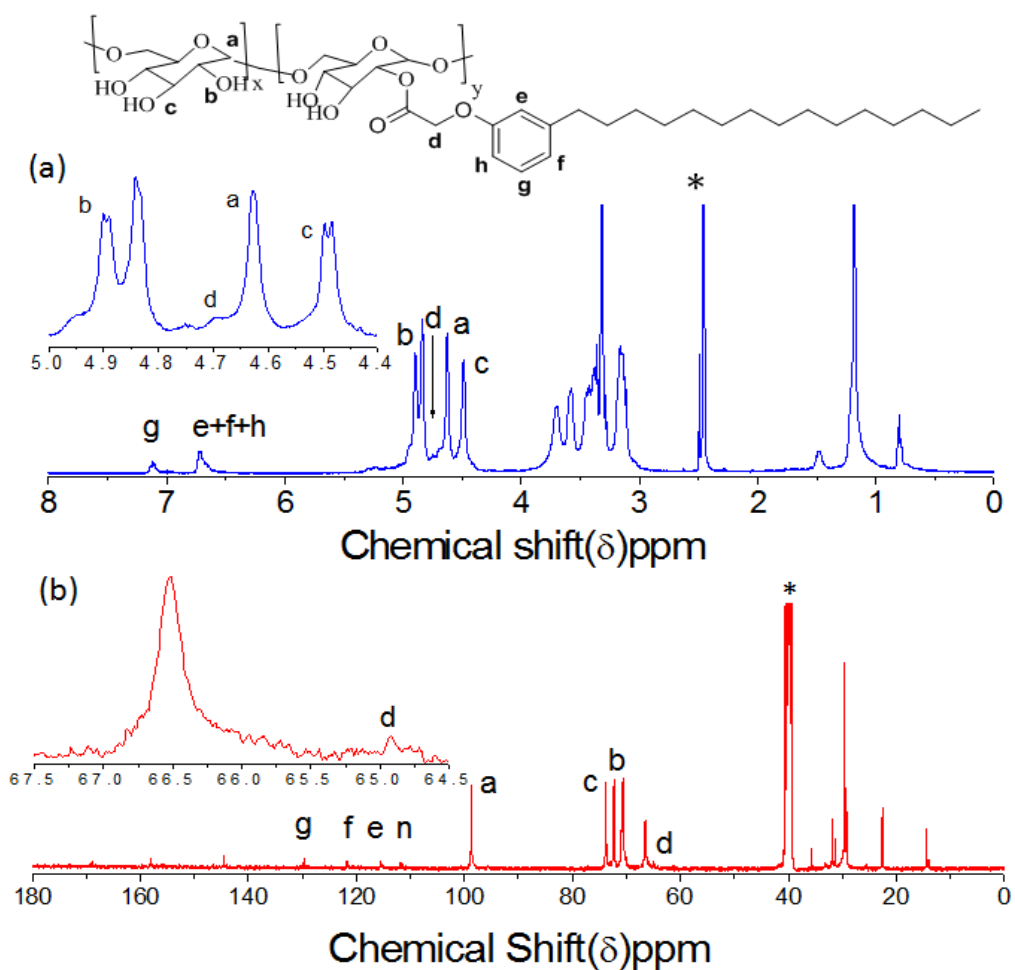


Figure 2.5. Synthesis of dextran derivatives

Figure 2.6. ^1H and ^{13}C -NMR spectra of dextran(DEX)-PDP polymers (in d_6 -DMSO)

^1H -NMR spectrum (see figure 2.6a) of the substituted dextran showed peaks at 6.50 -7.30 ppm and 0.5 to 3.00 ppm for the PDP-aryl and aliphatic protons, respectively. The protons from dextran units appeared at 3.00 to 5.00 ppm.¹⁸ Upon the formation of ester linkage, the protons $\text{Ar-OCH}_2\text{-COO-DEX}$ appeared at 4.64 ppm, which merged with the anomeric protons of dextran (see Figure 2.6a). Similarly in the ^{13}C -NMR spectrum (see figure 2.6b), the carbon atom $\text{Ar-OCH}_2\text{-COO-dex}$ appeared at 64.5 ppm. All the protons and carbons in the structure were assigned with the help of 2D NMR-HSQC technique (see Figure 2.7).

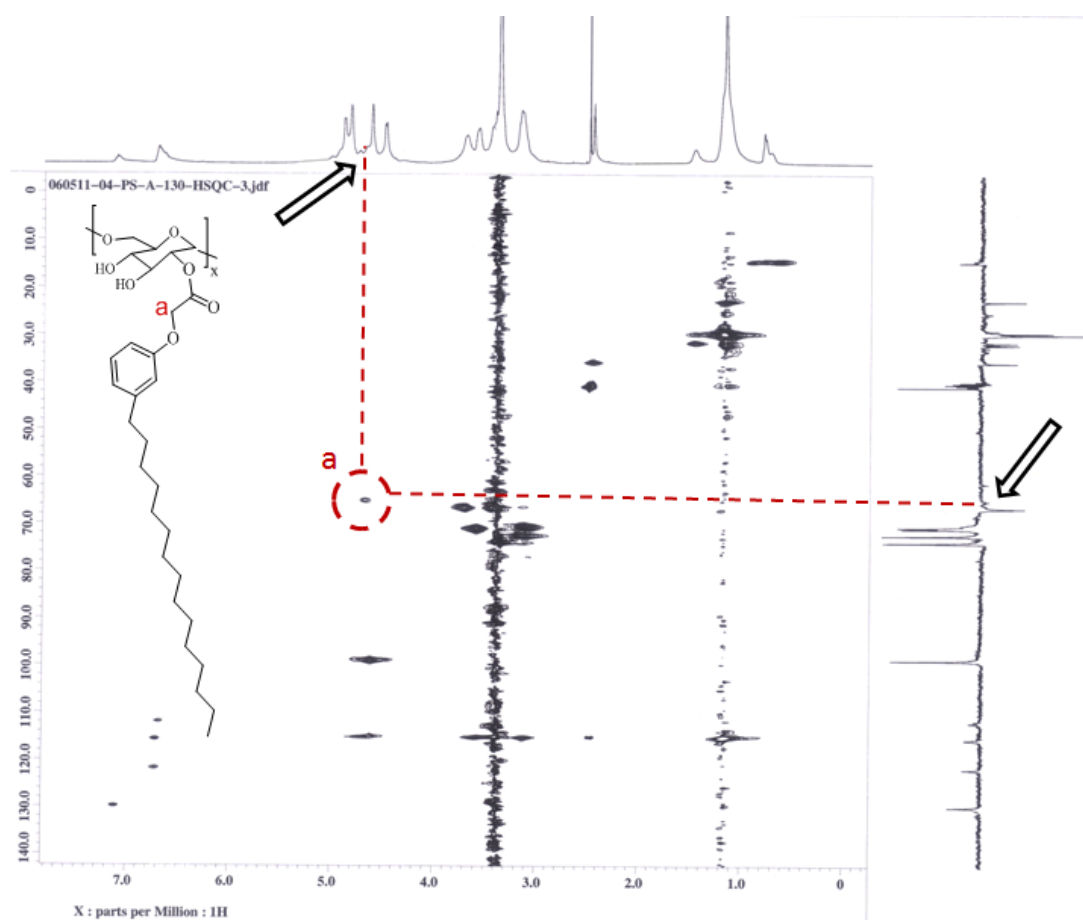


Figure 2.7. 2-D NMR-HSQC spectrum of DEX-PDP in d_6 -DMSO

The degree of substitution (DS) was estimated by comparing the peak intensities of anomeric proton in dextran at 4.62 ppm with the PDP aryl protons at 7.2 ppm in the hydrophobic part. The degree of substitution in dextran versus feed revealed that the incorporation of PDP hydrophobic units in the dextran linearly increased with increase in their feed (see figure 2.8 and 2.9).

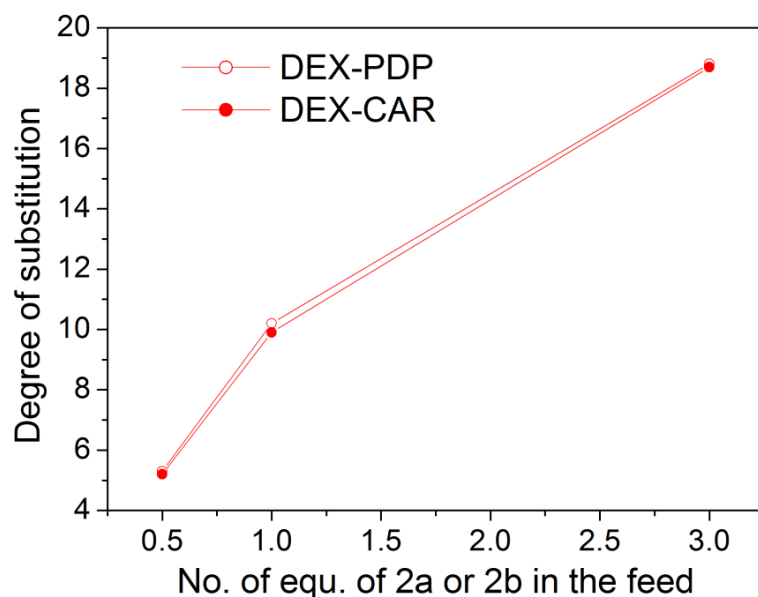


Figure 2.8. Plot of degree of substitution on dextran backbone versus no. of equivalents of compound 2a or 2b in the feed.

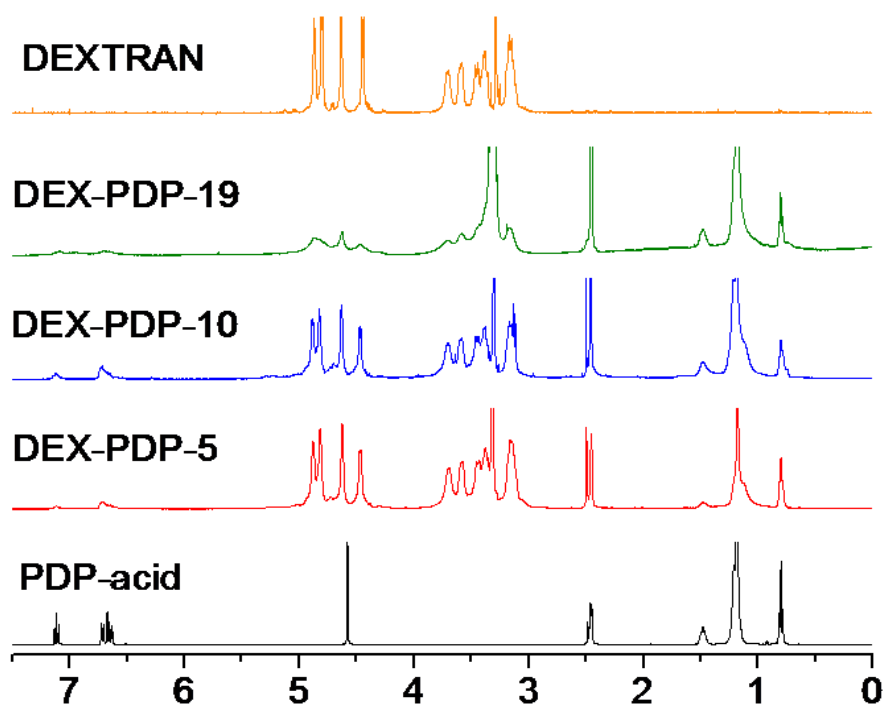


Figure 2.9. Stack plot of PDP-acid, DEX-PDP-5, DEX-PDP-10 and DEX-PDP-19 and Dextran NMR in d_6 -DMSO.

FT-IR spectrum of PDP-acid showed distinct stretching band at 1730 cm^{-1} with respect to -C=O stretching frequency (see Figure 2.10). Upon the formation of ester linkage between dextran and PDP-units, the -C=O stretching frequency shifted to higher wave number at 1765 cm^{-1} .¹⁸ Additionally, the free hydroxyl groups in the dextran were also showed broad peaks at 3400 cm^{-1} in DEX-PDP-5.

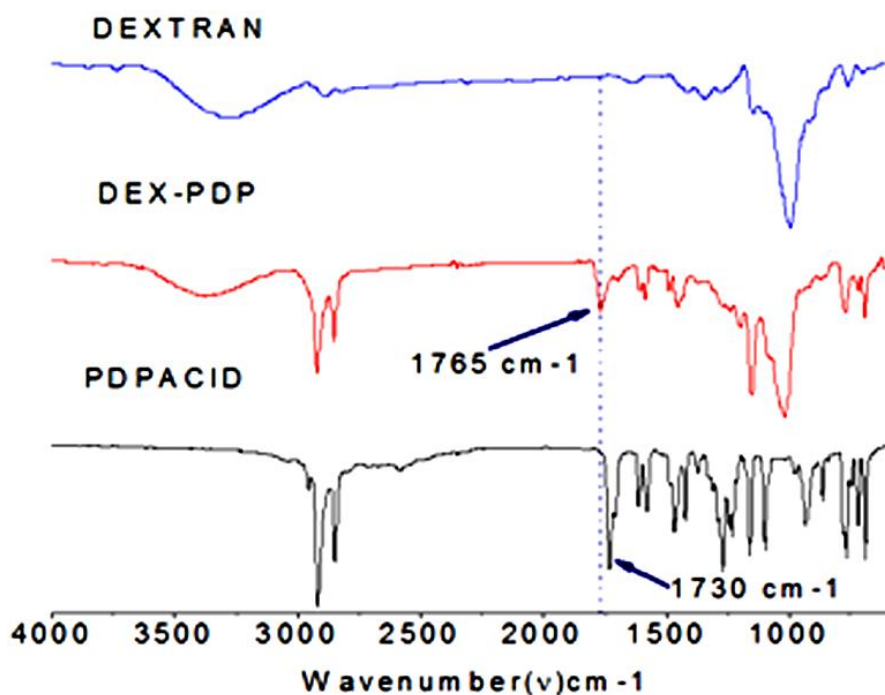


Figure 2.10. FTIR spectra of Dextran, DEX-PDP and PDP-acid (compound 2a)

The gel permeation chromatograms (GPC) of the modified dextran showed mono-modal distribution with respect to pure samples and they were completely devoid of starting materials (see Figure 2.11 and inset table). The shift in the chromatogram of DEX-PDP-5 and DEX-PDP-10 in the low retention time compared to dextran further validates the substitution of PDP-acid on the dextran backbone.

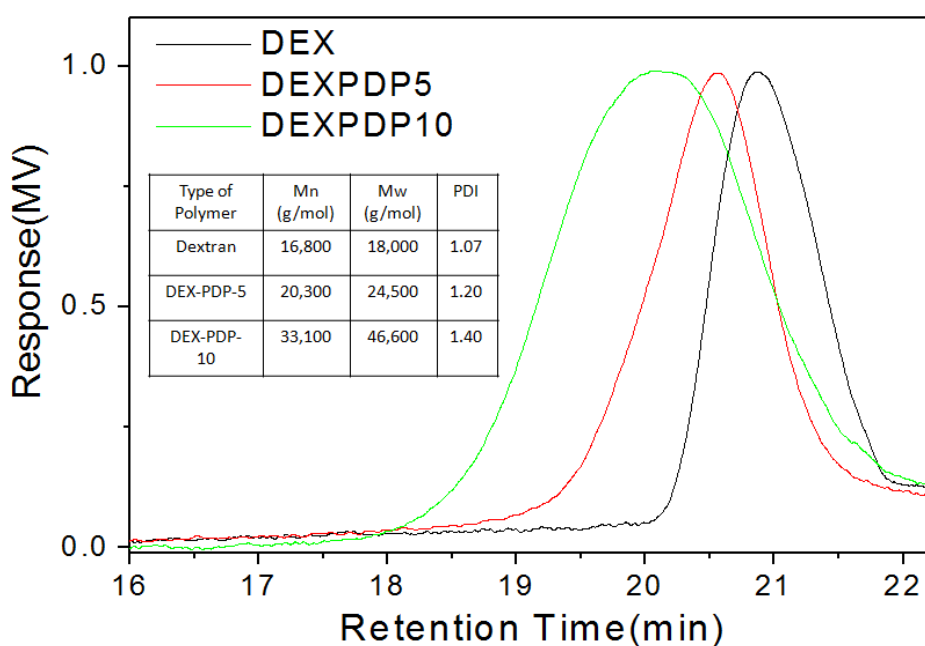


Figure 2.11. GPC Chromatograms of dextran, DEX-PDP-5, DEX-PDP-10 and PDP-acid in dimethyl formamide at 25 °C.

Thus, both NMR and FT-IR analysis confirmed the formation of the expected structure. Similarly, the structures of cardanol and SA substituted dextrans were also confirmed by NMR and FT-IR techniques (See Annexure figure 2.1 to 2.4).

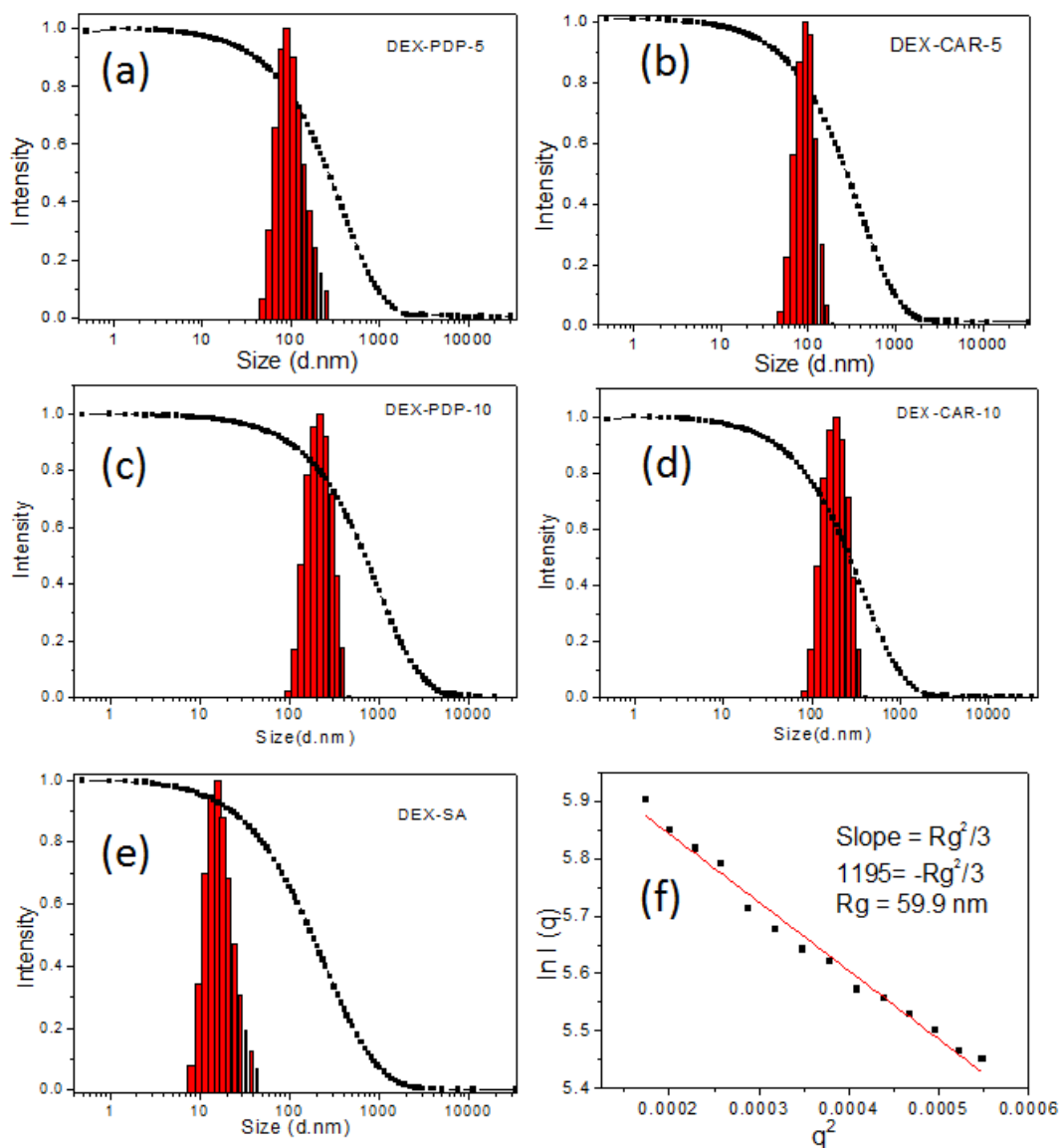


Figure 2.12. DLS histogram of (a) DEX-PDP-5 (b) DEX-CAR-5, (c) DEX-PDP-10, (d) DEX-CAR-10, (e) DEX-SA. SLS plot of DEX-PDP-5 (f).

2.3.2 Self Assembly of Amphiphiles- Light Scattering Analysis:

The newly designed dextran derivatives had unique amphiphilic structure and they self-organized in water (also PBS) through hydrophilic and hydrophobic interactions. The amphiphiles were found to be easily dispersible in water (or PBS buffer) at ambient conditions and produced clear solution. Dynamic light scattering (DLS) analysis of DEX-PDP-x was carried out in PBS buffer and the histogram for DEX-PDP-5 is shown in figure 2.12a. A very good auto-correlation with respect to

self-assembled structures having uniform size distribution of 120 nm (radius = 60 nm) was obtained. Similarly DLS histogram of DEX-CAR-5 is shown in figure 2.12b. Hydrodynamic radius of DEX-CAR-5 was calculated as 45 nm. The average sizes of the self assembled structures of dextran amphiphiles was found to be increased with increase in the PDP or CAR substitution. DLS histogram of PDP and CAR with degree of substitution 10 % in the dextran backbone are given in Figure 2.12c and 2.12d. The hydrodynamic radius for DEX-PDP-10 and DEX-CAR-10 was obtained as 110 nm and 90 nm respectively. However, the samples with 15-20 % degree of substitution were found to be partially soluble in water for self-organization. The stearic acid substituted dextran derivative DEX-SA-7 showed the formation of small aggregates of 25 nm in the DLS (See Figure 2.12e).

Static light scattering (SLS) technique was further employed to analyse the structure of the dextran polymer (see figure 2.12f). Typically, in the SLS technique the variation of scattered intensity with scattering angle was exploited to measure the size of a particle which directly provides the radius of gyration (R_g) of polymer structures in solution. The SLS data for DEX-PDP-5 in PBS at 25.0 ± 0.1 °C was carried out and the intensity (in $\ln$ scale) of scattered light at different angle was plotted against q^2 , where q is scattering vector magnitude (Guinier plot).^{16,19-21} It is given by the equation:

$$q = \frac{4\pi n \sin(\theta/2)}{\lambda_o}$$

Here n is the solution refractive index and λ_o is the incident wavelength, ie: 632.8 nm. The radius of gyration (R_g) was calculated from Guinier plot, given by:

$$\ln(I(q)) = \ln(I(0)) - \frac{q^2 R_g^2}{3}$$

Here slope of the graph is $R_g^2/3$.

The hydrodynamic radius (R_h) obtained from DLS was used to calculate the ratio R_g/R_h .^{16, 19-21}

The hydrodynamic radius (R_h) of the dextran polymer derivative was obtained by the dynamic light scattering data (see figure 2.12a, typically one measures the hydrodynamic diameter and half-of this value is R_h). The R_h was determined from the plot in figure 2.12a as 60 nm. The ratio of R_g/R_h will directly provide the information on the solution assemblies of the polymer: $R_g/R_h = 0.77$ for hard spheres, $R_g/R_h = 1$

for vesicles and $R_g/R_h = 1.7$ for coil-like structures.¹⁹⁻²¹ This methodology was recently reported by Houga et al. for polymer vesicles structures.^{16, 19-21} Adopting a similar methodology and using the SLS and DLS data the R_g/R_h value for the dextran polymer assemblies was determined as $R_g/R_h = 1$ which was exactly matched with the existence of vesicular structures.

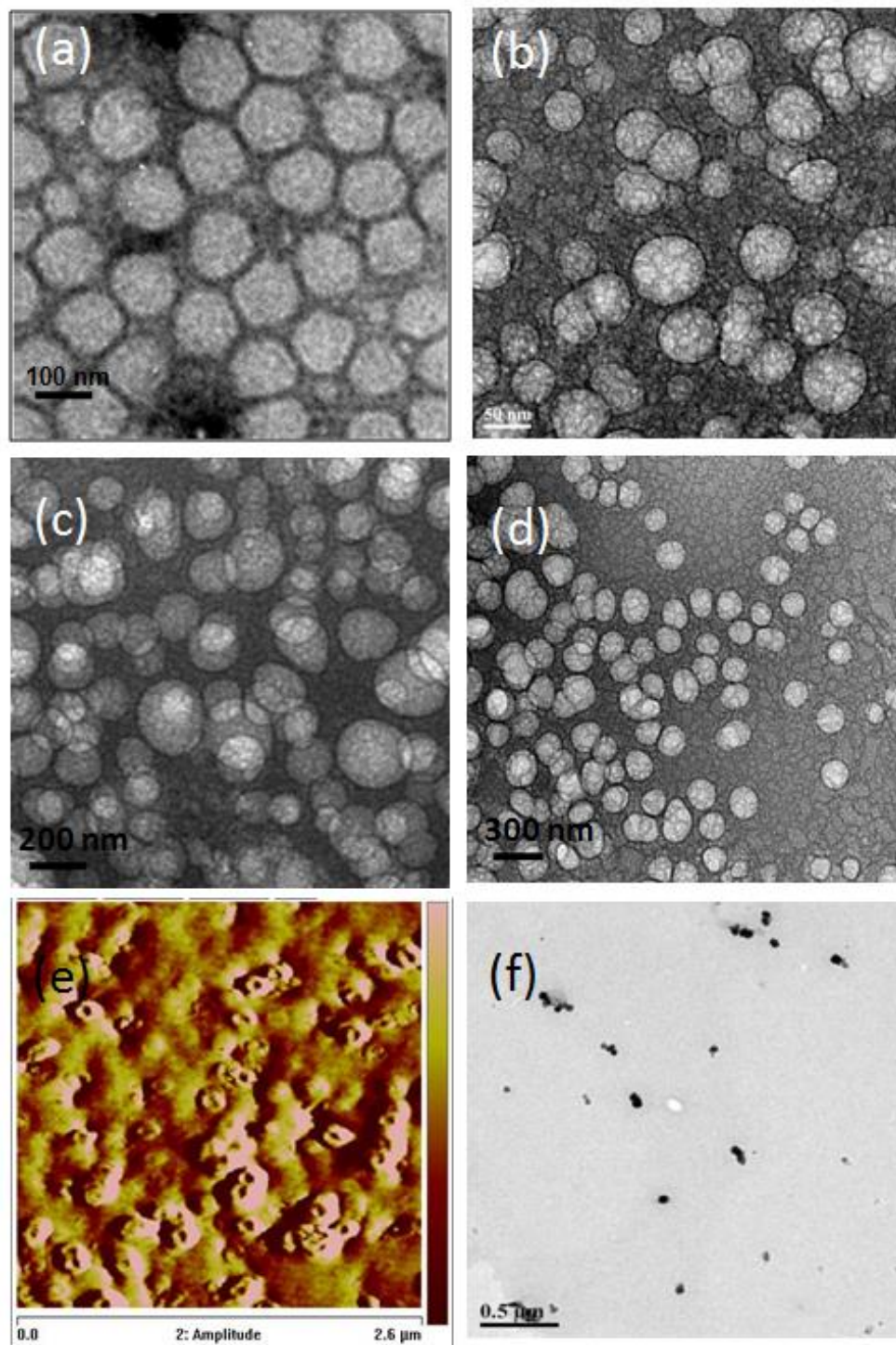


Figure 2.13. HR-TEM image of (a) DEX-PDP-5 (b) DEX-CAR-5, (c) DEX-PDP-10, (d) DEX-CAR-10. AFM image of DEX-PDP-5(e). HR TEM image of DEX-SA (f).

2.3.3. Self Assembly of Amphiphiles- Microscopic Analysis

HR-TEM image of DEX-PDP-5 (figure 2.13a) further confirmed that these self-organized objects were nothing but spherical shaped vesicles with an average diameter of 110 ± 10 nm ($n = 50$). The sizes of the TEM image of vesicles were almost identical to that of DLS data (see figure 2.12a). A similar observation was made in the HR-TEM image of DEX-CAR-5 (see figure 2.13b). Interestingly, PDP and CAR with higher degree of substitution (10 %) on dextran back bone was also found to be preserved vesicular geometry as seen figure 2.13c and 2.13d.

The vesicular structure of DEX-PDP-5 was also confirmed by AFM in both phase image (see Figure 2.13e) as well as in height image. The phase relied upon the energy that dispersed from the vesicular structure to the AFM tip. The diameter of the vesicles were obtained as 140 ± 10 nm which is very well matched with HR-TEM and DLS data. In typical vesicle geometry, center part of the structure was assumed to be softer than peripheral region and the elevated phase was an indication of harder structures and vice versa (see figure 2.13e).²² This surface difference in the vesicular self assembly dissipated the different responses to the force of AFM tip; as a result, the phase image of DEX-PDP was observed as high periphery (harder surface) and lower center (soft interior).²³ The AFM images provide direct proof for the vesicular structure of the DEX-PDP-5. Thus both DEX-PDP and DEX-CAR forms vesicular structures. Remarkably, HR-TEM image of the DEX-SA-7 resembled as nanoparticle²⁴ (see Figure 2.13f) rather than hollow vesicles as observed in the dextran modified with renewable resource hydrophobic tails (see figure 2.13a to 2.13d). This directly provides evidence that the nature of the hydrophobic segments played a crucial role in determining the size and shape of the vesicular self-assemblies in dextran backbone. The role of hydrophobic unit on self assembly was studied separately using crystal structure analysis and by calculating enthalpy of packing.

2.3.4. Crystal Structure and Enthalpy of Packing

Interestingly, in the present investigation, the renewable resource hydrophobic units PDP and CAR were capable of producing exclusively vesicular shapes. The reasons for the renewable resource hydrophobic units directing the dextran backbone toward vesicular self-assemblies were analyzed based on by two independent methods: (i) resolving the single crystal structures for the PDP units and (ii) thermal

analysis of hydrophobic units to measure their degree of packing. To trace the nature of the chain packing in the vesicular structures, single crystal X-ray structure of the PDP-acid was resolved. Crystals were grown for PDP-acid in dichloromethane/acetone and its unit cell structure and ORTEP diagram are given in Figure 2.14.

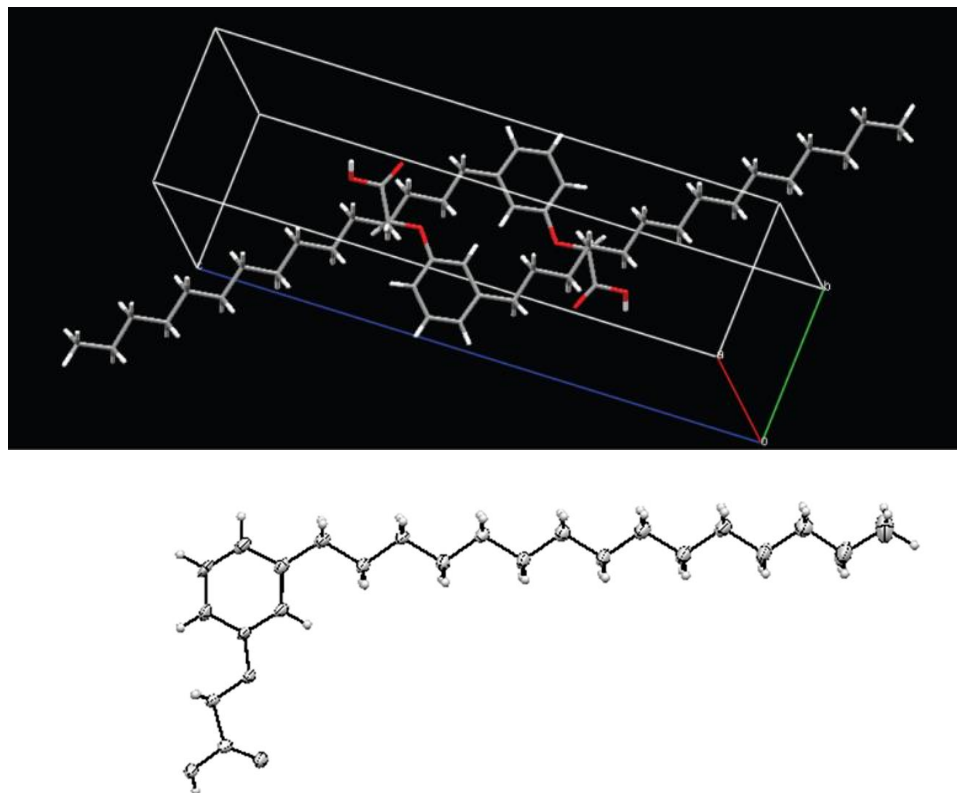


Figure 2.14. Unit cell and ORTEP diagram for the single crystal of compound PDP-acid (compound 2a).

This molecule crystallized in triclinic lattice in the space group of P-1 and their unit cell parameters were determined as: $a = 5.382 \text{ \AA}$, $b = 6.929 \text{ \AA}$, $c = 29.95 \text{ \AA}$, $\alpha = 85.615^\circ$, $\beta = 89.872^\circ$ and $\gamma = 73.155^\circ$. PDP-acid was planar and the pentadecyl chains were orientated towards each other to form a hydrophobic layer of thickness $23.6 \text{ \AA}$ (or 2.36 nm). The wall thickness of the dextran vesicles ($\sim 2.45 \pm 0.18 \text{ nm}$ ($n = 50$), from TEM images) was almost identical to that of the hydrophobic layer distance ($\sim 2.36 \text{ nm}$, from the crystal structure) indicating that the thin hydrophobic walls in the vesicles were constituted by the long tails in the PDP units. Based on the crystal structure, it could be concluded that the dextran vesicles were typically unilamellar in nature (see figure 2.15).

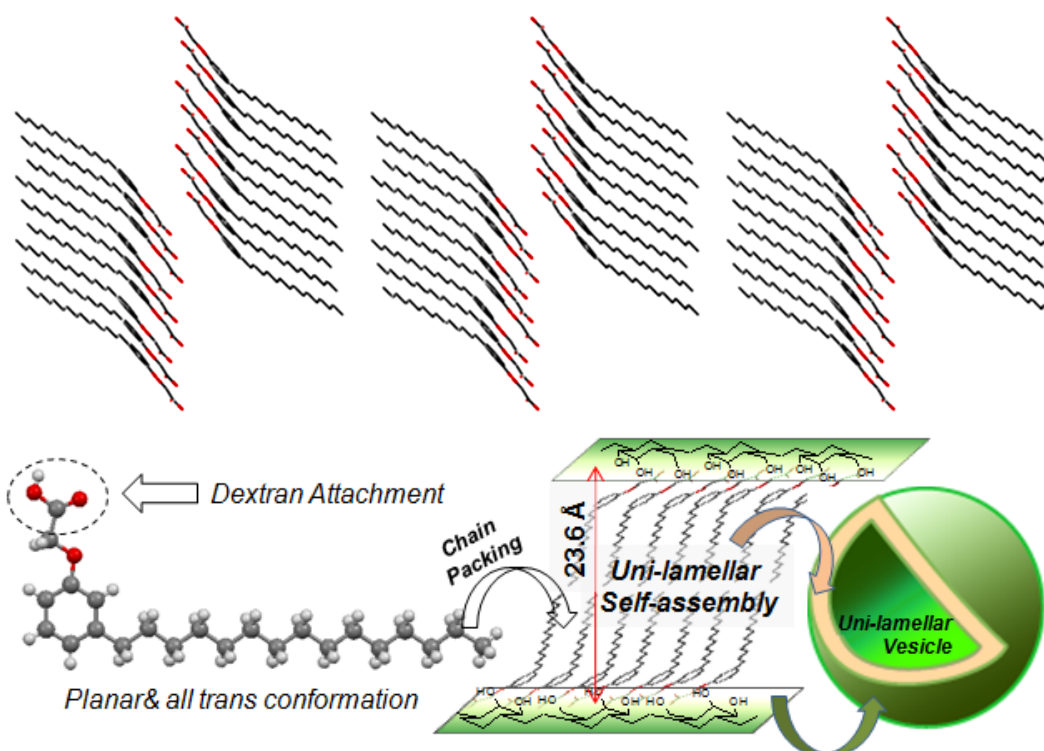


Figure 2.15. Molecular packing diagram for compound PDP-Acid along b^* axis and schematic representation of crystal packing of hydrophobic unit in the unilamellar vesicle formation.

Differential scanning calorimetry (DSC) thermograms of the hydrophobic units were recorded in heating/cooling cycles to understand their chain packing abilities (See Figure 2.16 and Table 1). The enthalpies of the crystallization (ΔH_c) and melting transitions (ΔH_m) of the PDP-acid was obtained as $\Delta H_c = 49.5$ kJ/mol and $\Delta H_m = 52.8$ kJ/mol, respectively. The enthalpies of the stearic acid was found to be almost similar to that of PDP-acid ($\Delta H_c = 47.7$ kJ/mol and $\Delta H_m = 54.2$ kJ/mol). Despite both PDP-acid and stearic acid have similar enthalpies values (percentage of crystallinity); they showed large difference in their self-organization in the dextran backbone (PDP-acid produced vesicles and SA induced nano-particles). This was attributed to the lack of chain inter packing as observed from the SA- crystal structures.²⁵

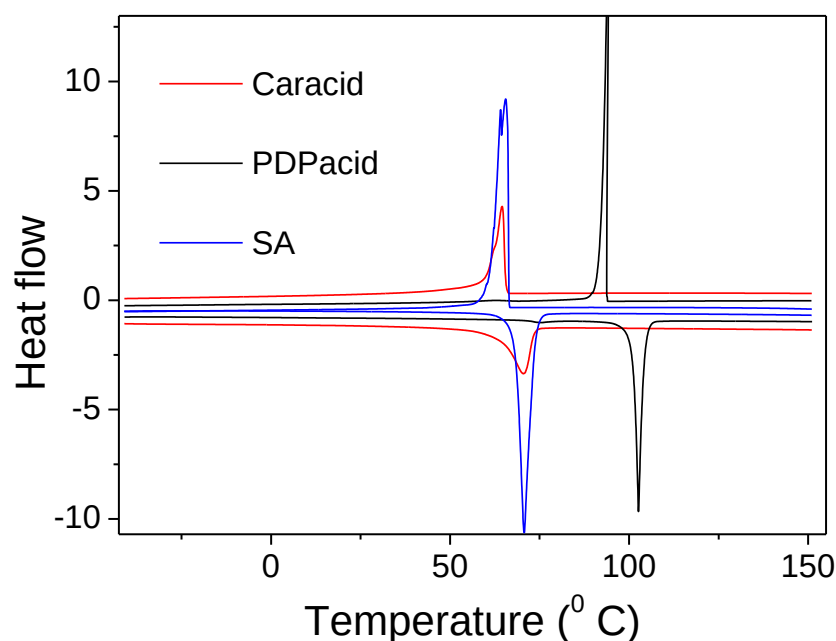


Figure 2.16. DSC thermograms of CAR acid (compound 2b), PDP acid (compound 2a) and SA(stearic acid) in the heating and cooling cycle at 10° C /min.

Table 1. Enthalpies of Melting and crystalline transitions of PDP acid, CAR acid and SA

Sample	T _m (°C)	ΔH _m (KJ/mol)	T _c (° C)	ΔH _c (KJ/mol)
PDP-acid	101.54	52.8	95.28	49.5
CAR-acid	65.48	23.3	63.94	17.9
SA	68.88	54.2	65.13	47.7

On the other hand in PDP-acids, the alkyl chains were inter-digitized along the c-axis as seen in their crystal packing (see figure 2.15). Hence, it may be concluded that the strong inter-digitations of the chains in the renewable resource hydrophobic tails facilitated the vesicular structures. This is for the first time that such a good correlation has been established between the polysaccharide vesicles and their chemical structure using single crystal structure. The unsaturated tail molecule CAR-acid showed very less packing with lower energy terms ($\Delta H_c = 17.9$ kJ/mol and $\Delta H_m = 23.3$ kJ/mol) compared to that of its saturated counterpart. Typically, strongly packed chains showed higher crystallinity and needed higher energy for melting and released more heat during crystallization. It confirmed that the unsaturated chains

could be packed relatively weak in the DEX-CAR-x vesicles compared to that of DEX-PDP-x vesicles.

2.3.5. CVC determination and Drug/Dye Encapsulation

The critical vesicular concentrations of the vesicles (CVCs) were determined using pyrene as fluorophore (See figure 2.17a and 2.17b). A fixed pyrene amount (0.6×10^{-6} M) was encapsulated in the amphiphilic polymer scaffolds and subjected to photoexcitation using monochromatic light at 337 nm.²⁶ Upon increasing the concentration of amphiphilic DEX-PDP-5 and DEX-CAR-5 scaffold in aqueous medium, pyrene molecules got encapsulated in the hydrophobic layer of the vesicles.

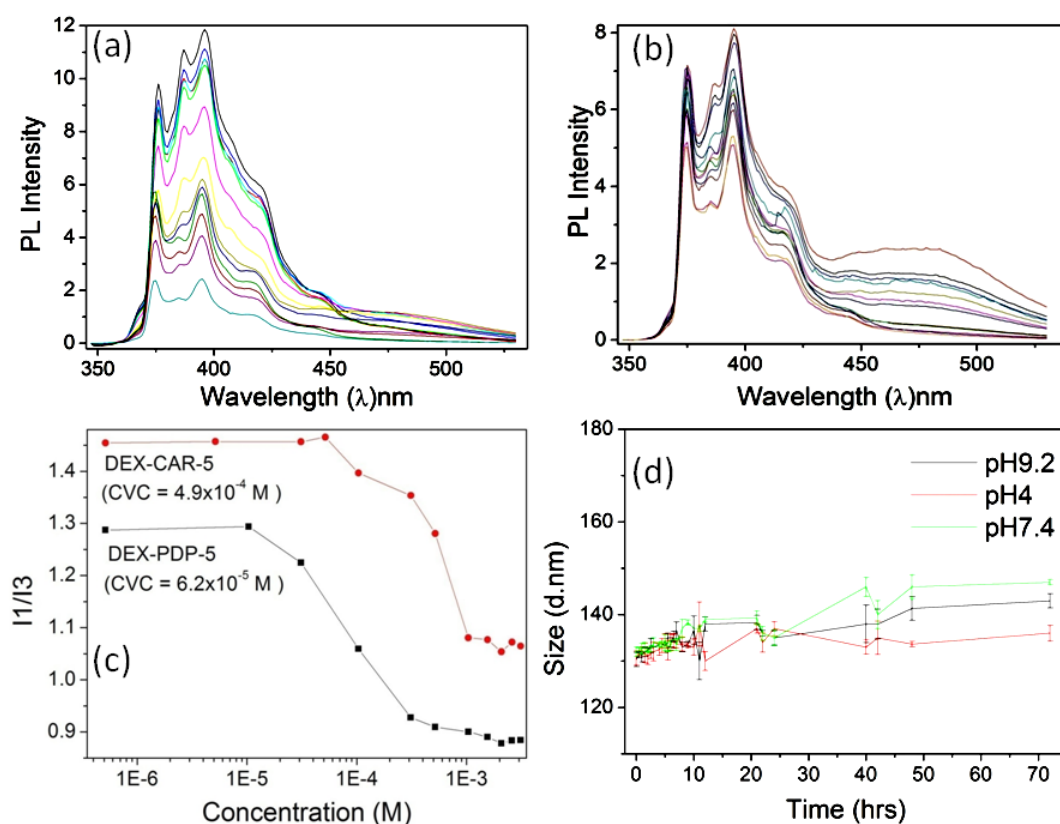


Figure 2.17. Pyrene emission spectra in the presence of (a) various concentrations of DEX-PDP-5 and (b) various concentrations of DEX-CAR-5. (c) I_1/I_3 Vs concentration plot of DEX-PDP-5 and DEX-CAR-5. (d) Size Vs time plot of DEX-PDP-5 obtained from DLS histogram at various pH conditions.

Pyrene emission spectra in the presence of DEX-PDP-5 and DEX-CAR-5 were shown in figure 2.17a and 2.17b. Based on the pyrene fluorescence peak characteristics I_1/I_3 ratio (375/386 nm),²⁶ the CVC of the DEX-PDP-5 and DEX-CAR-5 were determined as 6.2×10^{-5} M and 4.9×10^{-4} M, respectively (see figure 2.17c). The concentration of the polymer samples were calculated based on the repeating unit mass of the anhydro-glucose unit in dextran since the low degree of

substitution of PDP (5 %) do not contribute significantly to the concentration of the entire mass. The one order less concentration of CVC in DEX-PDP-5 confirmed the presence of strong packing of PDP units in their vesicular membranes compared to the unsaturated DEX-CAR-5 vesicles. This observation again supported the similar trend as seen in the DSC data for higher enthalpy values of PDP-units with respect to higher degree of packing. The precipitation of pyrene and polymer from the PBS hampered the CVC determination of the higher degree substituted dextran derivatives. This indicated that at higher degree of substitution, the encapsulation of polyaromatic molecules like pyrene in the unilamellar vesicles became almost impossible. Interestingly, less than 5 % incorporation of PDP in dextran was sufficient enough to self-organize dextran- backbone into very stable vesicular assemblies. Moreover the stability of these vesicles was studied at various pH and long storage period. The vesicles were found to be stable for long duration (3 days) and also in wide pH ranging from 4.0 to 9.2 (See figure 2.17d). It confirmed that the vesicles were stable in both acidic and basic medium.

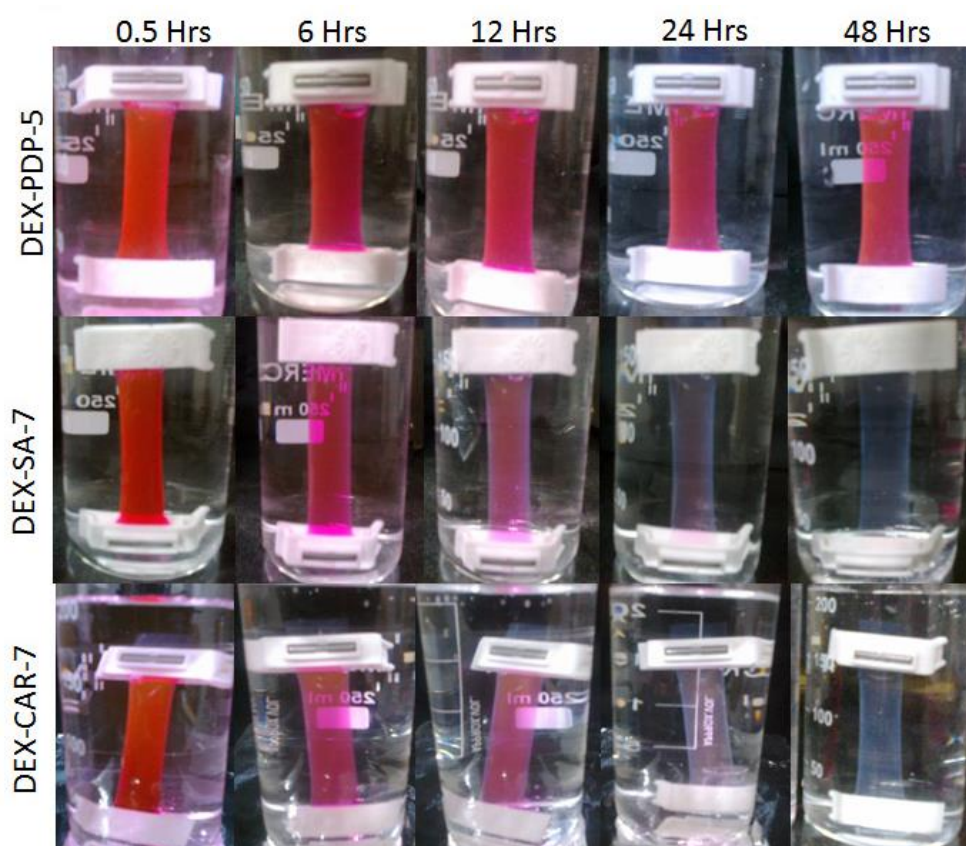


Figure 2.18. Dialysis photographs of rhodamine B (RhB) with DEX-PDP-5, DEX-CAR-5 and DEX-SA-7 taken at regular interval of time.

One of the important experiments which could differentiate the nanoparticles from the vesicular assemblies is that the encapsulation of the hydrophilic dyes (water soluble dye, like Rhodamine B (RhB) in the interior of the vesicles.^{27,28} Since the interior of the nanoparticle is hydrophobic, the encapsulations of water soluble dyes do not occur. To confirm the nature of these dextran assemblies, the derivatives DEX-PDP-5, DEX-CAR-5 and DEX-SA-7 were subjected to RhB encapsulation in water. The scaffolds and RhB were dissolved in DMSO + water and stirred for 12 h. Subsequently, the content was dialyzed in semi-permeable membrane for 48 h against water. The photographs are taken at regular interval during the dialysis are shown in figure 2.18.

The stability of the RhB was checked up to 7 days against dialysis in water and the results were almost same. The schematic representations of RhB encapsulation in DEX-PDP-5 are shown in figure 2.19a. DEX-CAR-5 did not stabilize the RhB and only very faint amount got trapped (see figure 2.19b for vials). This suggests that unsaturated tailed layers did not produce stable vesicular structures for stabilizing water soluble dye during dialysis. This observation was matching with DSC and CVC data for the packing of the tails in the saturated and unsaturated tails. As expected, the leaching out of RhB was faster in DEX-SA-7 due to its particle nature (See figure 2.18 and 2.19b).

The dialyzed sample vials were further exposed to photo-excitation using hand held UV-lamp (see figure 2.19c). The photograph provided clear evidence for the presence of RhB (orange emission) in the encapsulated vesicles only in DEX-PDP-5. DLS histograms of the RhB encapsulated DEX-PDP-5 vesicles indicated that the vesicles were little larger (170 nm, see figure 2.10d) in size compared to the nascent vesicles (120 nm, see figure 2.12a). Additionally, the static light scattering (SLS) measurements with the combination of DLS data revealed that the ratio of the R_g/R_h as 1.11 [radius of gyration R_g = 76 nm (from SLS) and hydrodynamic radius R_h = 85 nm, from DLS, see figure 2.19d and 2.19e], which confirmed their existence of vesicular structure in the RhB loaded dextran vesicles.¹⁹⁻²² HR-TEM image of DEX-PDP-5 (see figure 2.19f) was found as solid spherical objects of 170 ± 10 nm with respect to the encapsulation of RhB in their vesicular interior compared to their nascent vesicles (see figure 2.13a). The confocal microscope image of the RhB loaded

vesicles also further confirmed their spherical shape and very good luminescent in the encapsulated form (see figure 2.19g).

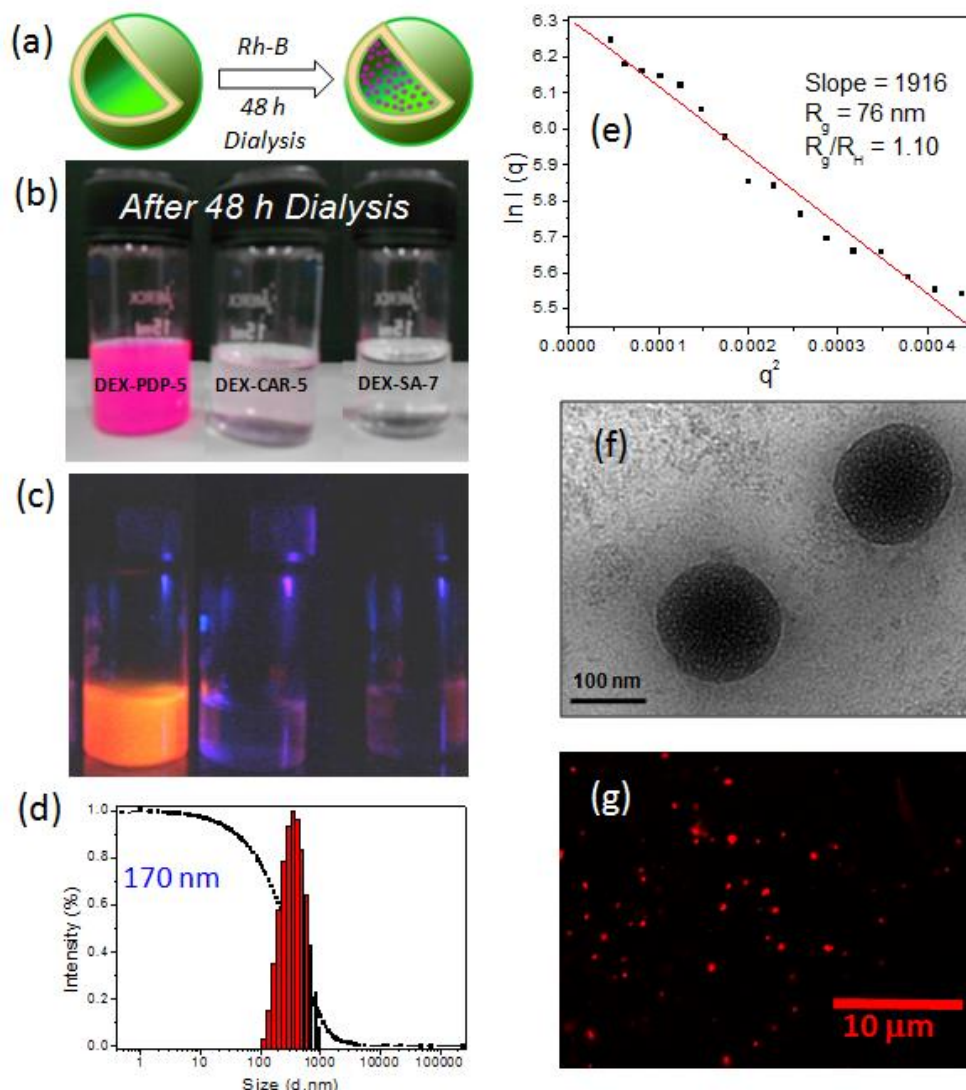


Figure 2.19. Schematic representation of the encapsulation of Rhodamine-B in the vesicle (a). Photographs of vials containing the dextran derivatives with RhB after 48 h of dialysis (b) and their solution color under photo excitation (c). DLS histograms (d) SLS plot (e) and HR-TEM (f) of RhB loaded DEX-PDP-5 in PBS (6.2×10^{-5} M). Confocal microscopic image of RhB loaded vesicles (g) in PBS (6.2×10^{-3} M).

Further, the multiple acquisition analysis by fluorescence microscopy revealed that RhB was indeed occupied at the interior of the vesicles and not on the periphery (see figure 2.20a). The AFM image of the RhB loaded dextran vesicle further proves the retention of the vesicular morphology (see 2.20b). Thus, the custom designed DEX-PDP-5 vesicular structure is very unique to encapsulate the water soluble dye (RhB). These RhB vesicles were found to be stable under stored conditions for more than a month for further usage.

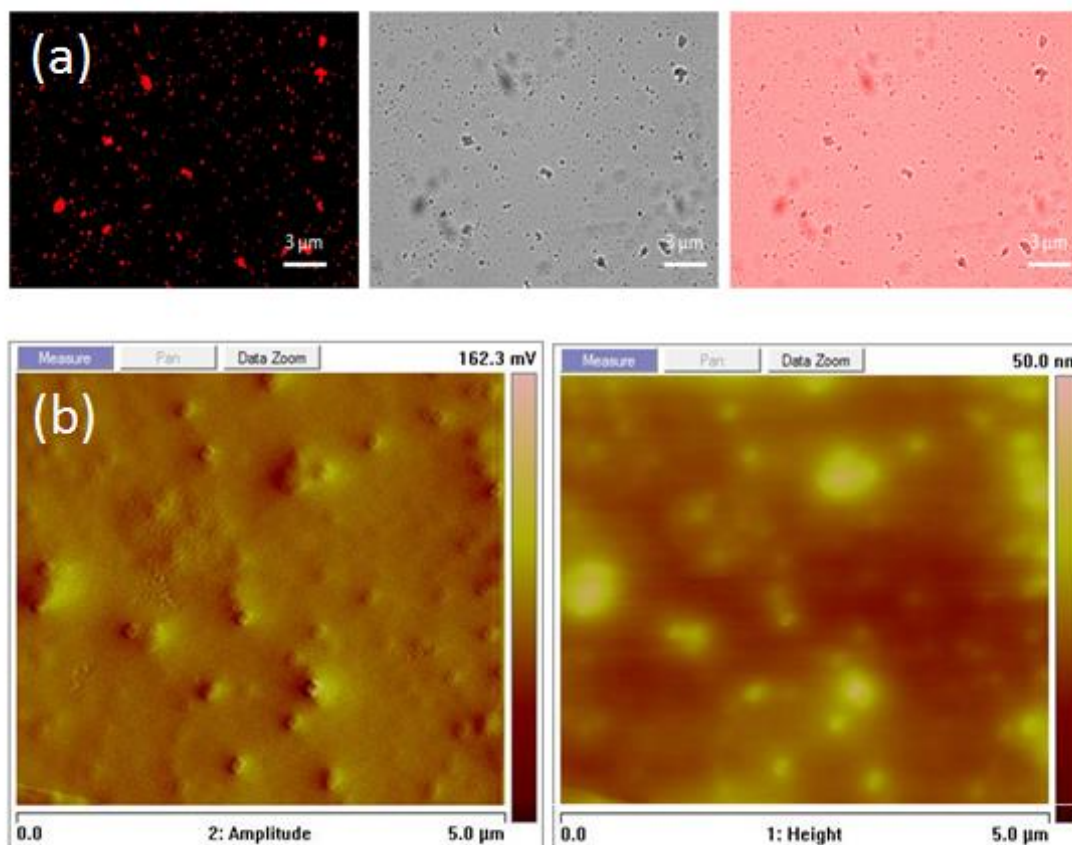


Figure 2.20. Multiple acquisition Fluorescent microscopic image of RhB loaded vesicles (a), AFM Phase image and Height image (b) of RhB loaded in the vesicle.

Anticancer drug camptothecin (CPT) was encapsulated in DEX-PDP-5 vesicles by dialysis method (See 2.21a). The polymer to drug ratio was maintained as 10 wt % in the feed. The drug loading content was estimated using absorbance spectroscopy as 5.8 wt % in the vesicles (see experimental section for details). DLS histograms and features of the resultant drug-loaded dextran vesicle conjugate are shown in figure 2.21b. DLS analysis indicated that the size of the drug-vesicle conjugate as 140 nm, which was almost similar to that of its nascent vesicle (120 nm, see figure 2.12a). TEM images of drug-vesicle conjugate (see figure 2.21c) confirmed the retention of the spherical vesicular structure. The thin-hydrophilic dextran as corona in the periphery was clearly evident from the expanded TEM image (see inset). Upon encapsulation of CPT in the hydrophobic sheet, the drug loaded vesicle increased in size slightly from 110 nm to 120 ± 10 nm. The TEM image of the vesicles in the CPT loaded (or RhB loaded vesicles in figure 2.19f) were different from the nascent hollow vesicles (see figure 2.13b) due to the occupancy of the loaded molecules in the layer (or interior).

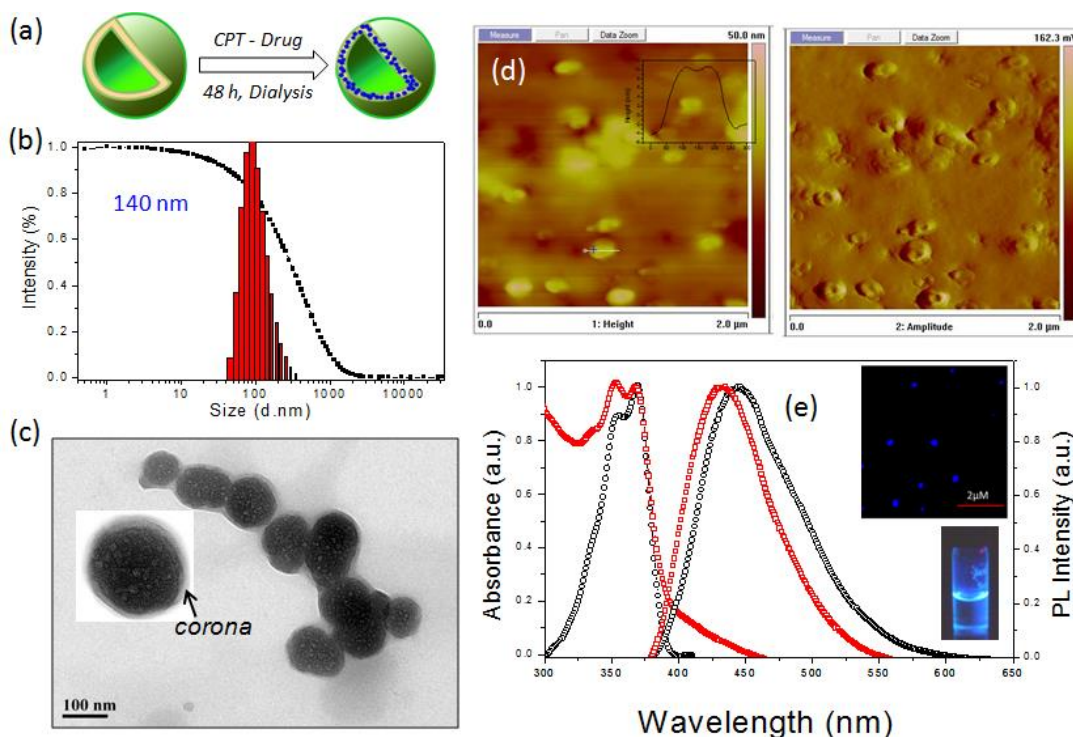


Figure 2.21. Schematic representation of the encapsulation of camptothecin in the vesicle (a) and its DLS histograms (b). HR-TEM image of the CPT loaded vesicles (c). The inset showed the expanded image of the vesicles with well defined corona with respect to vesicular assemblies. Phase and Height AFM images of CPT loaded vesicles (d). Absorbance and fluorescence spectra of CPT-loaded vesicles in PBS (e). Inset in (e) shows fluorescent microscopic image of CPT-loaded vesicles. Vial in (e) shows the CPT-loaded vesicles under hand held UV Lamp.

The AFM image of the CPT loaded dextran vesicle further proves the retention of morphology (see figure 2.21d). The diameter and the height of the aggregates were calculated as 140 nm and 10 nm, respectively. Further, the static light scattering (SLS) measurements with the combination of DLS data revealed that the ratio of The R_g/R_h as 1.11, which confirmed their existence of vesicular structure in the CPT loaded dextran vesicles. The absorbance spectra of CPT (see figure 2.21e) did not show any difference whether it was alone or encapsulated in vesicles. Upon photoexcitation (at excitation wavelength, 370 nm), the CPT showed deep blue emission and their emission maxima were varied with respect to its surrounding environment. The 10 nm red-shift in the emission spectra of CPT in PBS compared to CPT loaded vesicles was a clear indication of their stable form in the latter compared to former.²⁹ The photograph of CPT-drug loaded vesicle (in PBS) showed the blue bright fluorescence (see figure 2.21e). The fluorescence microscope images of the

same sample confirmed the existence of spherical objects with respect to their vesicular geometry (see inset in figure 2.21e)

2.3.6. Stability of CPT in Vesicles- HPLC Studies

Typically, the camptothecin pharmacophore, α -hydroxylactone functionality, is highly susceptible for hydrolysis in PBS or blood plasma to its inactive carboxylic form (see figure 2.22).³⁰ Therefore, it is very important to quantify the active form of the encapsulated CPT in the DEX-PDP vesicles under the physiological condition at pH = 7.4 (in PBS). Both CPT loaded vesicle and CPT (alone) in PBS were incubated at 37 °C for 24 h. The samples were retrieved at regular interval and subjected to reverse phase HPLC for quantification of the CPT in its active form (see figure 2.22)³¹

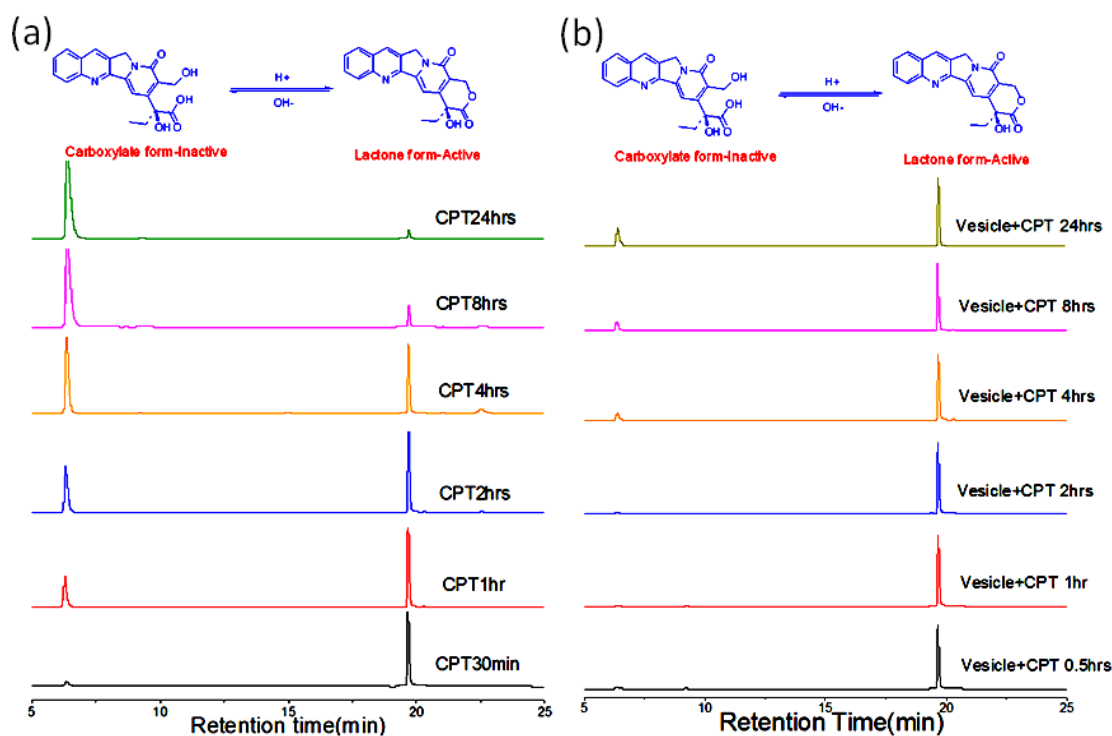


Figure 2.22. HPLC traces of free CPT (a) and CPT loaded in vesicles (b) at different time intervals.

CPT in carboxylic form (inactive form) and lactone form showed peaks at 7.2 min and 19.2 min, respectively. From the HPLC traces, it was evident that CPT in PBS underwent rapid hydrolysis from lactone to carboxylate (see figure 2.22a) whereas the CPT loaded vesicles preserved the active form even after 8 h (see figure 2.22b). The percentage of active lactone form was plotted over the incubation time and the data were fitted with first order exponential drug hydrolysis [fit-values are

given in the figure 2.23a, and 2.23b). In PBS, the hydrolysis of the drug was very fast with $\tau_D = 2.6 \pm 0.9$ h and more than 96 % of the CPT underwent fast decomposition into its inactive carboxylic acid form. On the other hand, the encapsulation of CPT in PDP-DEX-5 vesicles enhanced their stability as $\tau_D = 10.3 \pm 3.9$ h which was four times better compared to the drug alone. The Y-intercepts (59.4 %) revealed that more than 60 % of the drug could be stabilized while encapsulated in the vesicles in PBS. Hence, the stability of CPT in vesicle was ten times higher than that of CPT in PBS alone (even after 24 h). All the above studies confirmed that CPT was preserved in the active lactone form in the dextran vesicles.

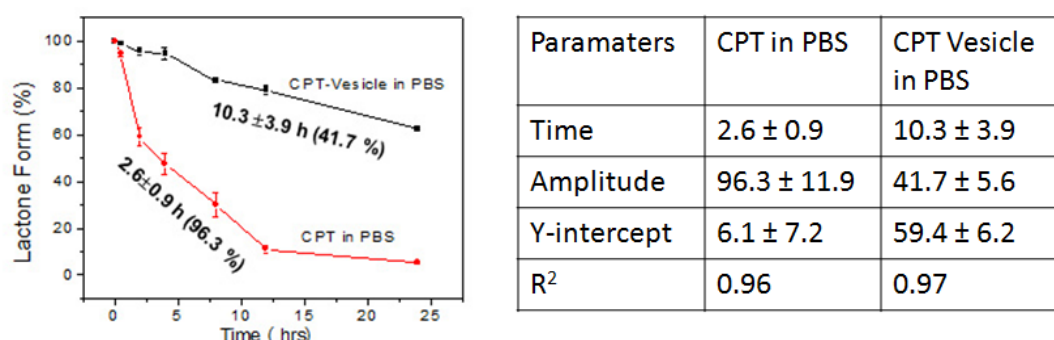


Figure 2.23. (a) Percentage of lactone form of free CPT and CPT in vesicle and (b) table depicts first order exponential hydrolysis data of CPT.

2.3.7. In-vitro Release Studies

The encapsulation of hydrophilic RhB and hydrophobic CPT were achieved in the interior and hydrophobic lamellar layer of the dextran vesicles through like-like interactions (for example hydrophobic layer has more preference for hydrophobic drug CPT loading and vice versa). Since these vesicular membranes were not cross-linked, the encapsulated molecules may be expected to release under dialysis over a period of time. Du *et al.*²⁴ and Shen *et al.*³² had reported a more than 60 % of drug release in their un-crosslinked dextran nanoparticles and CPT-conjugated PEG vesicles, respectively. In the present case, the structures of the scaffolds were designed with aliphatic ester-linkages between dextran and hydrophobic units. Hence, external stimuli such as esterase enzyme (abundant in cell cytoplasm) could also cleave the scaffold structures. Xu *et al.* had reported the estimation of esterase enzyme in liver microsomes and cytosols in the range of 9.8 – 30.5 U.^{33a} Alter *et al.* had reported that minimum of 10 U of esterase was required to cleave enzymatically polysaccharides derivatives.^{33b} Based on these literature reports, 10 U of esterase was used to study the release behaviors of *in-vitro* release of CPT and RhB loaded vesicles

either neat or in the presence of esterase (by dialyzing the at 37 °C). Aliquots were collected at regular interval and they were subjected to absorption spectroscopy for the estimation of drug release. These experiments provided opportunity to study the drug release from the vesicles either via natural leaching out under larger dilution or the chemical cleavage of membrane layer by esterase. The schematic representation and plots of cumulative CPT release (%) over incubation time of the CPT release in the presence of esterase (or its absence) are shown in figure 2.24a and 2.24b. In the absence of enzyme, the scaffolds undergo naturally leached of $60 \pm 5\%$ of the hydrophobic drug- CPT within 5 h. In the presence of enzyme, the release processes happened at two steps: (i) initial rapid release of 60-70 % of drug in 5 h and (ii) steady- release of remaining 30-40 % of the drug over a period of next 48 h. The faster drug release in the presence of esterase was correlated to its chemical action on the aliphatic-ester linkage which connects both the dextran and hydrophobic PDP-units.³⁴ The photographs of the vials taken (see figure 2.24a) in the presence of esterase clearly showed the precipitation (the precipitation occurred after 24 h) of samples with respect to the cleavage of vesicular membranes. In the absence of esterase, there was no precipitation (monitored up to one week) in the solution indicating that the vesicular structures could partially disassemble, however, not precipitated from the aqueous medium.

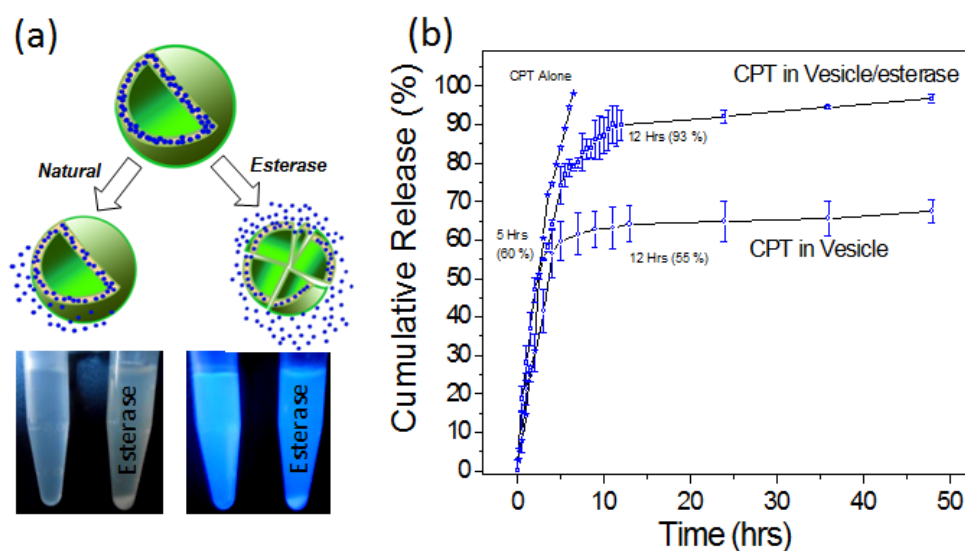


Figure 2.24. (a) Schematic drug (CPT) release of vesicular scaffolds under normal dialysis and in the presence of esterase enzymes. The photographs of the vials showed the formation of precipitate over a prolonged storage of the CPT loaded vesicles (b) In-vitro CPT release plot under normal and esterase conditions.

The chemical analysis of the precipitates by high resolution mass spectroscopy (HR-MS) confirmed that the cleavage of PDP-acid units from the dextran scaffolds (see HR-MS spectrum in annexure figure 2.5). Base peak is observed at 385.2709 is corresponding to PDP-acid +Na⁺. The PDP-acid was nothing but the renewable derivative initially used for the functionalization of dextran, and therefore, is not expected to be toxic.

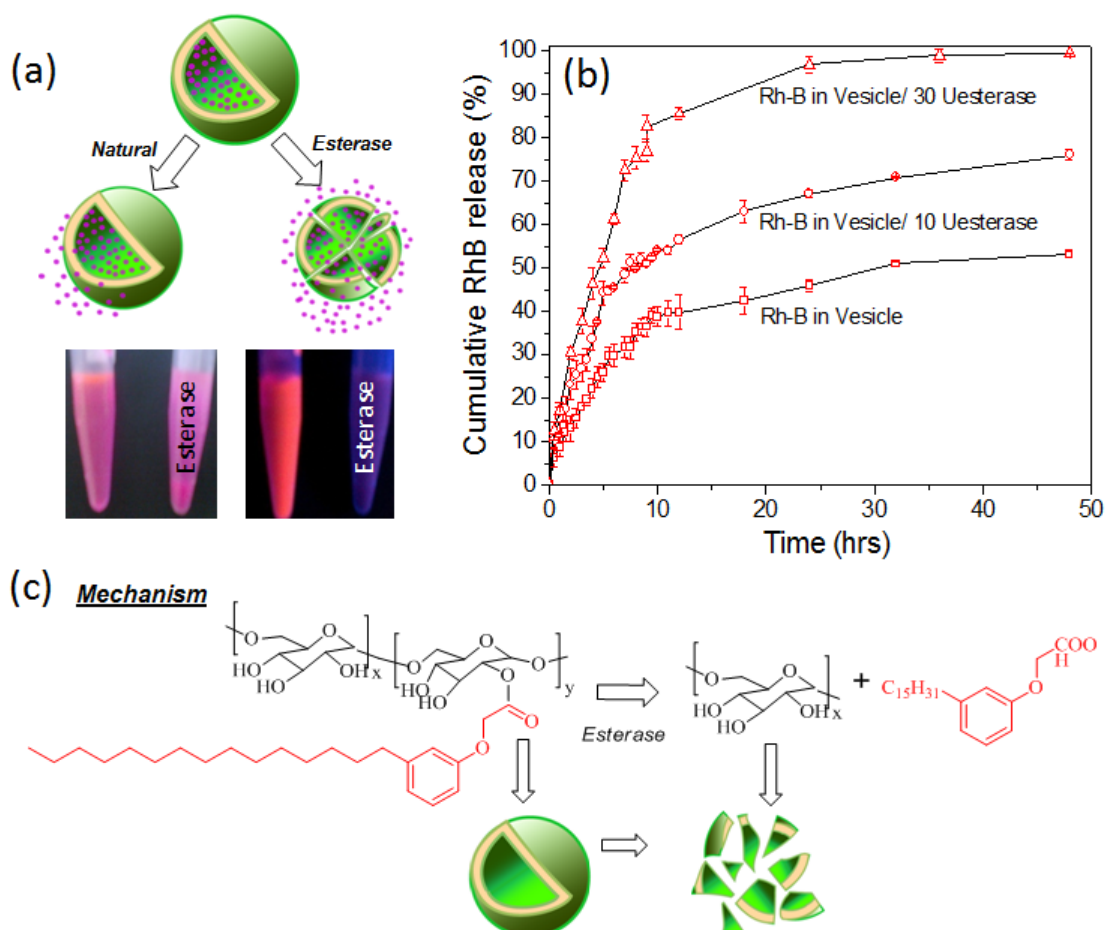


Figure 2.25. (a) Schematic RhB release from vesicular scaffolds under normal dialysis and in the presence of esterase enzymes. The photographs of the vials showed the formation of precipitate over a prolonged storage of RhB loaded vesicles in the presence of esterase. The left and right of the vials are corresponding to the RhB loaded vesicles in the absence or presence of esterase, respectively. In-vitro RhB release plot under normal and in the presence of esterase (10 U and 30 U) enzyme. (c) The schematic model represents the disassociation of vesicular scaffolds in the presence of esterase.

The *in-vitro* release studies of RhB loaded vesicles (see figure 2.25a and 2.25b) showed a similar release in the presence of the esterase enzyme. Up to 40 ± 5 % of the RhB was released under similar conditions in 12 h and this release was enhanced up to 70 ± 2 % in the presence of esterase. Surprisingly, the RhB release

was not completed for the 10 U of esterase enzyme. This could be correlated to the difference in the activity of the esterase enzyme towards the hydrophilic RhB loaded dextran vesicles compared to that of the hydrophobic CPT-loaded vesicles. In order to check this point, a control experiment was carried out under *in-vitro* release using excess amount of esterase (30 U) (see figure 2.25b).

At higher enzyme concentration, the RhB loaded vesicle released all the RhB dye within 24 h. Interestingly, the present scaffold with RhB was found to show more stability towards the external stimuli such as esterase. The CPT or RhB loaded vesicles produced turbidity in the presence of esterase which precipitated after 24 h. The natural release occurred only under the *in vitro* release studies at 37 °C during the prolonged dialysis against a large amount of water. The samples were found to be stable under storage conditions (at 4 °C, lyophilized dried sample in a vial or RhB vesicles in solution in the refrigerator). The unique advantages of the present design are that the scaffold could encapsulate both hydrophilic and hydrophobic guest molecules. Hence, based on above studies, it could be concluded that the custom designed dextran vesicular structure was selective to cleave under esterase and release entire drugs under physiological conditions as shown in figure 2.25c.

2.3.8. Cytotoxicity and Cellular Uptake Studies

Cytotoxicity of the polymer vesicle and its drug conjugate were tested on wild type mouse embryonic fibroblasts cells (WTMEFs) using the MTT assay.³⁵ Cytotoxicity of the polymer vesicle was tested for 0.05 μ M and 1.87 μ M in PBS (see figure 2.26a) and the polymer was found to be non-toxic to fibroblasts at both concentrations. Under identical conditions, 0.05 μ M of CPT in PBS and CPT encapsulated vesicles in PBS when tested showed a $\sim$ 2 fold increase in loss of viable cell numbers (detected using a crystal violet staining of cells, see figure 2.26b)³⁶ and $\sim$ 2.5 fold increase in cytotoxicity (detected by the MTT assay, see figure 2.26c) by CPT in vesicle as compared to CPT alone in PBS. Thus, CPT-loaded dextran vesicle was indeed more effective in killing cells than the drug alone.

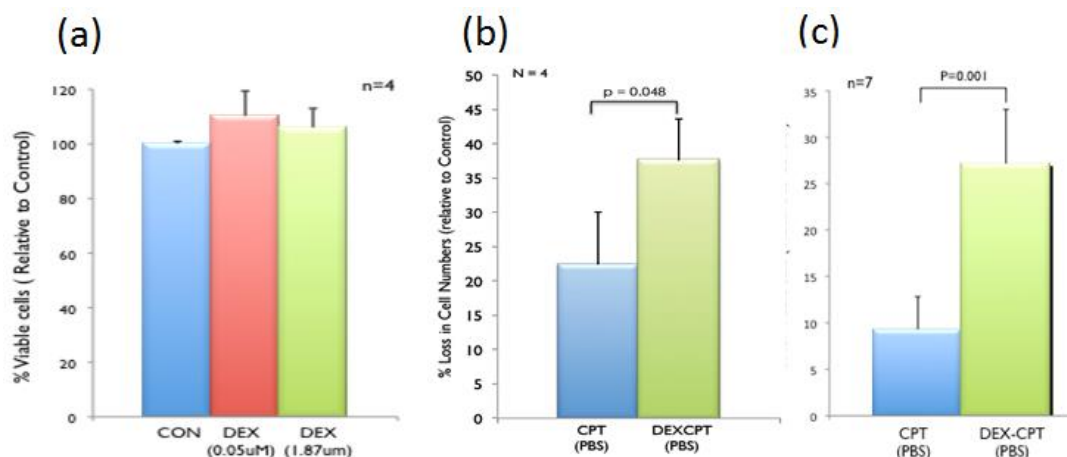


Figure 2.26. Cytotoxicity data of DEX-PDP-5 at various concentrations (a) in MTT assay. Cytotoxicity data of CPT and CPT loaded DEX-PDP-5 vesicles in crystal violet assay (b) and MTT assay (c).

In order to visualize the cellular up take of the drug-vesicle conjugate, the treated MEFs were analyzed by confocal microscope. WTMEFs were treated with 35 μ M concentration of either CPT alone or CPT loaded vesicles or equivalent amount of DEX without CPT for 2 h. This concentration of CPT was selected based on the ability to detect its fluorescence in cells using the λ 403 nm laser in the confocal microscope (see figure 2.27a and 2.27b). Cells were also stained with phalloidin conjugated to Alexa dye, which specifically binds F-actin and labels the actin cytoskeletal network in cells. This staining was visualized using a 568 nm laser and was used to define the morphology of the adherent spread cell (see Figure 2.27a and 2.27b). No major changes in morphology or actin cytoskeletal organization of cells were detected in the time the cells were treated. Phalloidin staining of cells was also used to map the spread area of individual cells to measure total CPT fluorescence in cells using the Image J-software.

Intensity thus calculated and equated to the area of the cell showed a significant increase in CPT fluorescence in cells treated with CPT loaded DEX-PDP-CPT vesicles as compared to CPT or DEX-PDP alone (see Figure 2.27a and 2.27b). CPT alone at the high 35 μ M concentration was seen to also form aggregates that were seen to be deposited on the cover slip as bright fluorescent spots (see Figure 2.27a). This was not observed in CPT loaded in DEX-PDP vesicles (see Figure 2.27a and 2.27b). Careful analysis of the confocal images, showed the presence of fluorescent CPT (observed at 403 nm) in distinct vesicles localized in the perinuclear region (marked by solid white arrows) (see Figure 2.27c). The nucleus in a

representative cell is marked with a dotted line and labeled as 'N'. These results confirmed the cellular uptake of the dextran vesicles and their localization in cells.

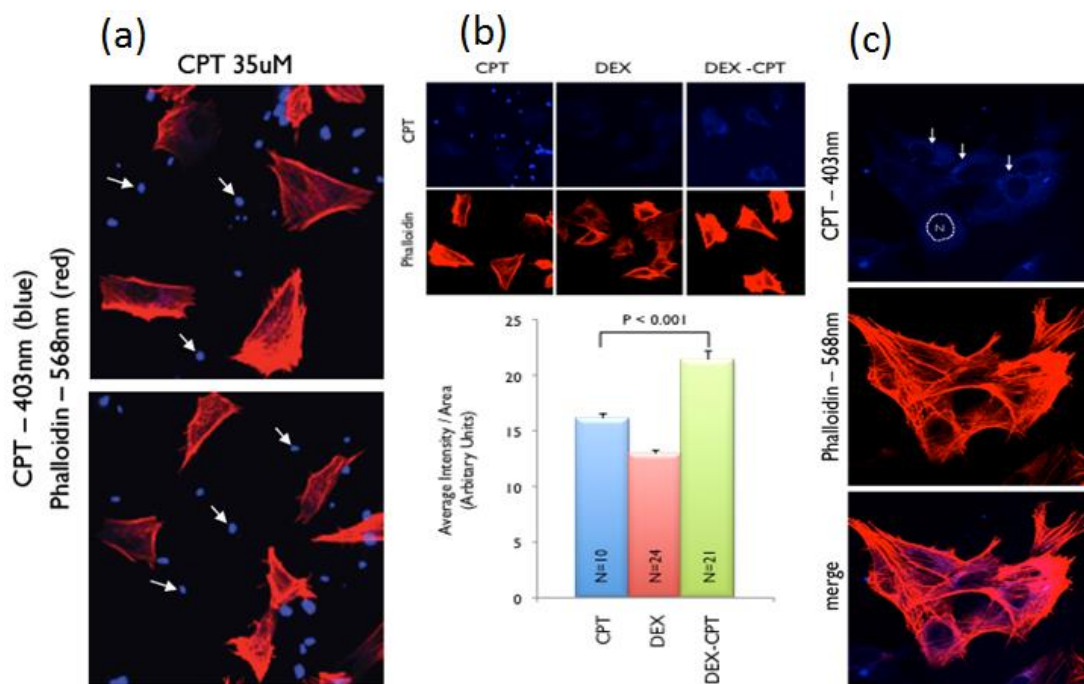


Figure 2.27. (a) Confocal image of free CPT treated cells. (b) confocal images DEX-PDP-CPT vesicles treated cells and its quantitation. (c) Localization of CPT loaded DEX-PDP-5 vesicles in cells.

The ability of DEX-PDP-5 scaffold to deliver hydrophilic molecules into cells was checked by analyzing the cellular uptake of rhodamine B. This hydrophilic dye molecule exhibits a strong emission at 580 nm enabling a direct visualization inside the cell. MEFs were hence treated with 200 μ M DEX-PDP-5 containing rhodamine B for two hours and analyzed by confocal microscope using the ($\lambda = 568$ nm) laser (figure 2.28a). This concentration of DEX-PDP-RhD-B was selected based on the ability to detect its fluorescence and hence uptake and localization in cells. Cells were also stained with phalloidin conjugated to Alexa 488 dye that specifically binds F-actin and labels the actin cytoskeletal network in cells. This staining was visualized using a 488 nm laser and was used to define the morphology of the adherent spread cell (see Figure 2.28a). DEX-PDP-RhD-B was seen to localize in vesicular structures (of μ m to sub μ m size) that showed distinct perinuclear localization. Mouse fibroblasts were further treated with 200 μ M concentration for increasing times (2hr and 24hrs) and the uptake of Rho B DEX compared.

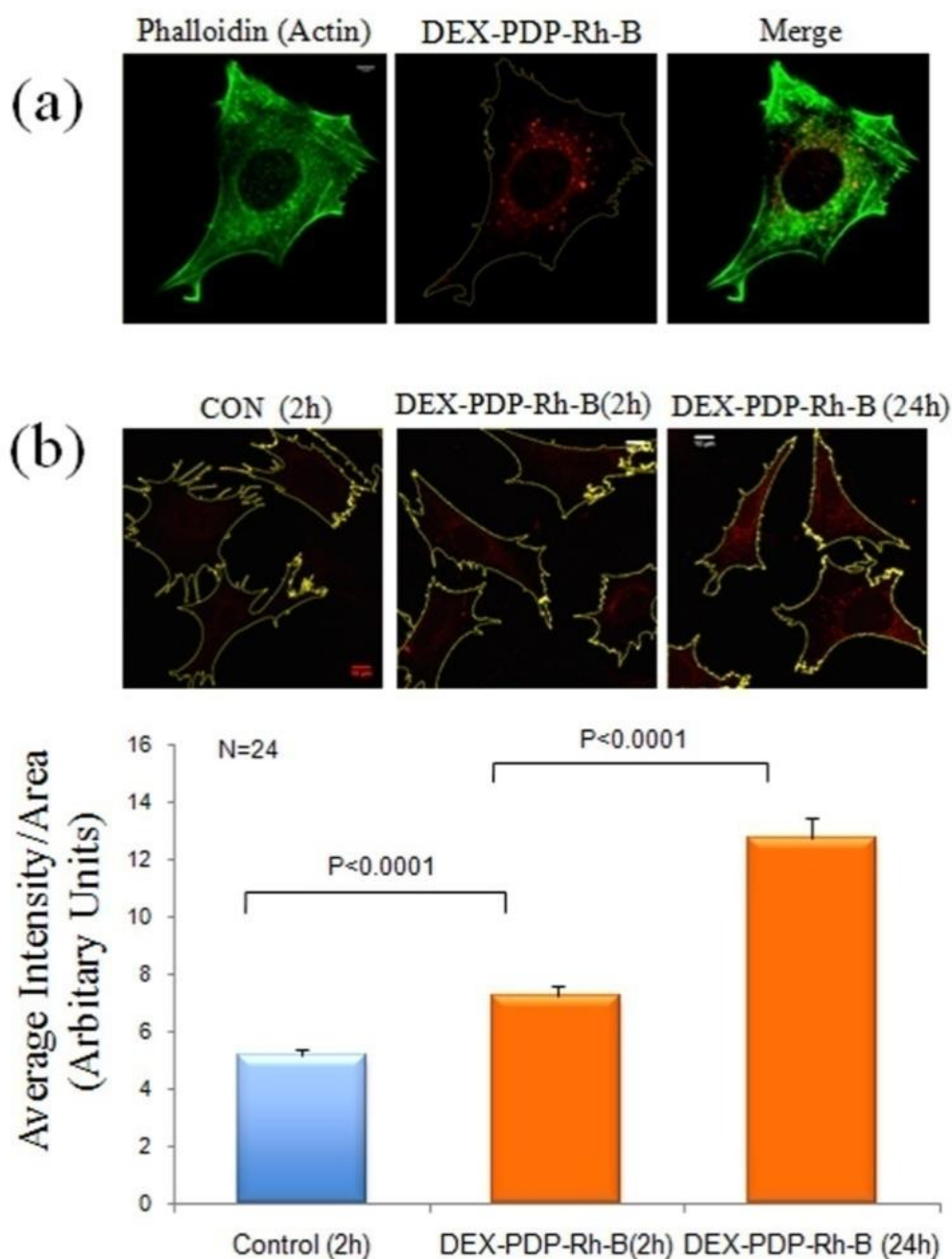


Figure 2.28. Endocytosis of DEX-RhB in mouse fibroblasts (a) Endocytosed DEX-PDP-RhB shows a distinct perinuclear localization in mouse fibroblasts. Actin cytoskeletal network in cells is stained with phalloidin. (b) Fluorescence confocal images of control untreated cells and cells incubated with DEX-PDP-RhB for 2h and 24hrs were recorded, their spread area mapped and fluorescence intensity in the cell area analysed using the Image J densitometric software. Mean of fluorescence intensities is represented in the graph.

As described earlier phalloidin staining of cells was used to map the spread area of individual cells and the red fluorescence in cells measured using the Image J software. Intensity thus calculated and equated to the area of the cell showed a significant increase in Rhodamine fluorescence in cells treated for 24hours versus

2 hours, both of which were significantly higher than the control (Figure 2.28b). This suggests that uptake of the DEX-PDP vesicles is rapid but also sustained showing a ~ 25 % increase after the first 2 hrs leading upto 24 hours. Moreover these studies suggest that the integrity of the scaffold is retained inside the cells as rhodamine fluorescence was seen localized in distinct vesicular structures. Disruption of the integrity of the scaffold would have resulted in release of rhodamine and its dispersion in the cytosol of the cell. Ongoing work is focused on identifying the subcellular compartment that these vesicles localize to and the endocytic pathway they use to enter the cell in normal and cancer cells.

2.4. Conclusion

In conclusion dextran vesicles based on renewable resource starting materials were successfully designed and synthesized as delivery vehicle for encapsulation of hydrophobic anticancer drug camptothecin as well as water soluble RhB molecules (or drugs). These newly synthesized dextran-PDP (DEX-PDP) amphiphilic polymers were self-assembled under physiological conditions as vesicles of ~120 nm in size. The vesicular structures very efficiently encapsulated CPT in the uni-lamellar pocket and also retained the active lactone pharmacophore in CPT more than ten times better than compared to CPT alone in PBS. The hydrophilic and hydrophobic segments in dextran vesicles were made up of aliphatic ester-linkage which was cleaved by esterase (as stimuli) for the large drug release under physiological conditions. *In vitro* drug releases studies confirmed that the vesicular scaffolds with water soluble dye (or drug) showed enhanced stability against esterase for more than 12 h. Cytotoxicity studies revealed that the polymer scaffold was non-toxic. The efficiency of the drug in killing the cells was found to be 2.5 times better when encapsulated in the vesicles compared to CPT alone. Compared to free CPT, the drug loaded vesicles showed significantly better cellular uptake and were seen to localize in a perinuclear region in cells. Additionally, the dual loading capability of the dextran vesicles for both hydrophilic and hydrophobic guest molecules (or drugs) provide new opportunity to study the combination of two drug loading in a single nano-scaffolds. The unique dextran vesicles is not only restricted to CPT delivery and is currently being explored for loading other fluorescent markers and other anticancer drugs. Understanding the cellular uptake mechanism, delivery and trafficking in normal and cancer cells are also being investigated.

2.5. References

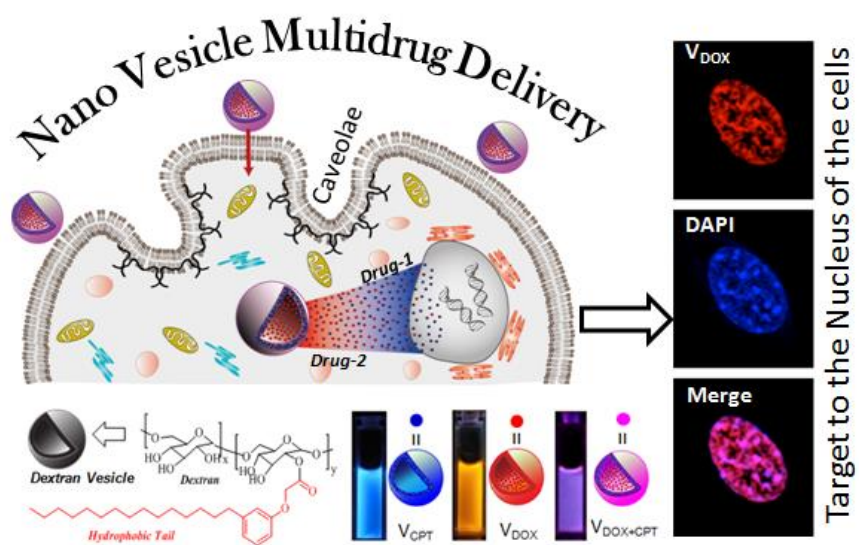
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Chapter 3

Polysaccharide Nano-vesicular Multidrug Carrier for Synergistic Killing of Cancer Cells



Chapter 3

Polysaccharide Nano-vesicular Multidrug Carrier for Synergistic Killing of Cancer Cells

Multi-drug delivery based on polymer nano-scaffolds is an essential protocol to be developed for better administration of anticancer drugs to enhance their therapeutic efficacies against cancer cells. This chapter describes dual delivery polysaccharide nano-vesicles that are capable of loading and delivering both water soluble and water insoluble drugs together in a single polymer scaffold. The selective rupture of the nano-vesicular assembly by intracellular enzyme conditions allowed the simultaneous delivery of hydrophobic drug camptothecin (CPT) and hydrophilic drug doxorubicin (DOX) supporting their synergistic killing of breast and colon cancer cells. The polysaccharide nano-vesicles have allowed us to address a few important questions regarding the need for multiple drug administration in cancer cells including a) the role of simultaneous drug release, b) antagonistic versus synergistic effects of drug combinations and c) how these are affected by the ratio of drugs. Further, evaluation of the role of caveolae in endocytosis of these polymer scaffolds was also made. The vesicular scaffolds were found to preserve and deliver DOX resulting in 50-60% better killing of cancer cells than the free drug. Additionally, dual loaded nano-vesicles when compared to drug cocktails with individual drugs in separate nano-vesicles (at comparable molar ratios) suggest the relative drug concentration following release and mode of delivery to be both important in cancer cell killing. Results from these experiments have revealed that newly developed polysaccharide nano-vesicles loaded with DOX and CPT drugs as potential candidates for improved breast cancer cell killing. Thus, these custom-designed polysaccharide nano-vesicles provide a new perspective on multi-anticancer drug delivery systems and their efficacy.

3.1. Introduction

Multi-drug delivery from single nano-carrier is an important approach for the administration of anticancer drugs to overcome tumor resistance to individual drugs and enhance their therapeutic efficacies¹⁻⁴ Mutation acquired by cancer cells during single drug therapy confers resistance to antitumor effect of drug.⁵ On the other hand, in multi-drug therapy, resistance acquired from one drug easily eliminated by second drug.⁶ Combination therapy also increases the number of cells exposed to cytotoxic effect and reduces the side effect of drug molecules.⁷ However, the choice of drugs for administration in combination therapy could be dependent among other things on their mode of action and their chemical properties.^{7,8} Drugs which have no cross resistances and overlapping safety profiles generally select for combination therapy to cancer cells.⁸

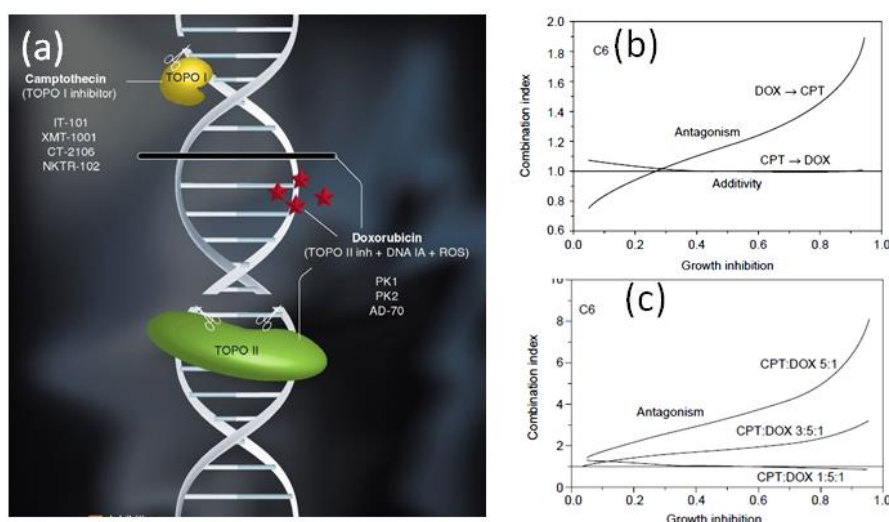


Figure 3.1. Mechanism of action of CPT and DOX drug molecules (a).Effect of sequential (b) and combination (c) delivery of CPT and DOX to cancer cells (adopted from Pavillard et al. *Br. J. Cancer* **2001**, 85, 1077-1083.).

Hydrophobic camptothecin (CPT) and hydrophilic doxorubicin hydrochloride (DOX.HCl) are most frequently employed therapeutic drugs in clinical cancer studies due to their high anti-tumor efficacy.⁹ DOX is DNA intercalating agent which prevent the DNA replication by stabilizing the DNA-Topoisomerase II complex¹⁰ and CPT is a Topoisomerase I inhibitor that stops DNA relegation by binding to Topoisomerase I-DNA complex¹¹ (See Figure 3.1a). Earlier studies have suggested that a combination of topoisomerases I (e.g. CPT) and topoisomerases II (e.g. DOX) inhibitors showed sequential delivery effect and it may be synergistic when administered together as is the administration of histone deacetylase inhibitors (HDACi) and topoisomerases II

(DOX).¹² (see figure 3.1b). Moreover, the concentrations and compositions of the drugs were also found to play a major role in the killing of cancer cells. Combination having more CPT exhibited antagonistic effect and vice versa (See Figure 3.1c).¹³ Drug combinations could also run into this problem of differential solubility that could affect their efficient delivery, eg. CPT - hydrophobic and water insoluble and DOX.HCl - hydrophilic and water-soluble.

Recently, Pacardo *et al.* reviewed nanocarriers based synergistic and sequential drug delivery systems for cancer therapy.¹⁴ Authors equated synergistic and sequential delivery to parallel and series electric circuits as shown in figure 3.2. In synergistic delivery, one component induces cytotoxicity even if second component is removed similar to parallel electric circuits. Conversely, removal of one component entirely impedes cytotoxicity action in sequential method, which is similar to series circuits. Studies carried out by Pavillard *et al.* revealed that the administration of CPT and DOX exhibited sequential effect, in which CPT followed by DOX showed synergistic killing effect, whereas the reverse caused antagonism (See Figure 3.1b).¹⁴ The study proved the importance of sequential delivery of CPT and DOX for obtaining synergistic effect.

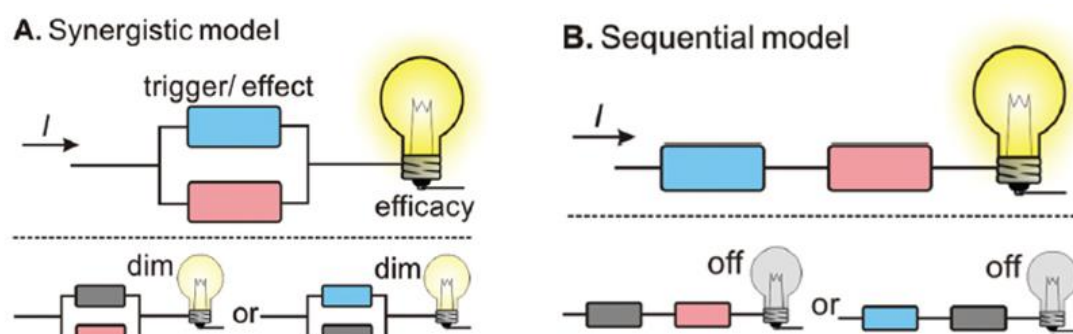


Figure 3.2. Schematic representation of synergistic (a) and sequential model (b). The blue and pink resistors stand for different drug/trigger and relevant effects; the gray resistor represents the failed trigger; the bulb denotes the efficacy of the treatment (adopted from Pacardo *et al.* *Chem. Soc. Rev.* **2015**, DOI: 10.1039/c4nr07677j).

Recently, few attempts were also made to exploit the dual drug carrier ability of polymer vesicles for combination therapy. Thambi *et al.* reported the simultaneous encapsulation of DOX.HCl and CPT in single polymersome prepared from PEG-b-PLys-SS-PCL triblock copolymer¹⁵ (See figure 3.3). The cytotoxicity assays showed that dual drug loaded polymersome exhibited comparable cell killing effect with that

of individual free drug and no synergistic effect were observed on combination of two drugs.

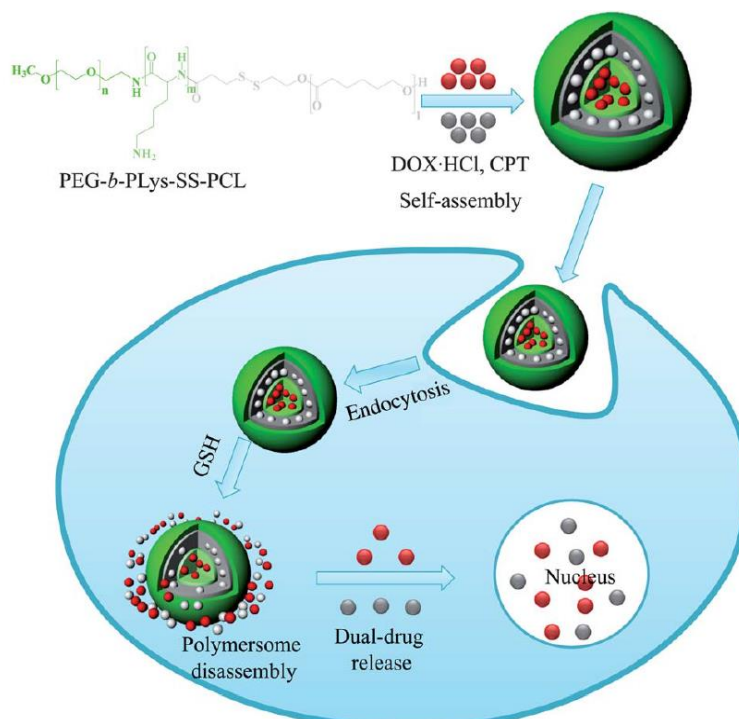


Figure 3.3. Schematic representation of CPT and DOX loaded polymer vesicles for dual drug release in cells (adopted from Thambi et al. *J. Mat. Chem.* **2012**, 22, 22028-22036).

Chen et al. reported the development of pH responsive polymersome based on PEG-PTMBPEC diblock copolymer for simultaneous encapsulation of paclitaxel and DOX.HCl anticancer drugs.^{2, 16} The polymersome showed faster release of loaded drugs under *in-vitro* conditions. However no cellular uptake or killing assays were performed to prove the combination effect of drug molecules. These preliminary studies highlight the potential of polymer vesicular assemblies that are only now being explored for clinical applications.¹⁷ The success of a polymer vesicular scaffold (or any other nano-carriers) for multi-drug delivery is dependent on several parameters including the ability of the polymer vesicle to load more than one drug under identical conditions, its ability to preserve their pharmacophore and its ability to release these drugs in a controlled manner. Their action could further be supported by an understanding of the target cells selectivity for the uptake of the polymer scaffolds and the cells ability to release the drug from the vesicle following their uptake.

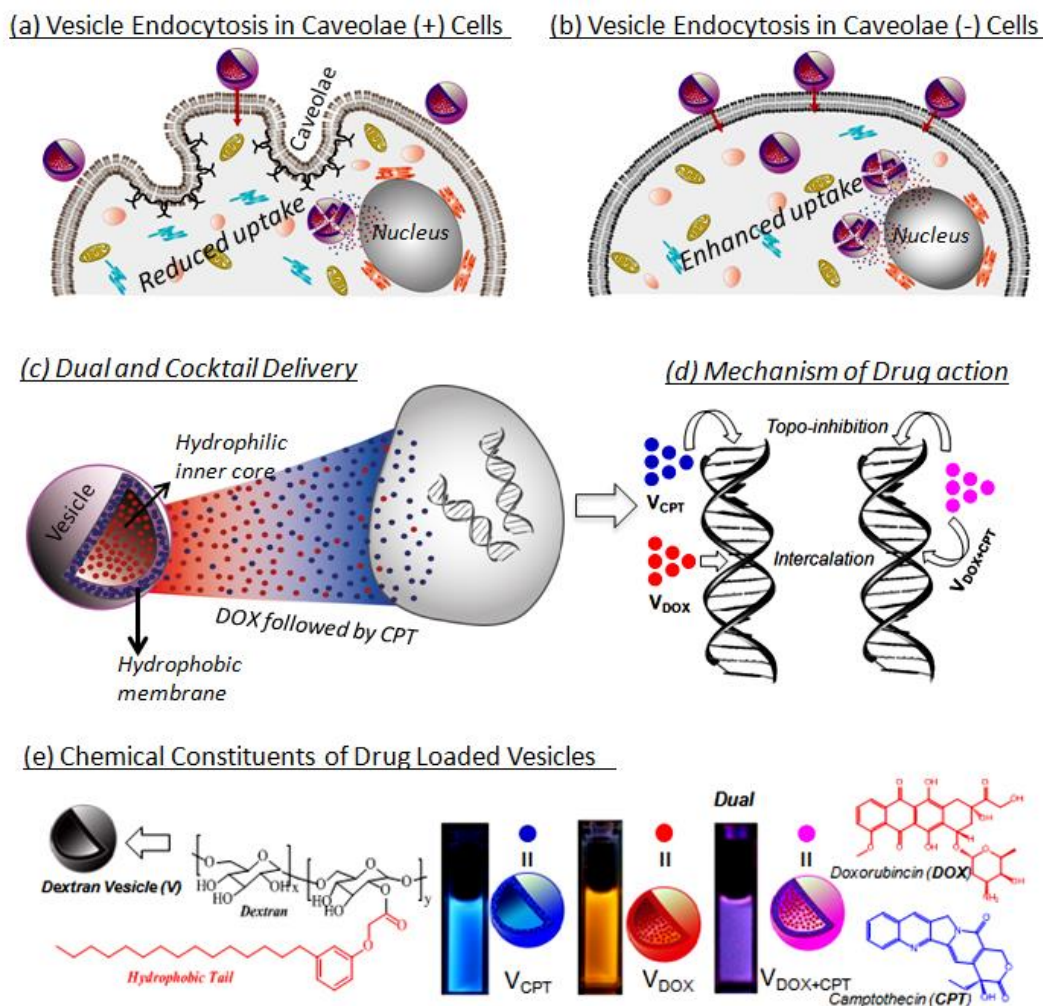


Figure 3.4. Endocytosis of vesicles in caveolae containing cell (a), caveolae lacking cancer cell (b). (c) Schematic representation of mechanism of dual drug loaded and individual cocktails of vesicle. (d) Mechanism of action of DOX loaded, CPT loaded and dual drug loaded nano-vesicles. (e) Chemical structure of hydrophobically modified dextran, vesicle solution of CPT loaded (V_{CPT}), DOX loaded (V_{DOX}), dual drug loaded ($V_{DOX+CPT}$) and Chemical structure of DOX and CPT. The colors of the vesicles represent their luminescent features under photoexcitation

Though, polymeric nano-scaffolds, especially polysaccharide derivatives are widely studied for anticancer drug delivery;¹⁸ their cellular uptake mechanism with respect to the type of cancer cells and the administration of multidrug delivery based on polysaccharide is not well understood. Typically, self-organized polymeric structures choose either endocytic (caveolae, clathrin), phagocytic or pinocytic pathways for cellular internalization.^{19,20} Depending on the kind of polymeric structures and the cell type the relative role of these different uptake mechanisms could vary (see figure 3.4a and 3.4b). Caveolin-1 for example is also a known tumor suppressor whose levels are down regulated in many cancers causing a loss of caveolae and affecting the cells endocytic uptake potential.²¹ A careful and complete

evaluation of the role of caveolar and other uptake pathways in polymer or nano-drug carrier delivery, particularly in context of specific cancers, remains to be made.

The work in this chapter aims to test the loading and administering of water soluble anticancer drug DOX (topoisomerases II inhibitor)¹⁰ and water insoluble drug CPT (topoisomerases I inhibitor) in a single polymer polysaccharide nano-vesicles (see Figure 3.4c to 3.4e). Esterase enzyme sensitive polysaccharide dextran nano-vesicle described in Chapter 2 (see figure 3.4e) were used for multidrug loading of hydrophilic and hydrophobic anticancer drug molecules. CPT loaded in the hydrophobic membrane and DOX loaded in the inner aqueous cavity allows the simultaneous or sequential delivery (CPT followed by DOX) (see figure 3.4c) from single polymer carrier, which could provide a synergistic killing of cancer cells (See figure 3.4d). Under intracellular conditions, the dextran nano-vesicular scaffold could rupture in the presence of the esterase enzyme (available in the lysosomal compartment) to deliver the loaded drugs (see figure 3.4e). This nano-vesicle design allows us to investigate the following questions associated with multiple drugs administration: (i) the synergistic / antagonistic role of these drugs in normal vs cancer cells, (ii) the role of drug ratios (ratiometric effect) in mediating cell killing in normal vs cancer cells, (iii) the effectiveness of dual drug loaded single-vesicle versus the cocktail of individual drug loaded nano-vesicles. The results provide significant new insights into polysaccharide loaded dual drug action of DOX: CPT drug combination in the breast and colon cancer cells.

3.2. Experimental Sections

3.2.1. Materials: DEX-PDP-5 reported in chapter 2 was used for the encapsulation of DOX and DOX + CPT dual loading. Wild type mouse embryonic fibroblasts (WT-MEFs), Caveolin1 knock out MEFs(Cav1-/-MEFs) and Human breast cancer cells (MCF7) were maintained in DMEM (phenol red free medium: Gibco) containing 10% (v/v) fetal bovine serum (FBS) and 1% (v/v) penicillin–streptomycin at 37° C under a 5% CO₂ humidified atmosphere. Under similar conditions, RPMI 1640, medium was used for colon cancer (DLDI) cell lines. Cells were washed with DPBS (Gibco), trypsinised using 0.05% trypsin (Gibco) and seeded in 96 well or 6 well (as per experiment) flat bottomed plastic plates (Costar) for all assays. Tetrazolium salt, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), DMSO, DAPI, Fibronectin (human Plasma) and paraformaldehyde was obtained from Sigma. Phalloidin

conjugated to Alexa 488 was obtained from Molecular Probes (Invitrogen) and fluoromount from Southern Biotech. Antibodies: Monoclonal anti- β -tubulin antibody (E7) was from Developmental Studies Hybridoma Bank (Iowa City, IA) and Anti-Caveolin antibody was from Santa Cruz Biotechnology (Cat# sc894)(Santa Cruz,CA). Goat anti-mouse HRP (Cat#115-035-003) and affinity purified Goat anti-rabbit HRP (Cat#111-035-003) were from Jacksons Immuno Research Labs,INC. Lysis Buffer: contains 1M Tris-Cl (pH-6.8) (Affymetrix, Cat#75825), 2% SDS (USB, Cat#75819), 5% β -mercaptoethanol (Sigma, Cat#M7154), Bromophenol blue (Sigma, Cat#B8206), 10% glycerol(USB,Cat#16374) and PVDF- membrane from MilliPore (Cat# IPVH0010).. Tris Buffered Saline (TBS) contains Tris (50mM) (Affymetrix, #75825), NaCl (150mM) (MP Blomed #194848), pH adjusted to 7.5 with 1M HCl, and used with 0.1% Tween-20 (USB, #20606). PDP ester, PDP acid and DEX-PDP were synthesized as described in chapter 2.²²

3.2.2. General procedures

The absorption and emission studies were done by a Perkin-Elmer Lambda 45 UV-Visible spectrophotometer and SPEX Fluorolog HORIBA JOBIN VYON fluorescence spectrophotometer with a double-grating 0.22 m Spex1680 monochromator and a 450W Xe lamp as the excitation source at room temperature. The excitation spectra are collected at 430 nm for camptothecin and 554 nm for doxorubicin and the emission spectra are recorded by exciting at the excitation maxima. The samples were purged with N₂ gas for at least 15-20 minutes prior to photophysical experiments. The size determination of the drug loaded DEX-PDP was carried out by dynamic light scattering (DLS), using a Nano ZS-90 apparatus utilizing 633 nm red laser (at 90 ° angle) from Malvern instruments. The static light scattering experiment (SLS) was carried out using 3D-DLS spectrometer, from LS instruments, Switzerland. The instrument consist of a He Ne laser having wavelength of 632.8 nm, in autocorrelation mode attached to computer using Lab view interface utilizing toluene as reference. The measurement was performed from 20° to 130° by steps of 5. SLS and DLS studies were done by using 0.2 mg/ml solution of drug loaded vesicular solution which was prepared by dispersing the required amount of lyophilized drug loaded polymer powder in PBS and filtered through 0.45 μ M filters. This whole procedure was done under laminar air flow cabinet to avoid the other contamination.

The reproducibility of the data was checked for at least three times using independent polymer solutions. Atomic force microscope (AFM) images were recorded using Veeco Nanoscope IV instrument. The sample was dropcasted on freshly cleaved mica surface. The imaging was carried out in tapping mode using TAP-190AL-G50 probe from Budget sensors with a nominal spring constant of 48 N/m and resonance frequency of 163.5. FEI, QUANTA 200 3D scanning electron microscope was used for recording FE-SEM image of the sample. For FE SEM analysis, the samples were prepared by drop casting on silicon wafers and coated with gold. TEM images were recorded using a Technai-300 instrument by drop casting the sample on formvar coated copper grid. The fluorescent micrographs were collected using Carl Zeiss Axiovert 200 microscope. LSM710 confocal microscope was used for imaging the cells.

3.2.3. Encapsulation of Doxorubicin.HCl into Dextran nano-vesicles

Water soluble anticancer drug, Doxorubicin.HCl was loaded into the DEX-PDP nano-vesicles by dialysis method. Briefly, 100 mg of DEX-PDP and 2 mg of DOX.HCl was dissolved in 5 ml of DMSO taken in light protected container. To a stirring solution of above mixture, 5 mL of deionized water was added drop wise. The resulting solution were stirred under dark for 12 hours and then dialysed against deionized water using a dialysis membrane having MWCO 3500 (Spectrapor). The water was exchanged 5 times within 24 hours of time period. The entire procedure of dialysis was done in a dark chamber. The dialysed solution were collected after 24 hours, filtered and then lyophilized.

3.2.4. Determination of Drug Loading Content (DLC) and Drug Loading Efficiency (DLE)

Drug loading content and Drug Loading efficiency were found out by absorption spectroscopy. For this purpose, 3mg of lyophilized DOX loaded DEX-PDP (polymer) was dissolved in 1 ml of DMSO. 100 μ l of this solution was diluted to 3 ml and the absorbance at 480 nm was determined using UV-Vis spectrophotometer. The amount of doxorubicin loaded in the vesicle was calculated by using Beers law, where molar extinction coefficient of DOX at 480 nm was kept as 11,500. The following equations were used for finding DLE and DLC.

$DLE (\%) = \{ \text{Weight of drug in vesicles} / \text{Weight of drug in Feed} \} \times 100 \%$

$DLC = \{ \text{Weight of drug in vesicles} / \text{Weight of Drug loaded vesicles} \}$

DLE and DLC were calculated as 63 % and 12.6 µg/mg of polymer respectively.

3.2.5. Encapsulation of Camptothecin and Doxorubicin.HCl into Dextran nano-vesicles

Dual drug encapsulation abilities of dextran nano-vesicles were confirmed by loading hydrophobic Camptothecin (CPT) and hydrophilic Doxorubicin.HCl (DOX) simultaneously. The amount of DOX and CPT required for getting 1: 4 and 4: 1 ratio was determined based on the pre-evaluation of drug loading capacity of dextran nano-vesicles. For obtaining 1: 4 (DOX: CPT) ratio, 2 mg of DOX.HCl , 3.5 mg of CPT and 100 mg of DEX-PDP (polymer) were dissolved in 5 ml of DMSO. Deionized water was added drop wise to above solution with moderate stirring. The content stirred for another 12 hours and transferred into a dialysis bag having MWCO 3,500. The dialysis was continued for 24 hours with five water exchange. The entire procedure was done under dark condition. The dialysed solution was filtered to remove unencapsulated drug content and lyophilized to receive dry powder of dual drug encapsulated system.

Drug loading content was determined by using absorption spectroscopy as 28.1 µg CPT and 12.0 µg DOX per milligram of polymer. Similarly, for attaining 4:1 (DOX: CPT) ratio, 2 mg DOX.HCl and 0.20 mg of CPT were loaded into 100 mg DEX-PDP (polymer) as per the protocol stated above. Final DLC was estimated as 1.72 µg CPT and 11.2 µg DOX per milligram of polymer.

3.2.6. In-vitro Release of DOX.HCl from Dextran nano-vesicles

The release of DOX from nano-vesicles under physiological condition was determined by dispersing 5 mg of drug loaded DEX-PDP in 3 ml of PBS (Phosphate Buffered Saline). This solution was taken in a dialysis bag and the entire tube was immersed in 100 ml of PBS. The whole solution was incubated at 37 °C. At definite time interval 3 ml of PBS was withdrawn from the dialysate and restore with equal volume of fresh buffer. The samples collected at different time interval were analysed by UV-Visible spectroscopy. The absorbance value at 480 nm was used to determine amount of DOX release from the nano-vesicles. The cumulative release of the drug was calculated by using following equation.

Cumulative Drug Release = (Amount of Drug release at time 't' / Total amount of drug in the vesicle taken in dialysis tube) * 100.

3.2.7. Esterase Assisted Release of DOX.HCl from Dextran nano-vesicles

The effect of esterase enzyme on the release of doxorubicin from the DEX-PDP nano-vesicles was studied by dialysis method. Briefly, 5 mg of DOX loaded DEX-PDP system were dispersed 3 ml PBS and mixed with 10 U esterase enzyme. This entire solution was taken in a dialysis tube and submerged in 100 ml PBS solution. The whole system was incubated at 37°C under dark condition. At specific time points, 3 ml of dialysate was collected and replace with fresh buffer solution. The sample collected were analysed by UV visible spectrometer to determine the percentage of DOX release at different time points as described previously. The experiment was also performed by adding esterase enzyme 8th and 36th hour. In this case, *in-vitro* release was started under normal physiological condition, then after 8th hour, or 36th hour, solution were recovered from dialysis tube and mixed with 10 U esterase enzyme and continued the release studies. The complete drug elution from the DOX loaded dextran nano-vesicles was also studied by adding excess amount (30 U) esterase enzyme to dialysis bag. The rest of the protocol was maintained same as stated above.

3.2.8. *In-vitro* Release of DOX.HCl and CPT from Dextran nano-vesicles

The release of DOX and CPT from dual loaded vesicle system (both 1:4 and 4:1) or individual cocktails (both 1:4 and 4:1) was studied by similar method as illustrated for only DOX loaded system. Here in absorbance at 480 nm and 370 nm were noted for DOX and CPT respectively. The concentration of CPT was calculated by using Beers Law, where molar extinction coefficient of camptothecin is kept as 11,250 at 370 nm. The effect of esterase on the release of DOX and CPT from dual loaded system was also studied by adding (10 U) esterase into the solution. The entire procedure was done in a light protected container.

3.2.9. Cell Viability Assay (MTT Assay)

To observe the effect of DOX in V_{DOX} , and DOX and CPT in dual loaded $V_{DOX-CPT}$, a cell viability assay was performed using wild type mouse embryonic fibroblasts, MCF7 and DLD1. In case of V_{DOX} , the concentration of DOX was

maintained as 1 μ M and 0.5 μ M for all cell lines used in the assay. While in dual loaded nano-vesicles, DOX concentration was retained as 0.5 μ M for both combinations. CPT concentration hence varied to according to the drug molar ratio required to 2 μ M (1:4 DOX:CPT) and 0.125 μ M (4:1 molar ratio DOX:CPT). WT-MEFs (1×10^3 cells/well), Cav1^{-/-} MEFs (1×10^3 cells/well), MCF7 (2×10^3 cells/well) and DLD1 (2×10^3 cells/well) were each seeded in a 96 well plate (Corning, USA) in 100 μ l medium with 10 % FBS (Fetal Bovine Serum) and allowed to adhere for 24 hrs. Medium from cells was aspirated and drug samples at the required concentrations diluted in the same medium were added to the cells. A blank 'medium only' control without cells and an 'untreated cells' control was used in each experiment. All control and treated experiment wells were set in triplicates. Cells were incubated for 24 hrs without a change in media and after 24 hrs, drug containing media was aspirated and freshly prepared MTT in sterile PBS (5 mg/ml) was diluted to 50 μ g/ml in 100 μ l media with FBS and added to each well. Cells were then incubated with MTT for 4 hrs at 37 °C in the dark in the CO₂ incubator where the cells were grown. Medium with MTT was then aspirated from wells and the purple formazan crystals formed as a result of reduction of MTT by mitochondrial dehydrogenase enzyme from cells were dissolved in 100 μ l of 100% DMSO (added per well). The absorbance from formazan crystals was immediately measured using microplate reader at 570 nm (Tecan Plate Reader) and is representative of number of viable cells per well.

Values from the triplicates for each control and treated set were noted and their means calculated. If all three values were variable the sample was not considered for the study. The mean of the absorbance values for the blank control samples was subtracted from the mean values of the untreated control and treated samples respectively. The values thus obtained for the untreated control samples were equated to 100 % and the relative percentage values for drug treated samples were calculated accordingly. Percentage values thus obtained for treated samples were subtracted from the untreated control (100%) to determine the percentage cell death (relative to control). Results thus obtained from a minimum of 3 to a maximum of 6 experiments were compiled evaluated statistically using a paired two tail T-Test.

3.2.10. Cellular Uptake of DOX and V_{RHO} by Confocal Microscopy

Wild type mouse embryonic fibroblasts (WT MEFs), Cav1^{-/-} MEFs, MCF7 and DLD1 were seeded at a density of 1×10^5 cells on cover slips coated with

fibronectin (2 $\mu\text{g/ml}$) to facilitate their attachment and spreading, in 6 well plates. Cells were incubated overnight and treated with the required concentration of free drug or nano-vesicle encapsulated drug (for a maximum of 4hours) or nano-vesicle encapsulated rhodamine (for 48hours). At this time drug-containing medium was aspirated from each well, cells were washed twice with PBS (1mL per wash) and fixed with 3.5 % paraformaldehyde solution in PBS for 15 min at room temperature. The cells were washed thrice with PBS (1 mL per wash) and stained for 35min with phalloidin conjugated to Alexa 488 (Invitrogen) diluted 1:100 in 5 % BSA solution in PBS. This incubation was done in the dark and the excess dye was washed from the cells with PBS. These cover slips were then incubated with DAPI (0.05 $\mu\text{g/ml}$) for 2 min each to stain the nucleus, and then mounted on slides using Fluoromount-G mounting medium (Southern Biotech). Slides was then dried overnight at room temperature in the dark and then imaged using a LSM710 confocal microscope with the λ 405nm (blue channel), λ 568 nm (red channel) and λ 488nm (green channel) lasers. Images thus obtained were analysed using the Image J analysis software (NIH). Image of the DAPI stained nucleus in cells (blue channel) was evaporated from the rest and the threshold plug in used to define the border of the stained nucleus. A mask mapping the edge of threshold nucleus was created and saved as a region of interest (ROI) in the ROI manager. This ROI was then imported into the image for DOX in the cell (red channel) and used to define the nucleus of the cell in this image. The intensity of fluorescence within this ROI (and hence within the nucleus) in the red channel was now measured using Image J. Recorded integrated density values were then divided by the area of the nucleus (defined by the ROI) to determine the intensity per unit area. Values thus obtained for each nucleus in a sample was used to calculate the mean intensity/area. Results thus obtained from minimum of 14 to maximum of 55 cells in different treatments were compiled and differences statistically evaluated using a paired two tail T-Test. To determine total fluorescence within the cell, the actin network of the cells was stained with phalloidin and the perimeter of the cell defined as above using the same and the mask and ROI created accordingly. The intensity of fluorescence within this ROI (and hence within the cell) in the red channel was now measured using Image J and quantitated as discussed above.

3.2.11. Western Blotting

4×10^5 cells each of WT-MEFs, Cav1^{-/-}-MEFs, DLD1 and MCF7 washed with PBS and then lysed in 100ul of 1X SDS-Sample buffer. Cell- equivalent amounts of lysate were resolved using 12.5% SDS-polyacrylamide gels and transferred to PVDF membrane. Blocking was done using 5% non-fat dry milk in TBS+0.1% Tween 20 (TBS-T). Blots were incubated with anti-caveolin antibody (1:5000) followed by anti-rabbit HRP conjugated antibody (1:5000) and anti-tubulin antibody (1:5000) followed by anti-mouse HRP conjugated antibody (1:5000). Tubulin was used as a loading control. Detection was done using West PICO chemiluminescent substrate (Fisher Scientific, Prod# 34080) on Image Quant- Las 4000 system (GE Healthcare Life sciences).

3.3. Results and Discussion

3.3.1. Dextran nano-vesicles loaded with DOX.HCl

Dextran (MW = 6000) amphiphile described in chapter 2 was used here to load hydrophilic DOX anticancer drug molecule.²² Encapsulation of DOX produced DOX loaded nano-vesicles (in the hydrophilic core) which is named as: V_{DOX} .

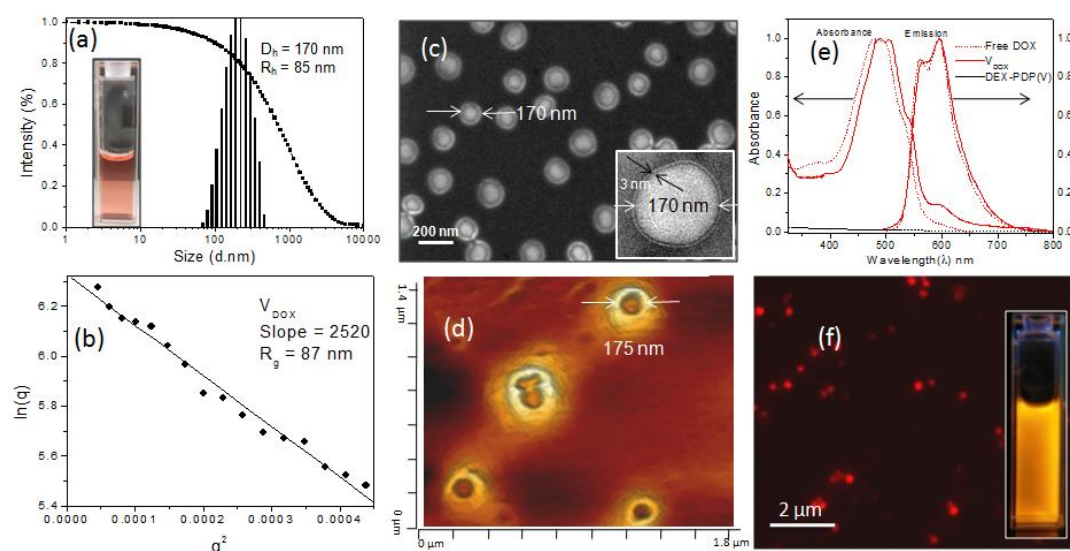


Figure 3.5. DLS histogram (a) SLS data (b) FE-SEM image (c) AFM image (d) of V_{DOX} . Absorbance and Emission plots of free DOX, DOX in Vesicle and Free polymer (e), Fluorescent micrographs of V_{DOX} (f). The vials insert in (a) and (f) show the color of the vesicles in PBS in visible light and under hand held UV lamp. Insert in (c) shows HR-TEM image of V_{DOX}

The dynamic light scattering (DLS) histogram of V_{DOX} showed mono-model distribution with the average diameter of the vesicles as 170 ± 10 nm (see Figure 3.5a). From this data, the hydrodynamic radius (R_h) of the V_{DOX} was calculated as 83 ± 5 nm. Static light scattering (SLS) analysis of the V_{DOX} (see figure 3.5b) provided the radius of gyration (R_g) from their Guinier plot, $R_g = 77 \pm 5$ nm. The ratio of R_g/R_h for the V_{DOX} was found to be 1.06, which is in good agreement for vesicular assemblies.²³⁻²⁶ Electron microscope images of V_{DOX} showed uniformly distributed 170 ± 5 nm spherical objects with a definite thin hydrophobic membrane which is a characteristic feature of vesicles (see Figure 3.5c). HR-TEM image (see figure 3.5c inset) further evident for the existence of the vesicular assemblies with distinct hydrophobic layer of 3.0 ± 1 nm. Tapping mode AFM images of V_{DOX} showed the appearance of donut-shaped objects with high periphery and low centre (See Figure 3.5d) as characteristics of vesicular assemblies.^{27,28} The size of the spherical shape was obtained as 170 ± 15 nm (see figure 3.5d). The height of these nano-vesicles was obtained as 13 ± 2 nm.

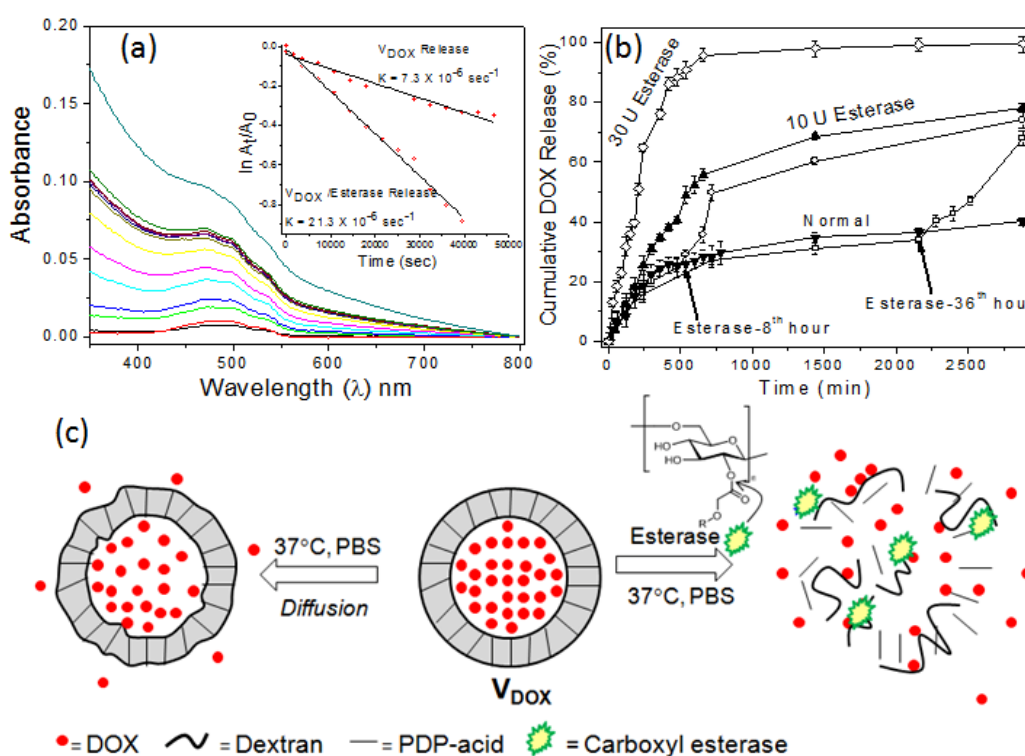


Figure 3.6. (a) Absorbance spectra of DOX release from V_{DOX} , (b) In-vitro drug release profile of V_{DOX} in the absence of esterase enzyme, 0 min, 8th hour and 36th hour of 10Uesterase addition and 30 U esterase addition, (c) Mechanism of drug release from vesicle under normal and esterase assisted cleavage. Inset figure in (a) shows $\ln A/A_0$ Vs time plot of $V_{DOX+CPT}$ for the determination of rate constant

DOX is a fluorescent anticancer drug and V_{DOX} had its absorbance maxima at 480 nm and emission maxima at 590 nm (see figure 3.5e). The comparison of V_{DOX} and free DOX indicate that the DOX photophysical characteristics were retained in the dextran nano-vesicle (see figure 3.5e). Fluorescence microscopic image of V_{DOX} captured using the RFP filter showed them to be red spherical luminescent objects (see figure 3.5f). Together this confirms the existence of DOX in the confined vesicular structures.

3.3.2. Release Kinetics

The *in-vitro* release of DOX from V_{DOX} was studied under physiological conditions to understand the drug release from the core of the vesicles. The drug loaded nano-vesicles were incubated at pH 7.4 at 37 °C in PBS and aliquots were collected at different time interval and analyzed by UV-Visible spectrometer. The absorbance spectra of DOX were used to plot its release profile from nano-vesicles (V_{DOX}) (figure 3.6a). This showed that about 25 % of drug was discharged in 12 hours and thereafter release occurred in a controlled fashion to release 40 % of the drug in 48 hours (see figure 3.6b). The capability of esterase enzyme present in cells to cleave the hydrophilic and hydrophobic segments of dextran nano-vesicles was studied *in-vitro* by treating vesicles with 10 U of the enzyme²⁹ (comparable to amount in some cells). This was seen to promote release of upto 70 % of DOX in 48 hours (Figure 3.6b). Additionally, if treated with excess of esterase enzyme (30 U) 100 % elution of DOX was observed in 10 hours, confirming the susceptibility of these dextran nano-vesicles to cleavage by the esterase enzyme.

Two additional experiments were performed to prove the drug retention capability and slow release capability of DOX containing dextran nano-vesicles. Normal release of DOX from the dextran nano-vesicle was interrupted by addition of esterase enzyme (10 U) at 8 hours and 36 hours after vesicles were incubated at physiological conditions (PBS at 37 °C). In both experiments (see figure 3.6b), an initial 25 to 30 % drug release was followed by an augmented release of the encapsulated DOX content on addition of the esterase enzyme at 8 hrs and 36 hrs time points. This suggests the availability of unreleased drug in nano-vesicles for release and action at the target site for upto 36 hrs, till it sees the esterase enzyme. A schematic model for the DOX release from the vesicular structure is shown in figure 3.6c. In PBS buffer at 37 °C, under larger dilution, the nano-vesicles undergo natural

leaching out to release about 25 % of the drugs. In the presence of esterase enzyme, the aliphatic ester linkage which connects the dextran to the PDP cleaved instantaneously to releases significant amount of the drugs.

3.3.3. Cytotoxicity and Endocytosis of V_{DOX}

The cytotoxic effects of the V_{DOX} nano-vesicle were first tested on wild type mouse fibroblasts (WTMEFs). The effect of DOX in V_{DOX} and as free (unloaded) drug was compared at 1.0 μM in these studies. WTMEFs showed $\approx 20\%$ killing with free DOX while V_{DOX} killed > 2 fold better (see figure 3.7). Empty dextran nano-vesicle (DEX) at concentrations used in the above studies did not significantly affect WT MEFs (figure 3.7). Earlier studies on DOX polymer vesicles based on amphiphilic di- and tri-block copolymers,³⁰⁻³³ poly(g-benzyl L-glutamate)-block hyaluronan,³⁴ and plasmonic vesicles having gold nanoparticles³⁵ were found to exhibit better kill cancer cells than DOX alone.^{36, 37} In our studies this difference is also observed be it more prominently with 2 fold increase in killing of WT MEFs when compared to free DOX (at 1 μM). One possible explanation for this enhanced killing could be that following the endocytosis of nano-vesicle their directed targeting to specific endocytic compartment and possible simultaneous release of the drug may impact the efficiency and localization of DOX released.

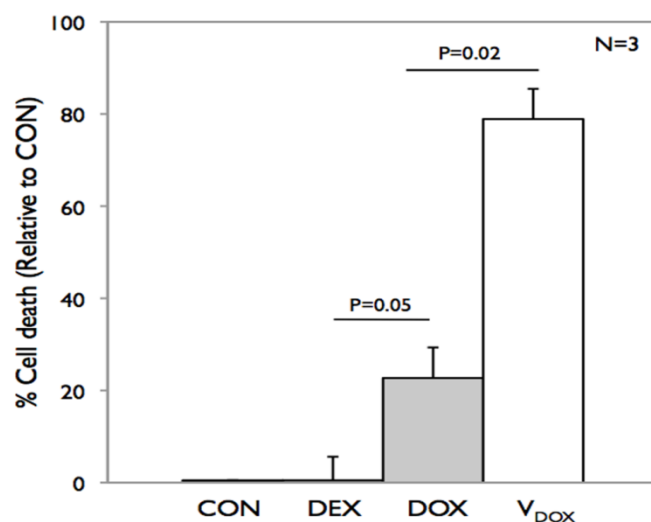


Figure 3.7. (a) Cytotoxicity of DOX vs V_{DOX} in WTMEFs. MTT assay was used to test the cytotoxicity of free DOX and V_{DOX} were compared to DEX-PDP empty vesicle alone and normalized to killing observed in untreated control (CON) at 1 μM concentration of drug. Graph represents Mean $\pm$ SE of percentage cell death relative to control in three independent experiments.

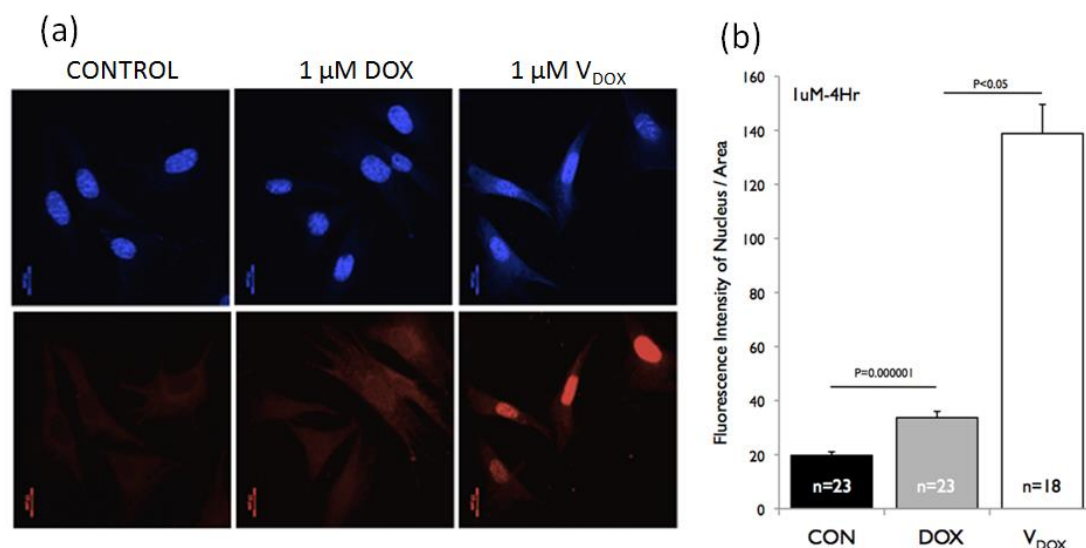


Figure 3.8. (a) Uptake of 1 μ M DOX and V_{DOX} was visualized by confocal microscopy in WTMEFs where the nucleus was counter stained with DAPI. (b) Graph represents Mean $\pm$ SE of DOX fluorescence in the nucleus from indicated number of cells. Data is representative of one in two independent experiments that gave similar results. Statistical analysis was done using standard T Test.

This is possibly reflected in better uptake and targeting of DOX in the nucleus as well (Figure 3.8a and 3.8b). To evaluate this we have looked at the fluorescence of DOX at $\sim$ 595nm on binding DNA³⁸ in treated cells. DOX, a DNA major-groove intercalating agent as well as topoisomerase type-II inhibitor is known to accumulate in the nucleus. The nucleus when mapped in these studies by staining for DAPI shows a close overlap with the DOX fluorescence (figure 3.8 and figure 3.9). DOX fluorescence in the nucleus in V_{DOX} treated cells was significantly more (> 4 fold) than DOX alone treated cells (figure 3.8 and figure 3.9). Free DOX localization in the nucleus has been ascribed earlier to diffusion³² while in the case of V_{DOX} improved uptake into the cell of the drug and / or better targeting to the nucleus could mediate the DOX accumulation seen. This increase in fluorescence is also seen despite the fact that DOX binding to DNA quenches some of its fluorescence.³⁵ The increased accumulation of the drug in the nucleus could hence result in better targeting of topoisomerase II in the nucleus supporting a better killing by V_{DOX} (Figure 3.7, figure 3.8 and figure 3.9).

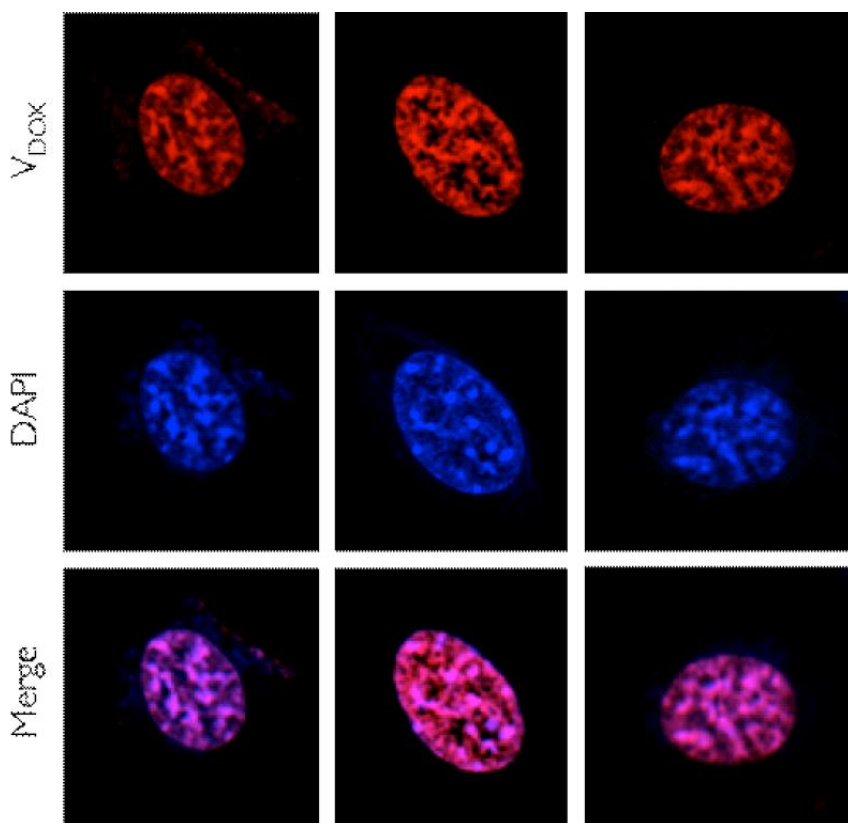


Figure 3.9. Nuclear Localization and overlap between DOX from V_{DOX} and DAPI staining

3.3.4. Membrane Uptake Mechanism Studies

Caveolae are cholesterol and sphingo-lipid rich plasma membrane invaginations formed by the presence of the structural transmembrane protein caveolin (isoforms caveolin-1, -2 and -3).³⁹⁻⁴¹ Loss of caveolin-1 has been seen to disrupt the formation of caveolae and affect caveolar endocytosis. Fibroblasts from Caveolin-1 knockout mice hence provide a clean system to test the role of caveolae in nano-vesicle endocytosis. Considering that V_{DOX} uptake is best visible following the nuclear localization of DOX, it may not be an accurate measure of cellular uptake. These studies were hence done using dextran nano-vesicles loaded with the fluorescent water soluble dye Rhodamine B (V_{RHO}) (details are given in chapter 2). Litter matched WT ($cav-1^{+/+}$) and caveolin-1 lacking ($cav-1^{-/-}$) MEFs obtained from the lab of Dr. Richard Anderson^{42, 43} were compared and their uptake of Rhodamine containing dextran nano-vesicle (V_{RHO}) studied over 48hours.

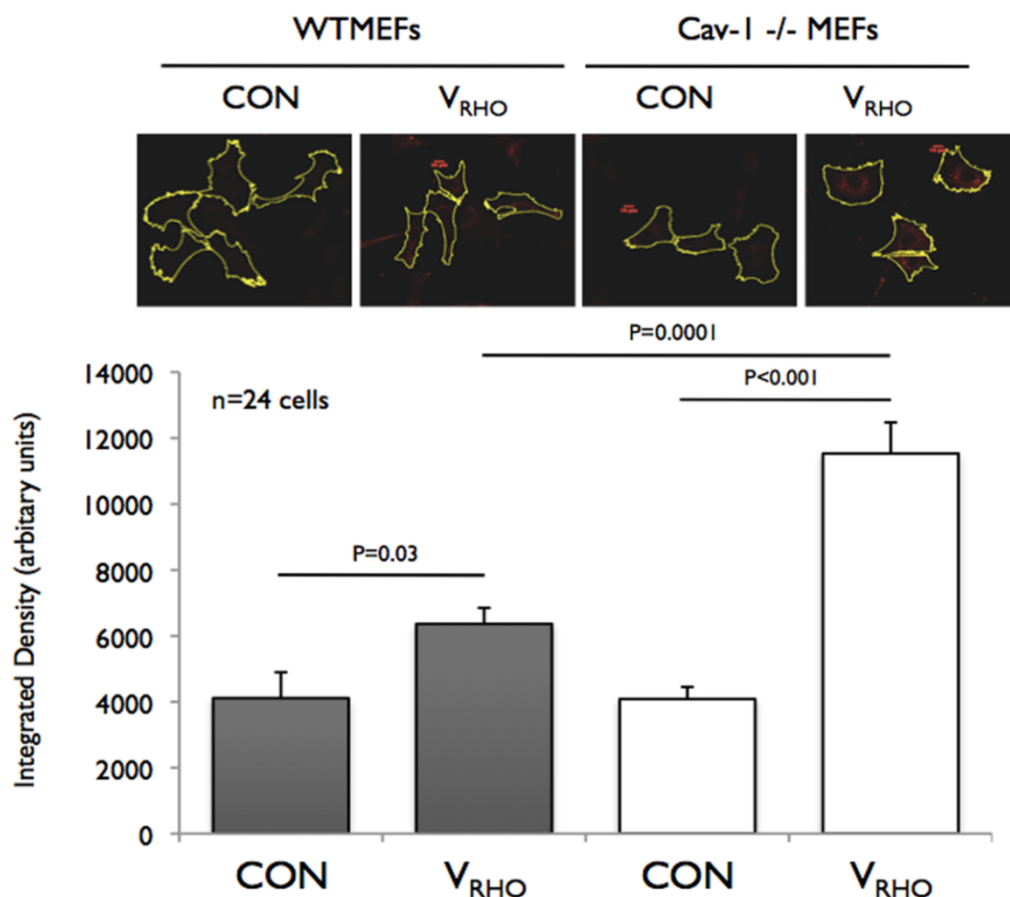


Figure 3.10. Nanovesicle uptake and killing in WT and caveolin-1^{-/-} MEFs.(a) Uptake of V_{RHO} in WT and Caveolin-1^{-/-} MEFs was visualized by confocal microscopy after 48hour treatment. Fluorescence intensity within in the cell perimeter marked by the yellow line was measured and compared between V_{RHO} treated and untreated control cells. Graph represents Mean $\pm$ SE of rhodamine fluorescence measured as integrated density from indicated number of cells for each treatment. Data is representative of two similar independent experiments that gave similar results.

Fluorescence intensity measurements for rhodamine at $\lambda = 596$ nm showed caveolin-1^{-/-}MEFs to have ~ 2 fold higher levels of rhodamine fluorescence (reflective possibly of vesicle uptake) than WT MEFs after 48hours (figure 3.10). Most of this fluorescence is seen to be in the cytosol, reflecting a better internalization of V_{RHO} into the cell. This shows caveolar endocytosis to not be a major pathway for uptake of these dextran nano-vesicles (V_{RHO}) and also suggests that loss of caveolin-1 (and caveolae) could (directly or indirectly) promote uptake of the dextran nano-vesicles in cells.

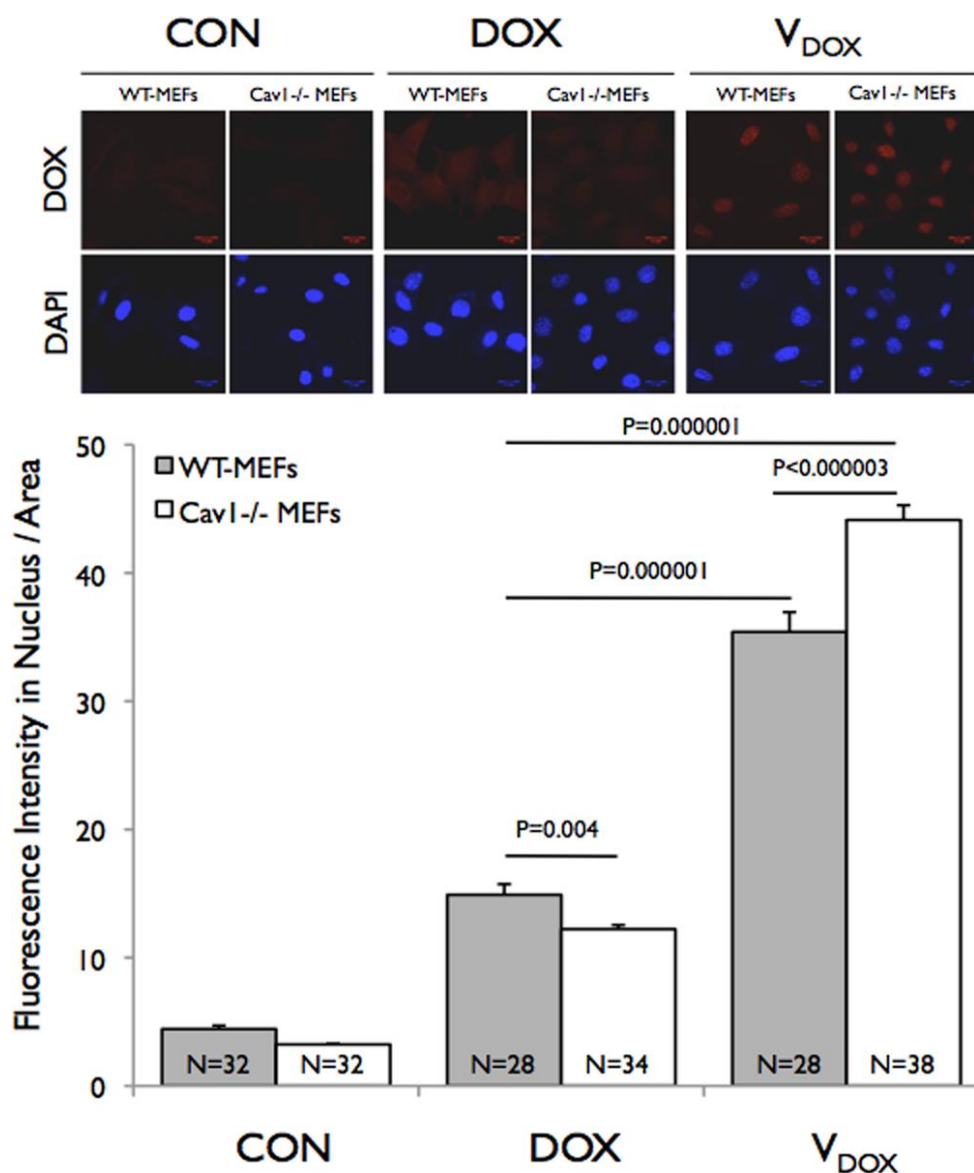


Figure 3.11. Uptake of $1\mu\text{M}$ DOX and V_{DOX} was visualized by confocal microscopy in WT and caveolin-1^{-/-} MEFs where the nucleus was counter stained with DAPI. Graph represents Mean $\pm$ SE of DOX fluorescence in the nucleus from indicated number of cells. Data is representative of one in two independent experiments that gave similar results.

Earlier studies characterizing cav-1^{-/-} MEFs have reported the plasma membrane order (detected using the membrane intercalating dye laurdan) and possibly cholesterol composition in these cells to be significantly altered as a result of the loss of caveolar endocytosis.⁴⁴⁻⁴⁶ This has been seen to regulate among other things cell spreading, migration and growth signaling in the cav-1^{-/-} MEFs^{32-44, 47}. Loss of caveolin in adipocyte cell line is also seen to promote endocytosis and degradation of insulin receptors.⁴⁸ Changes in membrane composition and order could similarly affect nano-vesicle uptake. To test if the better uptake of V_{RHO} is reflected in better

killing of cav-1^{-/-} MEFs cells, the uptake and hence nuclear accumulation of DOX and killing by free DOX vs V_{DOX} was tested in these cells and compared to WTMEFs. Free DOX showed significantly less nuclear fluorescence in cav-1^{-/-} MEFs while V_{DOX} showed significantly more fluorescence in cav-1^{-/-} MEFs (Figure 3.11) when compared to WTMEFs.

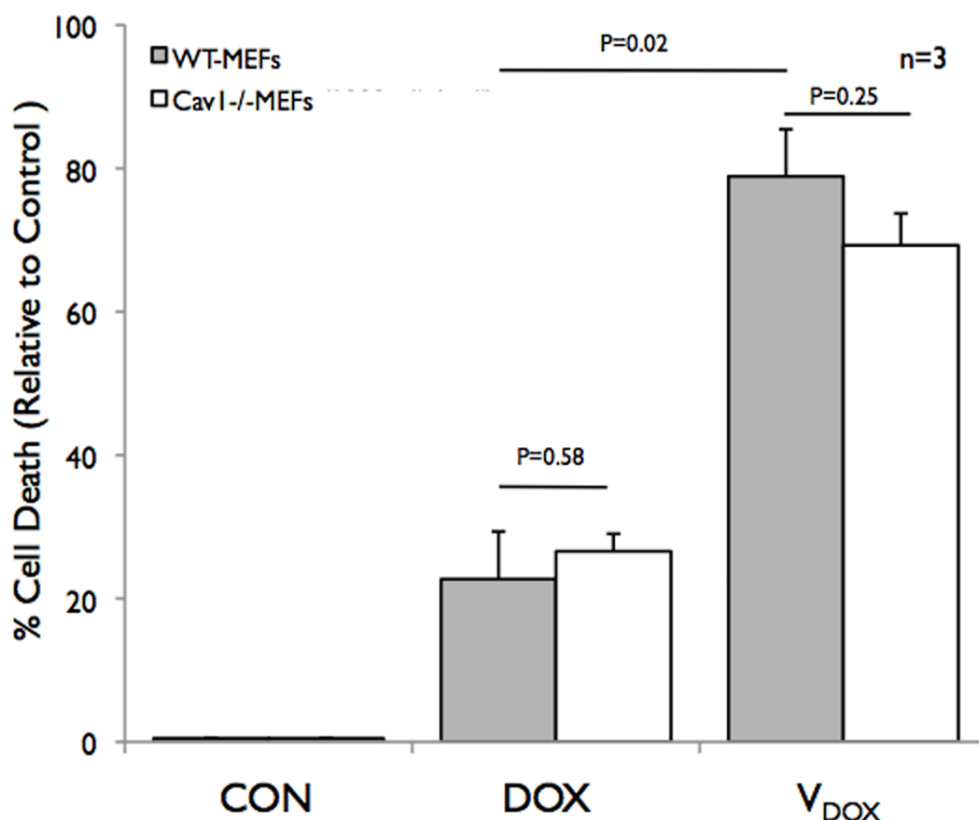


Figure 3.12. MTT assay was used to test the cytotoxicity of free DOX and V_{DOX} relative to untreated control (CON) at 1 μ M concentration. Graph represents Mean $\pm$ SE of percentage cell death relative to control in three independent experiments and statistically analysed using the standard T Test.

This suggests that the DOX nano-vesicle uptake into the cell and delivery of drug into the nucleus could both be differential in caveolin-1 lacking cells. While V_{DOX} was seen to kill both cell types better than free DOX, killing of cav-1^{-/-} and WT MEFs was not significantly different (Figure 3.12). One cause for this could be the differential quenching of DOX following its binding to DNA that contributes to its net fluorescence in the nucleus. This would affect the correlation of nuclear DOX fluorescence with DOX levels in the nucleus and hence cell killing. This effect might be more prominent for greater DOX uptake levels and smaller differences in DOX nuclear fluorescence observed. In case of larger differences in fluorescence such as between V_{DOX} and DOX treated cells (in WTMEFs and Cav-1^{-/-} MEFs) quenching

may not be a major influencing factor and hence a good correlation does exist between nuclear DOX fluorescence intensity and cell killing (Figure 3.12).

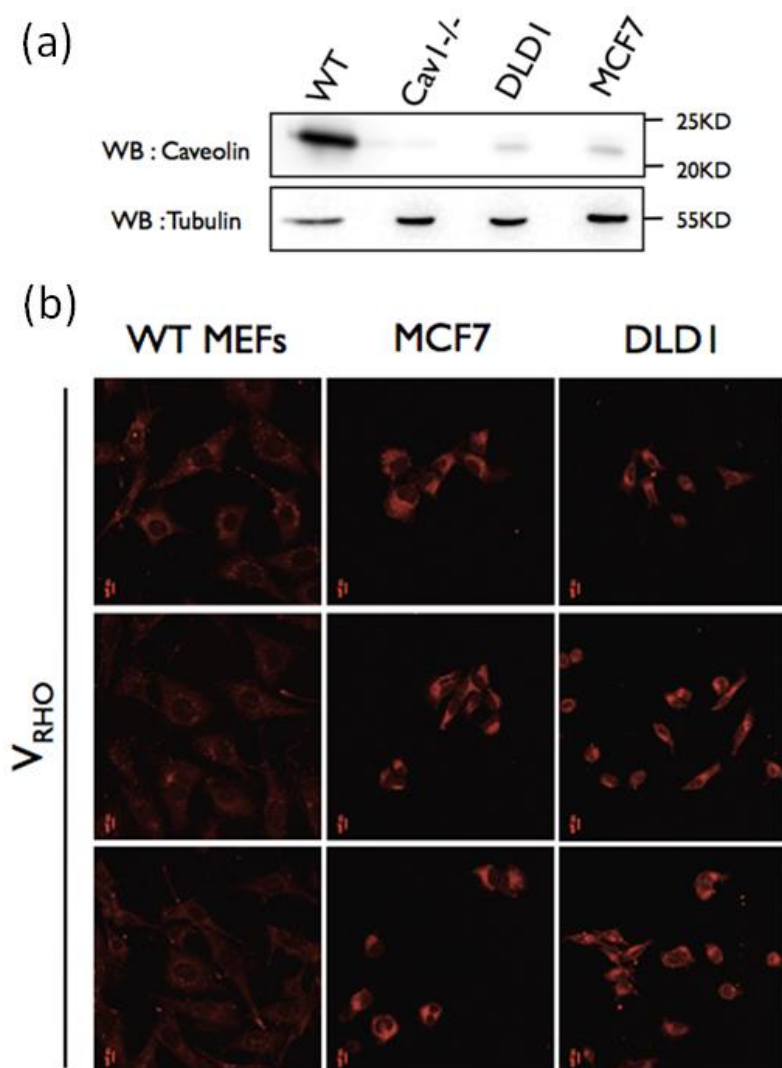


Figure 3.13. Caveolin-1 expression and uptake of V_{RHO} in cancer cells. (a) Western Blot detection of Caveolin-1 (~22 KD) in WT MEFs, cav1 ^{-/-} MEFs, DLD1 and MCF7, relative to the tubulin (~55 KD) loading control. Figure is representative of three independent experiments that gave similar results. (b) Uptake of 200 μ M V_{RHO} in WTMEFs, MCF7 and DLD1 was visualized by laser confocal microscopy.

To further understand the differential uptake vs killing seen in Cav-1^{-/-} MEFs these studies were extended to caveolin-1 lacking cancer cells as well. Caveolin-1 is a known tumor suppressor whose levels are down regulated in many cancers.²¹ Two caveolin-1 lacking cancer cell lines identified from literature were studied, MCF7 (breast cancer)³² and DLD1 (colon cancer).³³ Their caveolin-1 expression when compared to WTMEFs was seen to be less than 10 fold (WTMEF >>>>> MCF7 > DLD1 > Cav-1^{-/-}MEFs) (Figure 3.13a). Like in cav-1^{-/-} MEFs (Figure 3.12) cytosolic V_{RHO} fluorescence in MCF7 and DLD1 cells following their incubation with the

nano-vesicle is seen to be significantly more than in ‘normal’ WTMEFs (Figure 3.13b) suggesting better uptake by the caveolin-1 lacking cancer cells. Nuclear accumulation of free DOX while marginally better in cancer cells was significantly better when administered in V_{DOX} (Figure 3.14). This again was seen to be different between the two cancer cell types, with $MCF7 > DLD1 > WTMEFs$. This suggests that uptake/nuclear targeting could be influenced by more than just the loss of caveolin-1 levels in these cells.

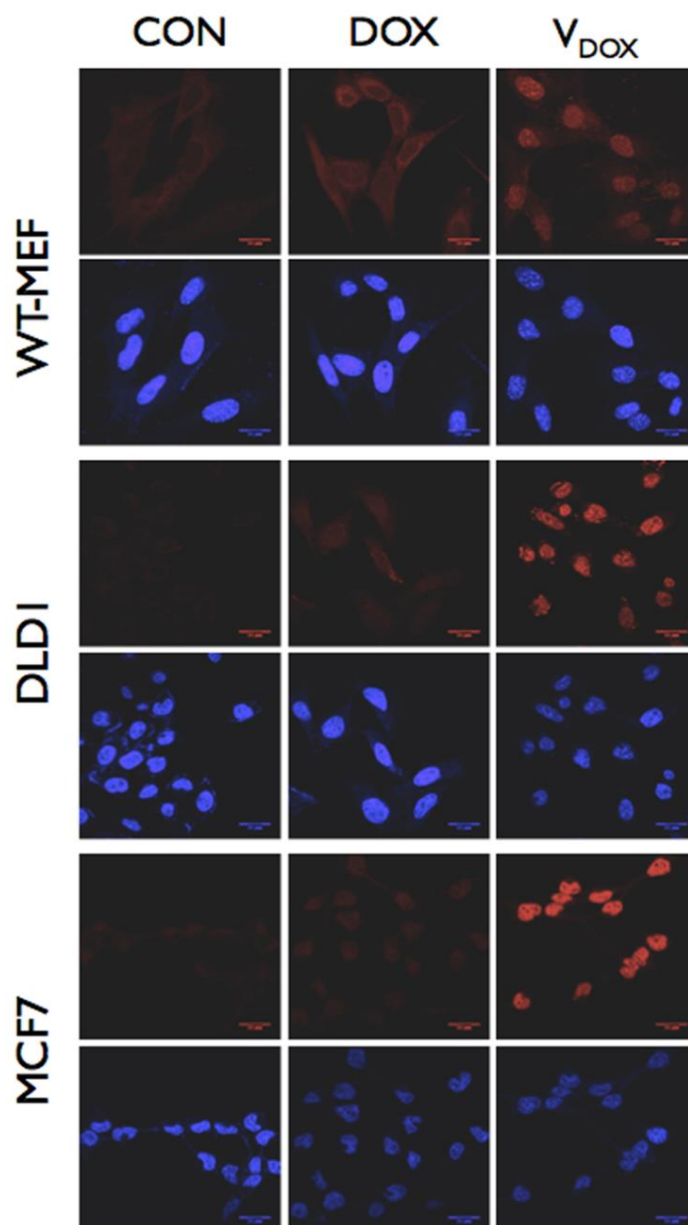


Figure 3.14. V_{DOX} uptake and killing in normal vs cancer cells. (a) Uptake of 1 μ M DOX and V_{DOX} (red) over 4 hours was visualized by confocal microscopy in WTMEFs, DLD1 and MCF7 cells, where the nucleus was counter stained with DAPI (blue)

The association between increased nuclear fluorescence and killing when tested in these cells (Figure 3.15a and Figure 3.15b) showed (a) V_{DOX} with better nuclear accumulation than free DOX does kills all three cell types better but (b) differences in nuclear accumulation of V_{DOX} between cell types did not directly correlate with better killing. MCF7 and DLD1 cells with significantly greater nuclear DOX fluorescence in V_{DOX} treated cells were killed almost as well as WTMEFs. To allow for better visibility and quantitation of differences in the nuclear DOX fluorescence uptake studies are done after a 4 hour treatment with the drug and nano-vesicle. MTT assays were done after a 24 hour treatment with drugs (at the same 1 μ M concentration). The above results could reflect this difference or be a result of the differential quenching of DOX fluorescence in these cell types at different time points making a direct correlation of DOX nuclear fluorescence at 4hours as a measure of its levels in the nucleus difficult, especially across different cell types. Difference in susceptibility to killing by DOX has been reported in cancer cells which could also contribute to this.

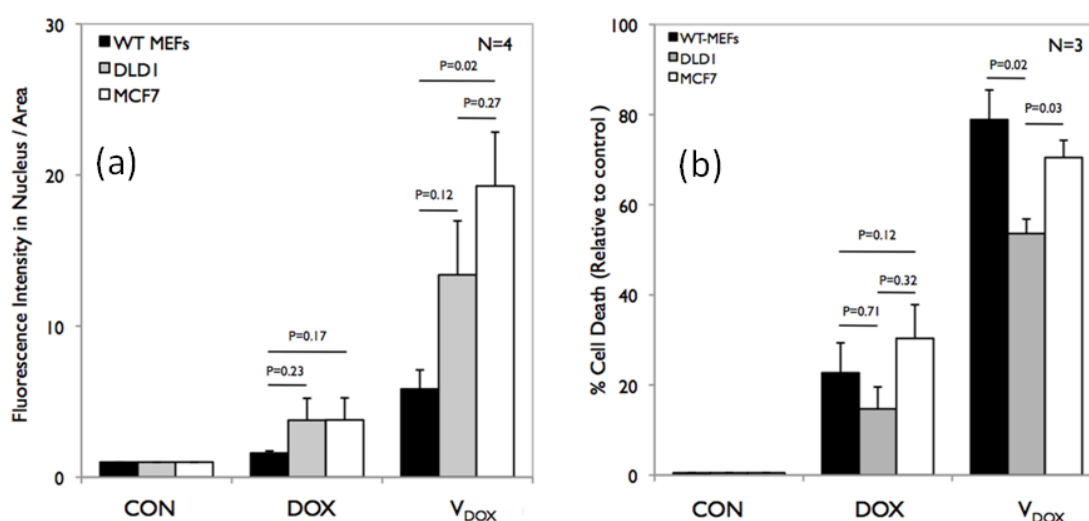


Figure 3.15. (a) Graph represents Mean $\pm$ SE of DOX fluorescence in the nucleus from ≥ 10 cells for each treatment in four independent experiments. (b) MTT assay was used to test the cytotoxicity of free DOX and V_{DOX} relative to untreated control (CON) at 1 μ M concentration over 24 hours. Graph represents Mean $\pm$ SE of percentage cell death relative to control in three independent experiments. Statistically analysis for this data was done using the standard T-Test

3.3.5. Dual Drug Loading

One way to improve the killing by DOX in cancer cells is to use it in a ratiometric combination with a drug that could support a synergistic action. Our earlier studies with the water insoluble topoisomerase type I inhibitor CPT have

shown the V_{CPT} nano-vesicle to kill WTMEFs significantly better than free CPT.²³ The improved killing of these WTMEFs by topoisomerase type II inhibitor V_{DOX} shown in the present studies raises the exciting possibility of creating a combined $V_{\text{DOX+CPT}}$ nano-vesicles for testing in cancer cells (see figure 3.16). Encapsulation of DOX and CPT produced pink or magenta fluorescent CPT and DOX dual loaded nano-vesicles ($V_{\text{DOX+CPT}}$). These nano-vesicles were produced in two different molar ratios of DOX:CPT, 4:1 and 1:4, to study their ratiometric effect.

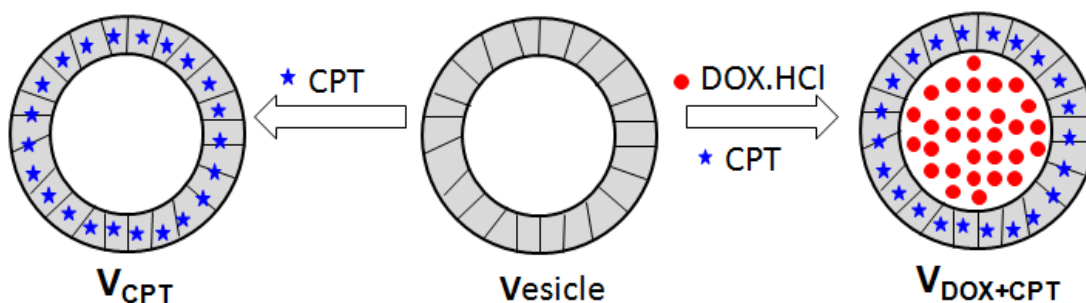


Figure 3.16. Schematic representation of CPT and DOX.HCl + CPT dual loading into vesicles.

The dual loaded nano-vesicle ($V_{\text{DOX+CPT}}$) was also found to show mono-modal distribution of average sizes of 220 ± 15 nm in the DLS (see figure 3.17a). The radius of gyration $R_g = 113$ nm was obtained from SLS studies and the ratio of $R_g/R_h = 1.05$ (see figure 3.17b) confirmed the vesicular assemblies in the dual loaded scaffold $V_{\text{DOX+CPT}}$.²⁶ Similarly, Electron microscopic images of the dual loaded nano-vesicle $V_{\text{DOX-CPT}}$ (1:4 and 4:1) was found to be spherical in shape with average size of 205 ± 10 nm (see figure 3.17c). The AFM image of $V_{\text{DOX+CPT}}$ (1:4 and 4:1) appeared as donut shaped with inner curvature as typically observed for vesicular assemblies.²⁷ The size of the vesicular structure was obtained as 200 ± 15 nm (see figure 3.17d). The height of these nano-vesicles obtained from the cross sectional analysis as 10 ± 3 nm. The sizes of objects in the images are in very good agreement with the DLS (and also with SLS) data. $V_{\text{DOX+CPT}}$, showed two absorbance maxima at 370 and 480 nm with respect to the presence of CPT and DOX, respectively (See figure 3.17e). Upon photo-excitation at 370 nm, only CPT emission was observed and there is no emission from DOX. The photo-excitation at 480 nm produced exclusively DOX emission at 590 nm in the $V_{\text{DOX-CPT}}$ (figure 3.17e). This suggested that the drug molecules were completely segregated in the nano-vesicle and no FRET was visible between them.

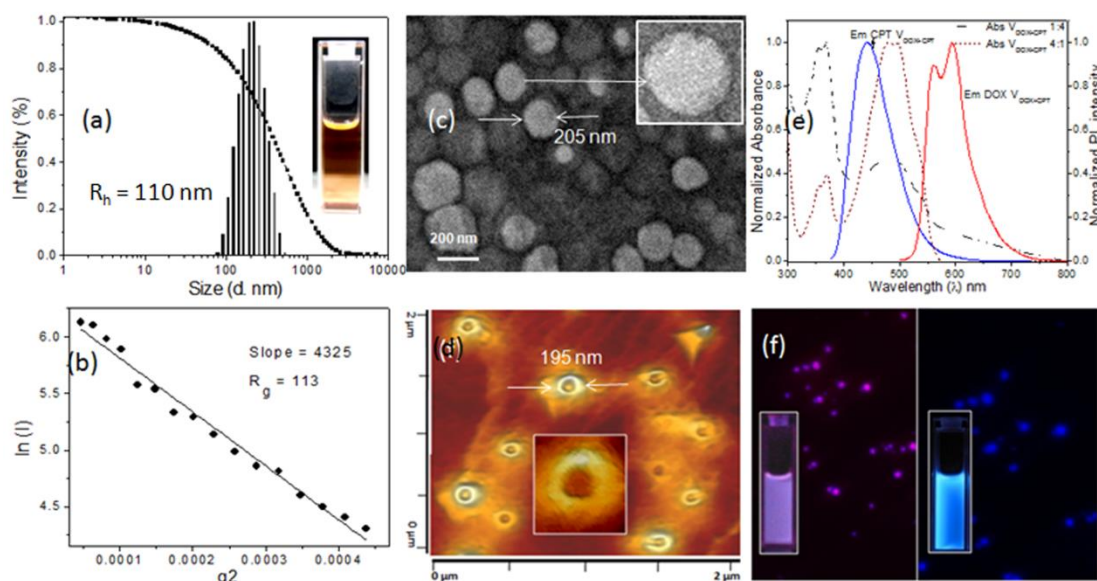


Figure 3.17. DLS histogram (a), SLS data (b), HR-TEM image (c), AFM image (d), and absorbance and emission plot (e) of $V_{DOX+CPT}$. (f) Fluorescent micrographs of $V_{DOX+CPT}$ (pink) and V_{CPT} (blue). Insert in (c) and (d) shows magnified HR-TEM and AFM image of $V_{DOX+CPT}$. The vials insert in (f) show the color of $V_{DOX+CPT}$ (pink) and V_{CPT} (blue) in PBS under hand held UV lamp.

Fluorescence microscopic images of V_{CPT} and V_{DOX} were observed as blue and red spherical luminescent objects (See Figure 3.5f and figure 3.17f), whereas $V_{DOX+CPT}$ appeared as pink colored spherical particles. (For details see figure 3.18). It was taken multiple acquisition modes, where DEX-PDP-DOX-CPT system drop casted on a glass slide was observed through blue and red channels. The blue channel helped to make out the CPT content inside the vesicle and the red channel for DOX. The images acquired through blue as well as red channels from the same field were merged together and was appeared as pink coloured spot. This indicates that both blue and red molecules, ie. CPT and DOX were entrapped in the same position. This is characteristic feature of dual drug loaded system.

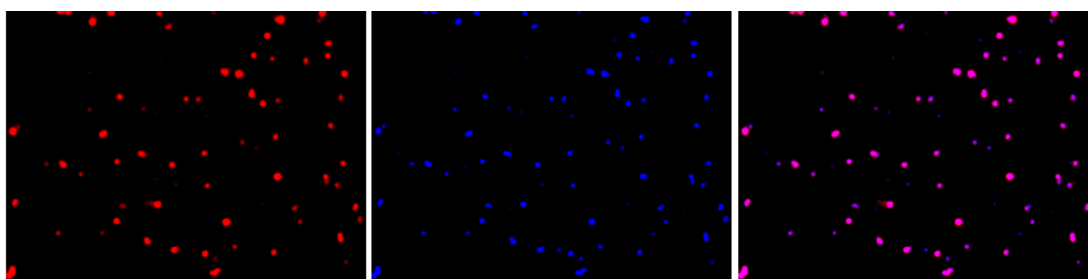


Figure 3.18. Fluorescent microscopic image of $V_{DOX+CPT}$

The absorbance spectra of the release profiles of dual loaded nano-vesicle $V_{DOX+CPT}$ (1:4) are shown in figure 3.19a. These show the release of both DOX as

well as CPT occurred simultaneously from the dual loaded nano-vesicles, but CPT levels released was much higher than DOX (see figure 3.19b for 1:4 and 3.19c for 4:1 ratio). Release from cocktail of individual drug loaded nano-vesicles $V_{DOX} + V_{CPT}$ (molar ratio 1:4 and 4:1) were also showed a similar trend (see figure 3.19d).

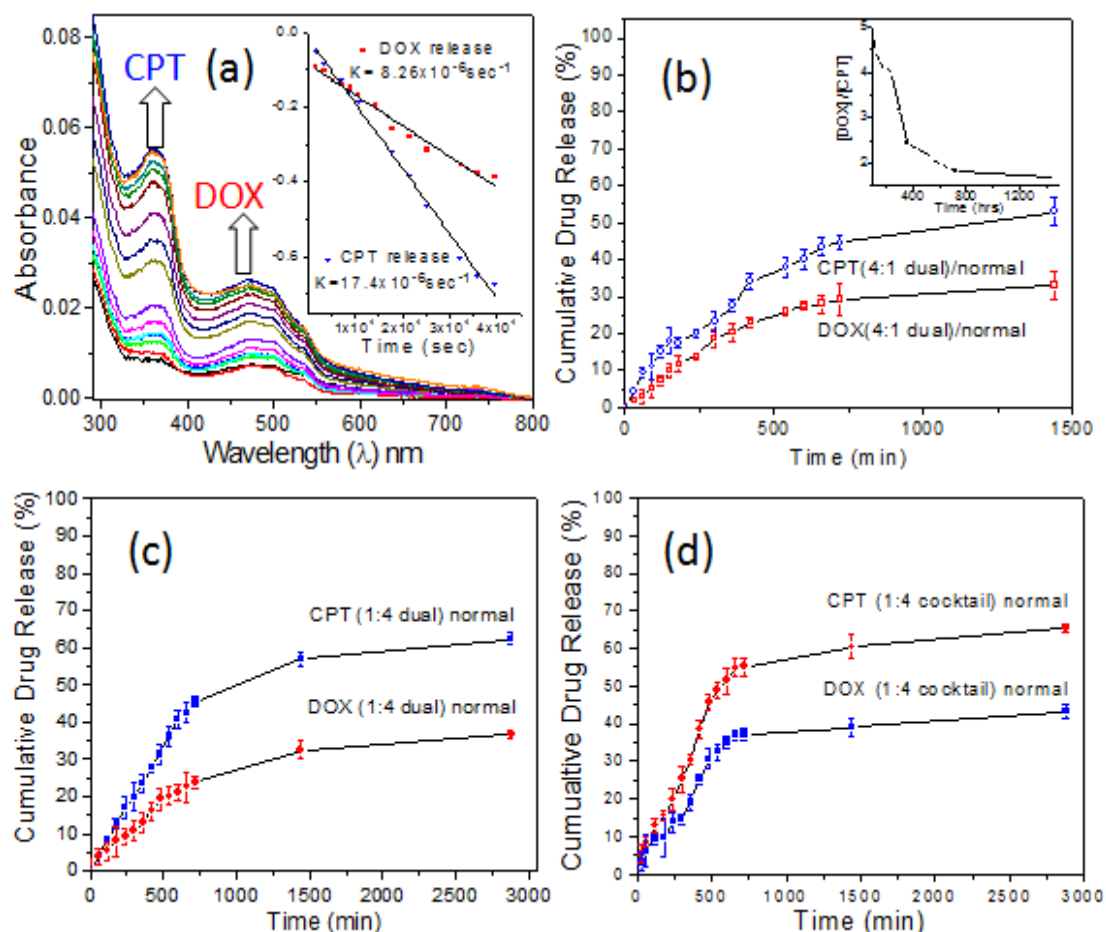


Figure 3.19. (a) Absorbance spectra of DOX and CPT released from $V_{DOX+CPT}$. (b) Cumulative drug release (b) of $V_{DOX+CPT}$ (4:1) (c) of $V_{DOX+CPT}$ (1:4) cocktail under normal physiological condition (pH 7.4 and 37 °C). Inset at (a) shows the $\ln A_t/A_o$ Vs time plot of $V_{DOX+CPT}$ (4:1).

Esterase enzyme mediated release of drugs from the dual loaded nano-vesicles $V_{DOX+CPT}$ (molar ratio 1:4 and 4:1) and cocktail of individual drug loaded nano-vesicles when compared were similar (see figure 3.20a to figure 3.20d for esterase assisted drug release). The release profile of the drugs from dual drug loaded nano-vesicles was followed in a similar manner as of their individual cocktails. Interestingly, both 1:4 and 4:1 molar ratio loaded nano-vesicles exhibited similar drug release pattern. Under normal conditions the release of DOX went upto $35 \pm 5 \%$ and that of CPT reached $55 \pm 3 \%$ in 48 hours in both type of nano-vesicles. As observed in DOX loaded nano-vesicles, esterase enzyme helped the breakdown of these nano-

vesicles causing better release of DOX ($65 \pm 4\%$) and CPT ($\sim 100\%$) in 48 hours. The ratio of the number of moles of DOX released to number of moles of CPT released were determined and plotted against time (see inset in figure 3.20a to figure 3.20d) to study the actual ratio of the drug that will reach the target cell. The 4:1 (DOX:CPT) dual drug loaded and individual cocktails of nano-vesicles attained the DOX to CPT discharge ratio as 3:1 in 6 hrs time period and maintained this ratio. But in the case of 1:4 (DOX:CPT) dual drug loaded nano-vesicles, the drug discharge ratio reached 1:4 in 8 hrs (1:4 in 6 hours also) while the individual 1:4 cocktails reached a 1:6 discharge ratio in 6 hrs. This suggest that the layer loaded CPT is released faster than when compared to nano-vesicles where the core is loaded with DOX.

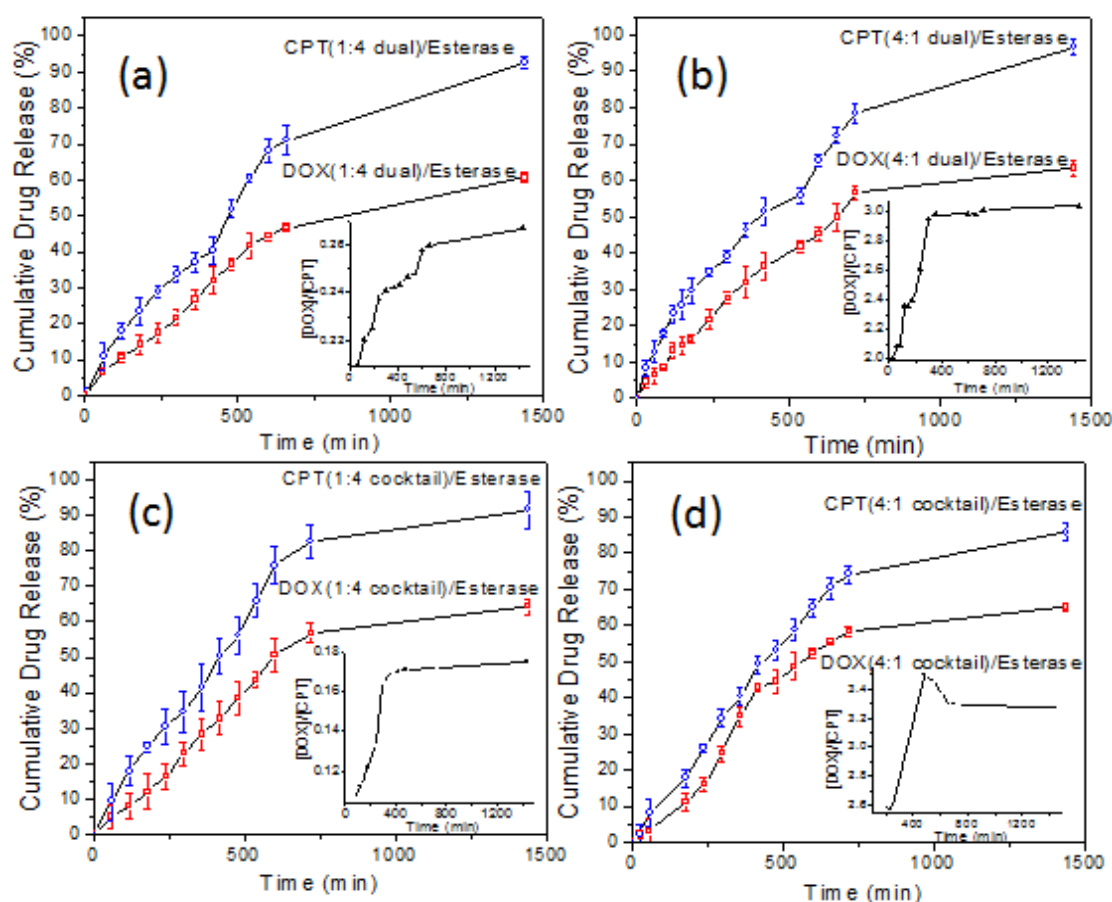


Figure 3.20. Cumulative release of DOX and CPT in the presence of esterase enzyme for 1:4 dual drug loaded (a), 4:1 dual drug loaded (b), 1:4 cocktail (c) and 4:1 cocktail (d). Inset at (a) (b),(c) and (d) shows the corresponding mole ratio of DOX to CPT released at various time points.

Earlier studies have suggested that a combination of topoisomerases I and II inhibitors may be synergistic when administered together.¹² Many cancer cell types seen to be resistant to camptothecin when treated in combination with DOX were seen to be additive or antagonistic depending on their molar ratio.^{14,49} Schedule-dependency is also reported with topoisomerase inhibitors, treatment with topoisomerase 1 inhibitor (such as CPT) followed by a topoisomerase 2 inhibitor (such as DOX) seen to be synergistic.⁴⁹⁻⁵¹

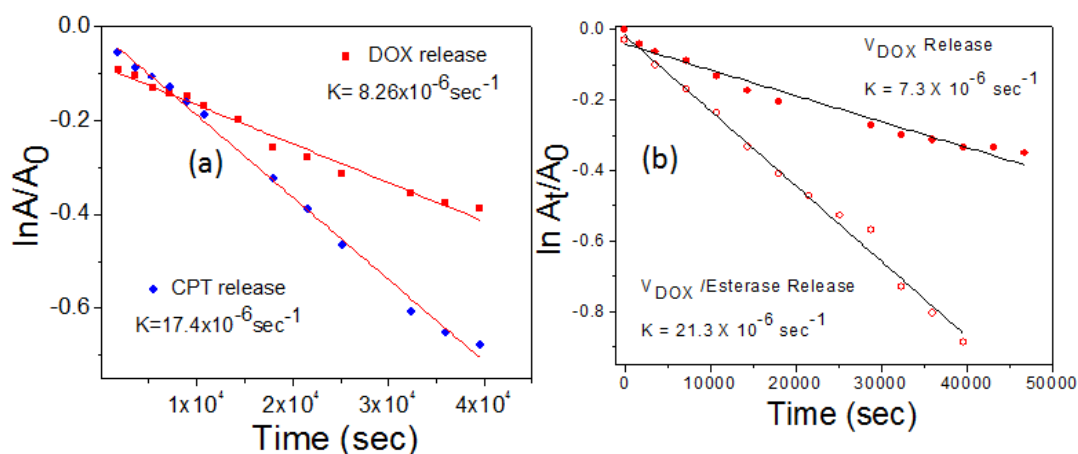


Figure 3.21. $\ln A/A_0$ vs Time plot of DOX and CPT from $V_{\text{DOX+CPT}}$ 1:4(a) and DOX normal and DOX in the presence of esterase(b)

Considering the same, the rate constant (k) for the drug release from the nano-vesicles was calculated for CPT and DOX using first order rate equation,⁵² $\ln(A_t/A_0) = -kt$, where A_t and A_0 are corresponding to drug in the nano-vesicles at time 't' and initial, respectively (see inset in figure 3.20a, figure 3.21 and table 3.1 and for details). These kinetic parameters revealed that the rate of CPT release from the individual loaded nano-vesicle (V_{CPT} , $15.3 \mu\text{s}^{-1}$) was two-fold faster than that of DOX from V_{DOX} ($7.3 \mu\text{s}^{-1}$). A similar trend was noted in both the dual loaded nano-vesicles [1:4 and 4:1 DOX: CPT, see table 3.1]. In the presence of esterase enzyme, the release rate of CPT and DOX became three fold faster than their normal release from both individual and dual loaded nano-vesicles.

Since molar ratios were reported to be important for this synergistic / antagonistic action between CPT and DOX, we also decided to study and compare the action of the 1:4 DOX: CPT (known to be antagonistic in earlier free drug studies¹⁴) and 4:1 DOX: CPT molar ratio treatments. These studies were initiated with a 4:1 $V_{\text{DOX+CPT}}$ nano-vesicle, testing for its action on WTMEFs vs DLD1 vs MCF7 cells,

while comparing it to $V_{DOX} + V_{CPT}$ (administered at comparable molar concentration but in separate nano-vesicles) and individual drug containing nano-vesicles (V_{DOX} and V_{CPT}).

Table 3.1. Drug release kinetic parameters

Type of Loaded Vesicle	Release Profiles	
	Normal	Esterase
	k (μs^{-1})	k (μs^{-1})
V_{CPT}	15.3	43.4
V_{DOX}	7.3	21.3
$V_{DOX+CPT}$ (4:1)	13.5 (CPT)	32.1
	8.3 (DOX)	20.8
$V_{DOX+CPT}$ (1:4)	12.9 (CPT)	30.7
	6.3 (DOX)	20.3
$V_{DOX+V_{CPT}}$ (4:1)	15.5 (CPT)	45.2
	8.9 (DOX)	22.9
$V_{DOX+V_{CPT}}$ (1:4)	14.9 (CPT)	44.1
	8.4 (DOX)	21.8

3.3.6. Cytotoxicity Studies of Dual Drug Loaded Vesicles

Results from these studies confirmed V_{DOX} (at 0.5 μM) to kill all three cell types significantly better than V_{CPT} (at 0.125 μM) (See Figure 3.22a). Further, in ‘normal’ mouse fibroblasts using these drugs in a 4:1 ratio (DOX:CPT) in separate or in the same nano-vesicle did not kill cells significantly better than V_{DOX} . This may in part have to do with the fact that in ‘normal’ fibroblasts V_{DOX} by itself kills cells well. In both cancer cells V_{DOX} mediated killing is significantly less than that seen in normal cells (See Figure 3.22b and 3.22c). Interestingly administering a DOX:CPT drug combination in a 4:1 ratio in these cancer cells killed them significantly better than V_{DOX} . Having this drug combination in the same nano-vesicle was also seen to kill cells significantly better than if they were in separate nano-vesicles. Earlier *in-vitro* release studies under physiological conditions have shown the DOX:CPT 4:1 drug nano-vesicle to achieve a final ratio of 3:1 in 6 hours, irrespective of if they are in same or separate nano-vesicles (See figure 3.20). This suggests that it is unlikely that major differences in release rates and hence final concentrations achieved may be

causing the differential killing observed. This leaves us to speculate that the release of both drugs in close proximity (achieved better with the combined nano-vesicle) might (at this ratio) be important for their improved action in the combined nano-vesicle. $V_{DOX+CPT}$ nano-vesicles were thus able to achieve ~80% killing in MCF7 cells and ~60% killing in DLD1 cells.

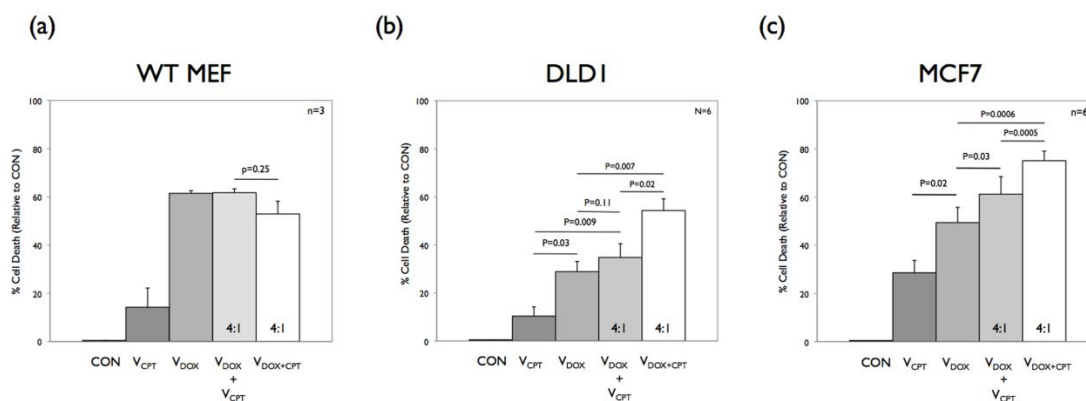


Figure 3.22. Killing by DOX:CPT combination at the 4:1 molar ratio. V_{DOX} (0.5 μ M) and V_{CPT} (0.125 μ M) were administered together in separate nano-vesicles ($V_{DOX}+V_{CPT}$) or the same nano-vesicle ($V_{DOX+CPT}$) in WTMEFs (a), DLD1 cells (b) and MCF7 cells (c). Cell killing was analyzed using the MTT assay and percentage cell death relative to untreated control calculated. Graph represents the mean $\pm$ SE data from three (WTMEF) and six (DLD1, MCF7) independent experiments.

To further establish that the ratio of the drugs (DOX:CPT) is vital to the enhanced killing observed in cancer cells, we also decided to compare drugs at molar ratio of 4:1 to 1:4 in separate and same nano-vesicle. Contrary to published reports¹⁴ the 1:4 DOX: CPT ratio was not seen to be antagonistic in these studies, killing MCF7 and DLD1 as well or better than 4:1 (Figure 3.23a and 3.23b). Interestingly if administered together in one nano-vesicle, both 1:4 and 4:1 showed almost comparable killing. However, when administered in separate nano-vesicles the 1:4 ratios killed both MCF7 and DLD1 cells better than 4:1 (See Figure 3.23a and 3.23b). Molar DOX concentrations at both these ratios (1:4 and 4:1) are similar (0.5 μ M) while molar CPT concentration increased from 0.125 μ M (in 4:1) to 2 μ M (in 1:4). This suggests that having more CPT in the DOX: CPT combination could promote their joint action. This coupled with the two fold faster release of CPT from individual nano-vesicle (V_{CPT}) than the combined ($V_{DOX+CPT}$) nano-vesicle causes the 1:4 $V_{DOX} + V_{CPT}$ treatment to achieve the best killing among all the four combinations tested in both cancer cell lines.

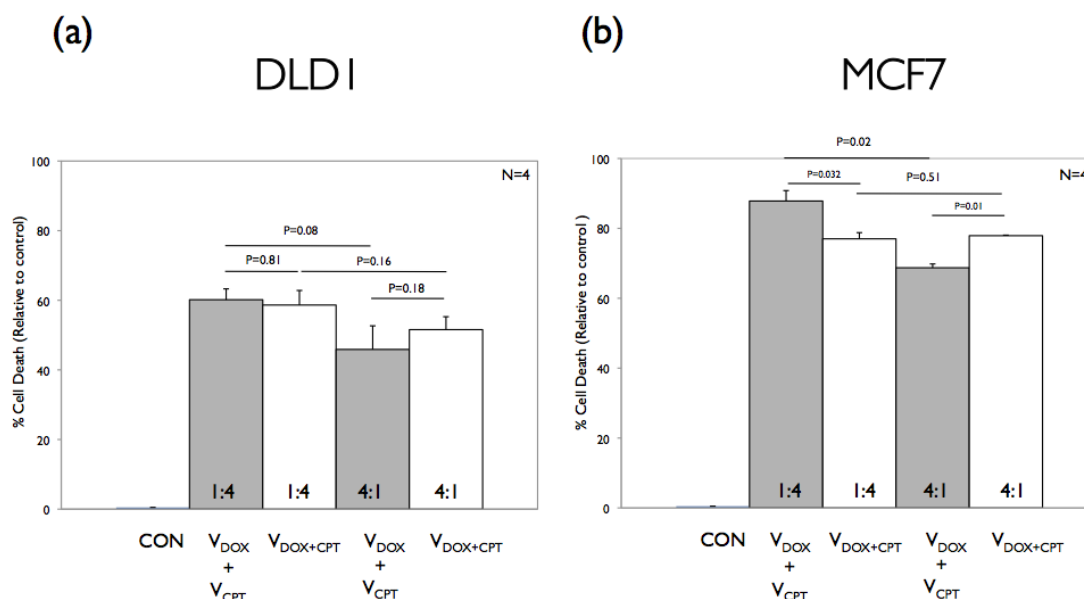


Figure 3.23. Killing by DOX:CPT combination at the 1:4 vs 4:1 molar ratio. DOX and CPT were administered together (in separate nano-vesicles – $V_{DOX}+V_{CPT}$) or the same nano-vesicle ($V_{DOX+CPT}$) in DLD1 cells (a) and MCF7 (b) cells at 1:4 (0.5 μ M: 2 μ M) molar ratios and 4:1 (0.5 μ M: 0.125 μ M). Cell killing was analyzed using the MTT assay and percentage cell death relative to untreated control calculated. Graph represents the mean $\pm$ SE data from four independent experiments

This study provides an insight into the polysaccharide nano-vesicle as multidrug carrier for synergistic killing of cancer cells also highlighting the role ratiometric drug composition could have in the effectiveness of this treatment. Though, this study focuses on only two topoisomerase targeting drugs (DOX and CPT); in principle, the polymer vesicle approach could be applicable to wide range of hydrophobic and hydrophilic drugs which are commercially available. Ongoing work focuses on expanding these studies to other possible drug combinations as well.

3.4. Conclusions

In summary, the present work in this chapter demonstrates the ability to utilize polymer vesicular nano-scaffolds for loading and delivering a topoisomerase I and topoisomerase II targeting drug combination against breast and colon cancer cells. Polysaccharide nano-vesicles custom designed using renewable resource pendent unit was employed as this multidrug carrier. These dextran nano-vesicles were seen to be capable of loading both hydrophilic DOX and hydrophobic CPT in the core and layer, respectively. These vesicular scaffolds were capable of preserving the anticancer drugs (DOX AND CPT) and release in the presence of the esterase enzyme under

physiological conditions. Though vesicular scaffold endocytosis can be mediated by caveolae, endocytosis of this nano-vesicle in caveolae lacking (Cav-1^{-/-}) MEFs was seen to be better. This differential uptake is also seen in caveolin-1 (and hence caveolae lacking) breast cancer (MCF7) and colon cancer (DLD1) cells. This is further seen to affect the uptake and nuclear localization of DOX released from V_{DOX} in caveolin-1 lacking MCF7 and DLD1 cells supporting their killing. The dual loaded polysaccharide nano-vesicles containing DOX and CPT act synergistically to promote killing of normal MEFs and cancer cells. These studies also revealed the relative ratio of DOX: CPT does affect the differential killing of cancer cells, though the 1:4 ratio reported to be antagonist in earlier studies³⁵ did not show a similar effect here. When administered as a cocktail having more CPT than DOX (1:4 ratio) synergistic killing of cancer cells was enhanced the most suggesting that a cocktail of these drugs could potentially be the best candidate for delivering multiple anticancer drugs with the best efficacy.

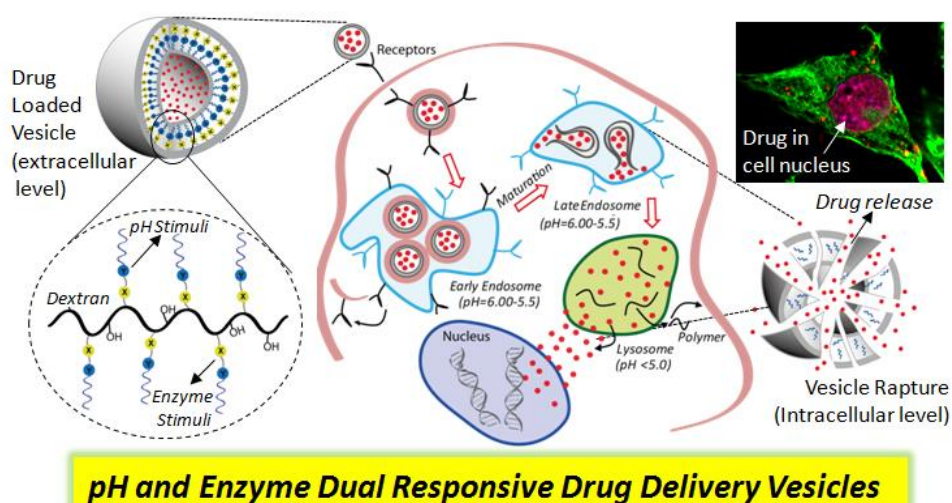
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Chapter 4

Dual Stimuli Polysaccharide Nanovesicles for Conjugated and Physically Encapsulated Doxorubicin Delivery in Breast Cancer Cells



Chapter 4

Dual Stimuli Polysaccharide Nanovesicles for Conjugated and Physically Encapsulated Doxorubicin Delivery in Breast Cancer Cells

The work in this chapter reports the development of pH and enzyme dual responsive polysaccharide vesicular nano-scaffolds for the administration of doxorubicin via physical loading and polymer-drug conjugation to the breast cancer cells. Dextran was suitably modified with renewable resource 3-pentadecyl phenol unit through imine and aliphatic ester chemical linkages that acted as pH and esterase enzyme stimuli, respectively. These dual responsive polysaccharide derivatives self-organized into 200 ± 10 nm diameter nano-vesicles in water. Water soluble anticancer drug doxorubicin (DOX.HCl) was encapsulated in the hydrophilic pocket to produce core-loaded polysaccharide vesicles whereas chemical conjugation produced DOX anchored at the hydrophobic layer of dextran nano-vesicles. In-vitro studies revealed that about 70-80 % of drug was retained under circulatory conditions under pH = 7.4 at 37 °C. At low pH=6.0 to 5.0 and in the presence of esterase; both imine and ester linkages were cleaved instantaneously to release 100 % of loaded drugs. Cytotoxicity assay on WTMEFs confirmed the non-toxicity of the newly developed dextran derivatives up to 500 $\mu\text{g}/\text{mL}$ in PBS. MTT assay on fibroblast cells revealed that DOX.HCl loaded nano-vesicles exhibited better killing abilities than DOX conjugated polymer nano-vesicles. Both DOX loaded and DOX conjugated nano-vesicles were found to show significant killing in breast cancer cells (MCF 7). Confocal microscopic images confirmed the uptake of DOX loaded (or conjugated) nano-vesicles by cells compared to free DOX. Thus, the newly developed pH and enzyme dual responsible polysaccharide vesicular assemblies are potential drug vectors for the administration DOX in both loading and chemically conjugated forms for efficient killing of breast cancer cells.

4.1. Introduction

In the recent years, considerable accomplishments have been witnessed in the field of stimuli responsive polymeric nanocarriers for drug delivery.¹⁻⁵ External stimuli such as temperature,^{6,7} radiation (for photodynamic therapy)^{8,9} and magnetic forces¹⁰ were demonstrated for disassembling the polymeric scaffolds to release the drugs at the tissue level. Unfortunately, these stimuli were less effective for polymer-drug delivery at the intra-cellular compartments which are regulated by either enzymes (like esterase and glutathione transferase) or pH variation in the endosomes/lysosomes.^{11,12} The pH of the endosomes and lysosomes have typically varied in the range of 6.0 - 5.5 which is much lower compared to that the cytosol (pH = 7.4).¹³ Moreover, recent *in-vivo* studies using optical imaging techniques, MR spectroscopy and PET radiotracers revealed the acidic pH of tumor tissue and its surrounding environment.¹⁴ Pagel's group has developed paramagnetic CEST (Chemical Exchange Saturation Transfer) agent which proved the pH of breast cancer (MCF7) as 6.0 to 6.5 compared to 7.4 as observed in normal breast tissues.¹⁵ (See figure 4.1).

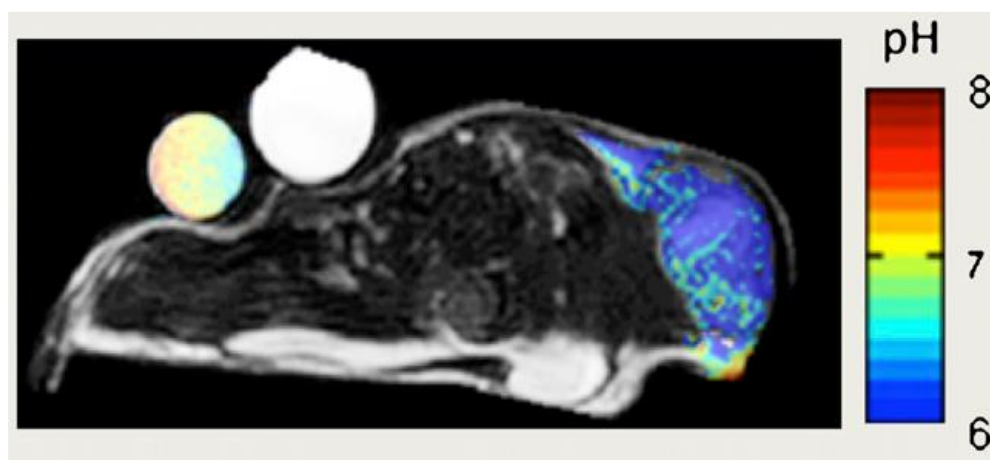


Figure 4.1. Acidic *pHe* of mouse MCF7 breast tumor model measured using paramagnetic CEST MRI (filtered parametric pH map) method (adopted from Liu *et al.* *Molecular imaging*. **2012**, 11, 47-57).

Thus, the developments of new polymer scaffolds that can undergo cleavage by pH variation at the cancer tissue level (in range of 6.5 to 5.0) and enzyme (like esterase or glutathione transferase) at intracellular compartments are potential dual stimuli vectors for efficient drug administration.¹⁶ Very recently Dai *et al.* had reported the development of disulfide linked block copolymer micelles that were cleavable under glutathione (GSH) and low pH = 5.0 at intracellular level in lysosomal

compartment of ovarian cancer cells.¹⁷ Authors performed ring opening polymerization (ROP) reaction to obtain triblock copolymer consist of poly ethylene glycol having monomethoxy functional group (mPEG), poly (L-aspartic acid) grafted with 2-mercaptoethylamine a(PAsp MEA) and poly (L-aspartic acid) grafted with 2-(diisopropylalmino) ethylamine). PEG chain in the layer was cross linked using disulphide linkage to yield highly packed interlayer crosslinked micelle (HP-ICM) (See figure 4.2). DOX were encapsulated in the micelle and released in the presence of acidic pH and glutathione reductase enzyme conditions.¹⁷

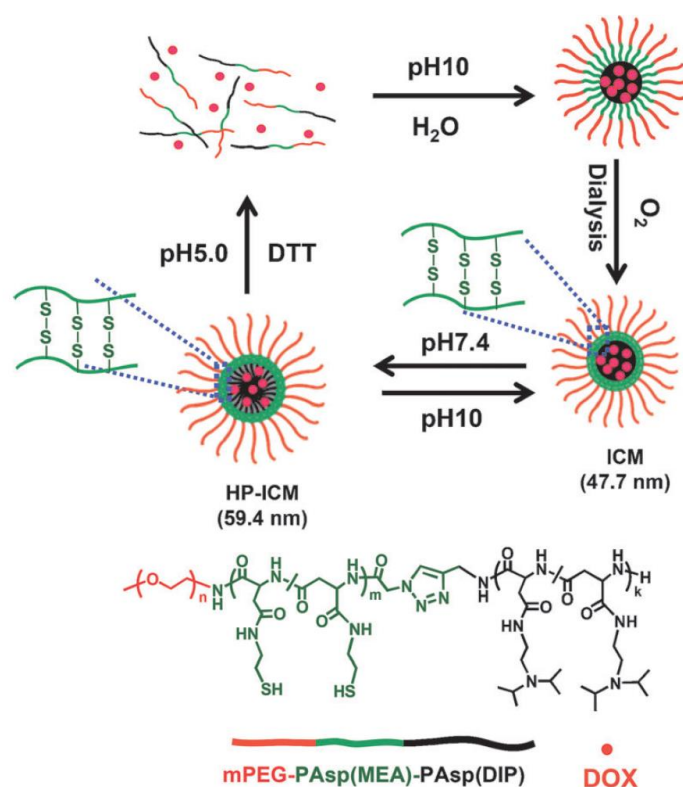


Figure 4.2. Highly packed interlayer cross linked micelle loaded with DOX and its release under pH and enzymatic conditions (adopted from Dai et al. *Angew. Chem. Int. Ed.* **2011**, 50, 9404-9408).

However, there is no effort has been put to exploit the pH and enzyme stimuli together in polysaccharide vesicles. Drug molecules can either anchor on the polymer back bone via pH sensitive linkage or physically load in the pH responsive vehicles. Earlier studies revealed that chemical conjugation of drug molecules in the polymer backbone prevents the premature leakage of drug from the formulation and thereby enhances the therapeutic activity and reduces the side effects.¹⁸⁻²³ More often the chemical conjugation of drug on the polymer backbone (to produce polymer-drug conjugates) was found to drastically change the nascent polymer self-assembly; as a

result, the drug loaded and conjugated scaffolds were found to be different in size and morphology.^{24,25} For instance Gou *et al.*, reported spherical micelles formed from miktoarm star copolymer composed of PCL and PEG arms on cyclodextrin. TEM studies proved that the ibuprofen drug conjugation altered the shape of spherical micelles to worm like aggregates (See figure 4.3).²⁴ A similar observation was made by Mikhail *et al.*, in docetaxel drug conjugated poly(ethylene glycol)-*b*-poly(ϵ -caprolactone) (PEG-*b*-PCL) copolymers. Upon changing the amount of drug conjugation on the polymer back bone, shape transformation happened from spherical to rod like morphologies. This morphology change lead to the ambiguity in the role of drug release profiles and their action in the drug conjugated versus loaded polymer nano-objects.²⁵

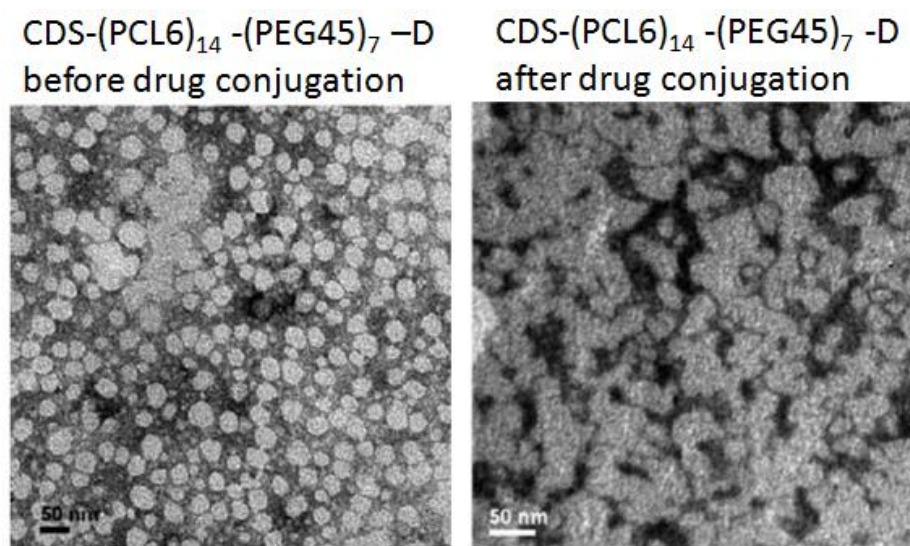


Figure 4.3. TEM image proving the shape transformation from spherical micelles to worm like aggregate upon drug conjugation on silylated cyclodextrin (CDS)-PEG-PCL based miktoarm copolymer (adopted from Gou *et al.* *Biomacromolecules* **2010**, 11, 934)

The studies on dextran vesicles described in chapter 3 revealed that polysaccharide vesicular scaffolds are potential drug vectors for targeting breast cancer.²⁶⁻²⁸ Thus, it would be appropriate to develop new polysaccharide scaffolds to address the following fundamental questions: (i) structural optimization for achieving identical size and shape in both polymer-drug conjugated scaffold and drug loaded polymer scaffold (this will facilitate the study of drug release profiles for physically loaded and chemically anchored anticancer drugs in identical nano-assembly) and (ii) pH and enzyme dual stimuli for drugs delivery at the intra-cellular lysosomal compartments for enhancing the therapeutic efficacy.

Therefore, the work in this chapter is emphasized to develop new dual pH and enzyme responsive polysaccharide vesicles with identical sizes and vesicular morphologies in both drug conjugated and drug loaded nano-assemblies (see figure 4.4). The pH responsiveness was achieved by the imine chemical linkage (stimuli-1, shown in blue color) and enzyme cleavable aliphatic ester bond that connected the hydrophobic segment at the dextran backbone (stimuli-2 shown in yellow color).

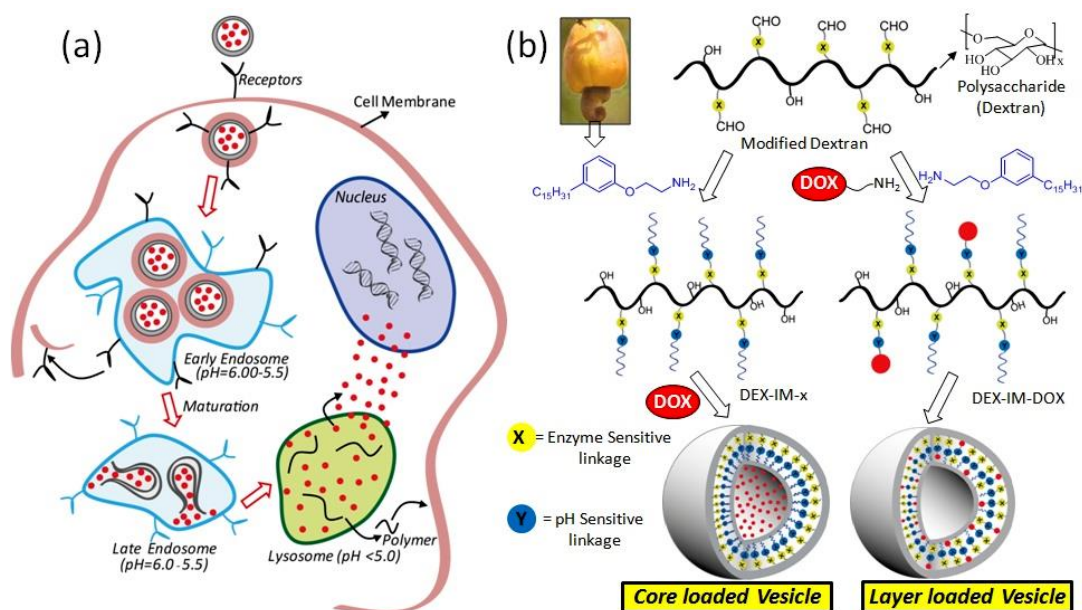


Figure 4.4. (a) Schematic representation of polymer-drug nanovesicle cellular uptake and their degradation in endocytic compartments. (b) Structural engineering of pH and enzyme dual responsive polysaccharide vesicles.

3-pendadecylphenol hydrophobic unit was suitably modified and utilized as structure director for achieving vesicular geometry in the dextran backbone as explained in chapter 2 and 3. Water soluble anticancer drug doxorubicin DOX.HCl, highly potent DNA intercalating agent, was chosen for loading in the vesicular hydrophilic core and its amine derivative (hydrophobic form) was anchored through imine chemical linkage. The current design provides two vesicular scaffolds: (i) DOX loaded at the core of the nascent polysaccharide vesicles and (ii) DOX-conjugated at the hydrophobic layer of the polysaccharide vesicles having identical morphology, pH responsiveness and enzymatic release kinetics. These DOX containing vesicular scaffolds were tested for cellular penetration and their cytotoxicity were tested in MCF7 cells. DOX physically loaded vesicles with about 30 % drug release under normal physiological conditions produced better cytotoxicity than that of DOX chemically conjugated vesicles with about only 10 % drug leaching under similar

conditions. These studies revealed that pH responsive polysaccharide vesicular scaffolds are potential drug vectors for breast cancer treatment.

4.2. Experimental methods:

4.2.1. Materials: Dextran ($M_w = 6,000$), ethanolamine, di-tert-butyl dicarbonate, 4-hydroxy benzaldehyde, diisopropyl azodicarboxylate, triphenyl phosphine, trifluoroacetic acid, 3-pentadecylphenol, dicyclohexylcarbodiimide, 4-dimethylamino pyridine, pyrene, doxorubicin.HCl (DOX.HCl), Nile Red, and rhodamine B were purchased from Aldrich chemicals. Dimethyl sulfoxide was dried over Calcium chloride and CaH_2 and distilled prior to use. Tert-Butyl bromoacetate, K_2CO_3 , KI, all other reagents and solvents were purchased locally and purified following the standard procedure. Wild type mouse embryonic fibroblasts were maintained in DMEM (phenol red free medium: Gibco) containing 10% (v/v) fetal bovine serum (FBS) and 1% (v/v) penicillin–streptomycin at 37° C under a 5% CO_2 humidified atmosphere. Cells were trypsinised using 0.05% trypsin (Gibco) and seeded in 96 well or 6 well (as per experiment) flat bottomed plastic plates (Costar) for all assays. Tetrazolium salt, 3-(4,5-dimethylthiazol-2-yl)-5-diphenyl tetrazolium bromide (MTT), DMSO and paraformaldehyde was obtained from Sigma. Phalloidin conjugated to Alexa 594 was obtained from Molecular Probes (Invitrogen) and fluoromount from Souther Biotech.

4.2.2. General procedures: NMR spectra were recorded using 400-MHz Jeol NMR spectrometer in CDCl_3 or DMSO (d_6) containing TMS as internal standard. FT-IR spectra of all compounds were recorded using Bruker alphaT Fourier transform infrared spectrometer. The mass of all small molecules were confirmed by using the Applied Biosystems 4800 PLUS MALDI TOF/TOF analyzer. High resolution mass spectra were obtained from Micro Mass ESI-TOF MS spectrometer. The purity of the modified dextran were determined by gel permeation chromatography (GPC) using a Viscotek VE 1122 pump, Viscotek VE 3580 RI detector, and Viscotek VE 3210 UV/Vis detector in dimethyl formamide. The absorption and emission studies were done by a Perkin-Elmer Lambda 45 UV-Visible spectrophotometer and SPEX Fluorolog HORIBA JOBIN VYON fluorescence spectrophotometer with a double-grating 0.22 m Spex1680 monochromator and a 450W Xe lamp as the excitation source at room temperature. The excitation spectra are collected at 375 nm and 420

nm (for pyrene experiment) and 626 nm (for Nile red experiment) and the emission spectra are recorded by exciting at the excitation maxima. The dye containing samples were purged with N₂ gas for at least 15-20 minutes prior to photophysical experiments. The size determination of the dextran derivatives were carried out by dynamic light scattering (DLS), using a Nano ZS-90 apparatus utilizing 633 nm red laser (at 90° angle) from Malvern instruments. The reproducibility of the data was checked for at least three times using independent polymer solutions. The static light scattering experiment (SLS) was carried out using 3D-DLS spectrometer, from LS instruments, Switzerland. The instrument consists of a He Ne laser having wavelength of 632.8 nm attached to computer using Lab view interface utilizing toluene as reference. The measurement was performed in autocorrelation mode from 20° to 130° by steps of 5°. Atomic force microscope (AFM) images were recorded by drop casting the samples on freshly cleaved mica surface, using Veeco Nanoscope IV instrument. The experiment was performed in tapping mode using TAP-190AL-G50 probe from Budget sensors with a nominal spring constant of 48 N/m and resonance frequency of 163.5. FE-SEM images were recorded using Zeiss Ultra Plus scanning electron microscope. For FE-SEM analysis, the samples were prepared by drop casting on silicon wafers and coated with gold. Thermal analysis of dextran derivatives was performed using TA Q20 differential scanning calorimeter (DSC). The instrument was calibrated using indium standards. TEM images were recorded using a Technai-300 instrument by drop casting the dialysed sample (0.1 mg/ mL) on formvar coated copper grid. The sample was vacuum dried overnight. The image was collected without negative staining. The fluorescent micrographs were collected using Carl Zeiss Axiovert 200 microscope. LSM710 confocal microscope was used for imaging the cells.

4.2.3. Synthesis of *tert*-butyl 2-(4-formyl phenoxy) acetate (1): 4-Hydroxy benzaldehyde (6.0 g, 49.1 mmol), anhydrous K₂CO₃ (13.5 g, 98.3 mmol) and a pinch of KI were taken in dry acetonitrile (60 mL) and stirred at 80 °C under nitrogen atmosphere for 30 minutes. The content was cooled and *tert*-butyl bromoacetate (7.9 mL, 54.0 mmol) added drop wise. The reaction was continued by stirring at 80 °C for 24 h under nitrogen atmosphere. Acetonitrile was removed by rota evaporation and the reaction mixture was poured into water (250 mL). Subsequently the product was

extracted with ethylacetate and the organic layer was washed with brine. The content was dried over anhydrous sodium sulphate and solvent was removed to obtain the product as low melting solid. It was further purified by passing through silica gel column using 3% ethyl acetate in hexane as eluent. Yield = 9.9 g (85 %). m.p. = 38 °C. ^1H NMR (400 MHz, CDCl_3) δ : 9.88ppm (Ar-CHO), 7.83ppm (d, 2H, Ar-H), 6.98ppm (d, 2H, Ar-H), 4.59ppm (s, 2H, O-CH₂), 1.47ppm (s, 9H, t-butyl). ^{13}C NMR (CDCl_3 , 100 MHz) δ : 190.84 (Ar-CHO), 167.22 (Ester C=O), 162.85, 132.03, 130.66, 114.93 (Ar-C), 83.02 (-C-(CH₃)₃), 65.61 (Ar-O-CH₂-), 28.09(-C-(CH₃)₃). FT-IR (KBr, cm^{-1}), 3069 (Aromatic C-H stretch), 2836, 2750 (=C-H stretch of aldehyde), 2978, 2935 (Aliphatic C-H stretch), 1743 (C=O stretch), 1573, 1506 (ring C=C stretch), 1215, 1149 (C(=O)-O stretch) MALDI-TOF-TOF, (MW:216), m/z = 274.3 ($\text{M}+\text{K}^+$). (see annexure for spectra).

4.2.4. Synthesis of 2-(4-formyl phenoxy) acetic acid (2): Compound 1 (5.0 g, 21.1 mmol) was dissolved in 25 mL dichloromethane and cooled to 0°C in an ice bath. Trifluoroacetic acid (16.2 mL, 211.6 mmol) was added dropwise into the above solution and reaction mixture was brought into room temperature. After 2 hours, dichloromethane was rota evaporated from the mixture and trifluoroacetic acid was removed by co-evaporation with dichloromethane (4X 15 mL). The product was collected as brownish solid which was further purified by passing through silica gel column using 2 % methanol in chloroform as eluent to get white solid. Yield = 3.1 g (81.3 %). ^1H NMR (400 MHz, d_6 DMSO) δ : 12.9ppm (s, 1H, CO-OH), 9.82ppm (s, 1H, Ar-CHO), 7.82ppm (d, 2H, Ar-H), 7.06ppm (d, 2H, Ar-H), 4.78ppm (s, 2H, O-CH₂). ^{13}C NMR (DMSO (d_6), 100 MHz) δ : 191.87 (Ar-CHO), 170.17 (-O-CH₂-COOH), 163.17, 132.22, 130.51, 115.52, (Ar-C), 65.08(-O-CH₂-COOH). FT-IR (KBr, cm^{-1}), 3638 (O-H stretch) 3093 (Aromatic C-H stretch), 2843, 2757 (=C-H stretch of aldehyde), 2690 (Aliphatic C-H stretch), 1750 (Aldehyde C=O stretch), 1714 (Acid C=O stretch), 1565, 1505 (ring C=C stretch), 1424 (O-H bending), 1210 (C-O stretch of COOH) 1266 (C (=O)-O stretch). MALDI TOF-TOF, (MW: 180.1), m/z = 218.2 ($\text{M}+\text{K}^+$) (see annexure for spectra).

4.2.5. Synthesis of DEX-CHO-x: Dextran (M_w =6,000, 1.0 g, 6.2 mmol of anhydroglucose unit) and compound 2 (1.1g, 6.2 mmol, for DEX-CHO-10) were dissolved in anhydrous DMSO (50.0 mL) and the solution was purged with dry nitrogen for 15 minutes. Dicyclohexylcarbodiimide (1.5 g, 7.4 mmol) in anhydrous

DMSO (3.0 mL) and 4-(Dimethylamino) pyridine (0.15 g, 1.2 mmol) in anhydrous DMSO (3.0 mL) and were added into the above reaction mixture. The reaction mixture was stirred for 1 hour under dry nitrogen purging and the reaction was continued at 25°C for 24 h under nitrogen atmosphere. The solution was filtered to remove dicyclohexyl urea and the solvent was removed under vacuum distillation (0.1 mm Hg). The thick viscous liquid was precipitated by adding into cold isopropyl alcohol (100 mL). The solid was filtered and washed several times with methanol. It was dissolved again in DMSO and purification by the precipitation technique was done at least twice. The product filtered out and dried under vacuum at 60 °C to get yellowish white solid as product. Yield = 65 %. ¹H NMR (400 MHz, d₆ DMSO): δ: 9.82ppm (s, 1H, -CHO), 7.82ppm (m, 2H, Ar-**H** adjacent to aldehyde group), 7.08ppm (m, 2H, Ar-**H**), 4.47, 4.82, 4.88 ppm (s, hydroxyl of dextran) 4.63ppm (s, dextran anomeric proton), 3.14-3.69ppm (dextran glucosidic protons). ¹³C NMR (DMSO (d₆), 100 MHz) δ: 191.98ppm (Aldehyde C=O), 168.45 (Ester C=O), 162.83, 132.26, 130.59, 115.54 (Ar-C), 98.63 (dextran anomeric C), 73.78, 72.29, 70.54, 66.43(Dextran glucosidic Carbons), 61.28ppm (O=C-CH₂). FT-IR (KBr), cm⁻¹), 3291 (O-H stretch), 2926, 2852 (Aliphatic C-H stretch), 1750 (Ester C=O stretch), 1513 (ring C=C stretch), 1422(O-H bending), 1209 (C (=O)-O stretch).

DEX-CHO-x with three different degrees of substitution were synthesized by changing the mole ratios of compound **2** to dextran as 0.5, 1.0 and 3.0 in the feed .

4.2.6. Synthesis of *N*-(*tert*-Butoxycarbonyl) ethanolamine (3**):** Synthesis was performed using reported procedures.⁶ Briefly, ethanolamine (5.0g, 81.0 moles) was dissolved in 173 mL of sodium carbonate (10%, 17.3 g, 163.7 mmol) solution and tetrahydrofuran (87 mL) was added into it. The reaction mixture was cooled to 0 °C in an ice bath and boc anhydride (19.6 g, 90.0 mmol) dissolved in tetrahydrofuran (86 mL) was added drop wise into it the reaction and it was stirred at 25 °C for 12 hours. The solvent was evaporated from the reaction mixture and the remaining aqueous solution was neutralized using 0.1M HCl solution. The product was extracted with ethyl acetate (100 mL) and the organic layer was washed with brine solution (2X 100mL) and dried over sodium sulphate. The combined ethyl acetate layer was rota evaporated to get the crude product as thick colourless liquid. It was further purified by passing through silica gel column, initially at 5 % ethyl acetate in hexane as eluent

to remove all unreacted boc anhydride and followed by isolating the product by passing ethylacetate. Yield = 10.6 g (80 %). ^1H NMR (400 MHz, CDCl_3) δ : 4.98ppm (s, 1H, N-**H**), 3.67ppm (t, 2H, OH-**CH**₂-**CH**₂-), 3.25ppm (OH-**CH**₂-**CH**₂-) 1.42ppm (s, 9H, t-butyl). ^{13}C NMR (CDCl_3 (d6), 100 MHz) δ : 156.99 (NH-**C=O**-), 79.78 (**C**-(**CH**₃)₃), 62.73 (OH-**CH**₂-**CH**₂-), 43.24 (OH-**CH**₂-**CH**₂-), 28.44 (**C**-**CH**₃)₃. FT-IR (KBr), cm^{-1} , 3342 (O-H stretch), 2975, 2934 (Aliphatic C-H stretch), 1683 (C=O stretch), 1393 (O-H bending), 1249 (C-O stretch) 1165 (C-N stretch). MALDI TOF-TOF, (MW: 161.1), m/z = 199.0 (M+K⁺) (see annexure for spectra).

4.2.7. Synthesis of 2-(3-pentadecylphenoxy) ethanamine (4): Synthesis was performed using reported procedures.⁶ Briefly, 3-Pentadecyl phenol (5g, 16.42 mmol), Compound **3** (2.6 g, 16.4 mmol) and triphenyl phosphine (4.3 g, 16.42 mmol) were dissolved in anhydrous tetrahydrofuran (50 mL). The mixture was taken in 3-neck round bottom flask and cooled in an ice bath purged with dry nitrogen. Diisopropyl azodicarboxylate (3.51 mL, 18.06 mmol) was added drop wise and the reaction was continued under nitrogen atmosphere for 24 hours at 25 °C. The solvent was removed from the reaction mixture and the remaining thick liquid was dissolved in ethyl acetate (80 mL). The organic layer was washed with brine solution for 3 times (3 X 50 mL) and dried over anhydrous sodium sulphate. It was purified by passing through silica gel column with 2 % ethyl acetate in hexane as eluent. Yield = 5.8 g (79 %). ^1H NMR (400 MHz, CDCl_3) δ : 7.19ppm (t, 1H, Ar-**H**), 6.80ppm (d, 1H, Ar-**H**), 6.72ppm (s, 1H, Ar-**H**), 6.70ppm (d, 1H, Ar-**H**), 5.01ppm (s, 1H, N-H), 4.02ppm (t, 2H, O-**CH**₂-**CH**₂-NH), 3.53ppm (t, 2H, O-**CH**₂-**CH**₂-NH), 2.57ppm (t, 2H, Ar- **CH**₂), 1.60ppm (m, 2H, Ar- **CH**₂-**CH**₂), 1.46ppm (s, 9H, t-butyl), 1.28ppm (m, 27H, Aliphatic protons), 0.87ppm (t, 3H, Ar-(**CH**₂)₁₄-**CH**₃). ^{13}C NMR (CDCl_3 , 100 MHz) δ : 155.99 (NH-**C=O**-), 58.62, 144.85, 129.30, 121.32, 114.75, 111.40 (Ar-**C**), 79.56 (**C**-(**CH**₃)₃), 67.08 (OH-**CH**₂-**CH**₂-), 40.26 (OH-**CH**₂-**CH**₂-), 36.10, 32.01, 29.77, 28.48, 22.78, 14.21 (aliphatic carbons). FT-IR (KBr), cm^{-1} , 3395 (N-H stretch), 2918, 2850 (Aliphatic C-H stretch), 1691 (C=O stretch), 1593, 1514 (ring C=C stretch), 1253 (C-O stretch) 1157 (C-N stretch). MALDI TOF-TOF, (MW: 447.6), m/z = 486.3 (M+K⁺).(see annexure for spectra).

4.2.8. Synthesis of tert-butyl (2-(3-pentadecylphenoxy) ethyl) carbamate (5) (PDP-amine): Compound **4** (5g, 11.1 mmol) was dissolved in dichloromethane (25

mL) and cooled to 0 °C in an ice bath. Trifluoroacetic acid (8.54 mL, 111 mmol) was dissolved in dichloromethane (20 mL) and added drop wise into the above solution. The reaction was continued at room temperature for 2 hours. The dichloromethane was removed by rota evaporation. The remaining trifluoroacetic acid was removed by co-evaporation with dichloromethane (4 X 15 mL) and the product was collected as yellowish brown low melting solid. Yield = 2.8 g (72 %). m.p. =40 °C. ¹H NMR (400 MHz, CDCl₃) δ: 7.16ppm (t, 1H, Ar-**H**), 6.76ppm (d, 1H, Ar-**H**), 6.72ppm (s, 1H, Ar-**H**), 6.69ppm (d, 1H, Ar-**H**), 3.98ppm (t, 2H, O-CH₂), 3.08ppm (t, 2H, O-CH₂-CH₂), 2.55ppm (t, 2H, Ar-CH₂), 1.58ppm (m, 2H, Ar-CH₂-CH₂), 1.28ppm (m, 27H, aliphatic-H), 0.87ppm (t, 3H, -CH₃). ¹³C NMR (CDCl₃, 100 MHz) δ: 157.44, 145.05, 129.31, 122.02, 114.59, 111.43 (Ar-C), 63.21 (Ar-O-CH₂), 39.52 (O-CH₂-CH₂-NH₂), 35.91, 31.90, 29.09, 29.34, 22.66, 14.08 (aliphatic carbons in the side chain). FT-IR (KBr), cm⁻¹, 3291 (N-H stretch), 2921, 2852 (Aliphatic C-H stretch), 1587 (N-H bending), 1554 (ring C=C stretch), 1257 (C-O stretch) 1158 (C-N stretch). MALDI-TOF-TOF, (MW:347.5), m/z= 385.2 (M+K⁺). (see annexure for spectra).

4.2.9. Synthesis of Dex-IM-PDP (DEX-IM): PDP-amine (0.23g, 0.674mmol) was dissolved in anhydrous DMSO (10 mL) containing sodium carbonate (0.1g) and purged with dry nitrogen for 10 min. DEX-CHO-10 (1g) was dissolved in anhydrous DMSO (80 mL) and added to the above solution. The reaction mixture was purged for another 15 min and then heated at 50 °C for 4 hours under nitrogen atmosphere. DMSO was removed from the reaction mixture under vacuum and the product was precipitated in cold methanol. The purification was done by dissolving in DMSO and precipitation in methanol for two times. The product was collected by filtration and dried under vacuum at 50 °C to get brownish yellow solid. Yield = 68 %. δ: 8.27ppm (s, 1H, benzylidene imine proton), 7.63ppm (d, 2H, Ar-**H** adjacent to imine linkage), 6.93ppm (d, 2H, Ar-**H**), 7.08ppm (s, 1H, Ar-**H** of PDP unit), 6.67ppm (m, 3H, Ar-**H** of PDP unit), 4.47, 4.82, 4.88 ppm (s, hydroxyl of dextran) 4.63ppm (s, dextran anomeric proton), 3.14-3.69ppm (dextran glucosidic protons), 4.14ppm (t, 2H, O-CH₂ of PDP unit), 3.81 ppm (t, 2H, O-CH₂-CH₂ of PDP unit), 2.45ppm (t, 2H, Ar-CH₂), 1.46ppm (m, 2H, Ar-CH₂-CH₂), 1.16ppm (m, 27H, aliphatic-H), 0.78ppm (t, 3H, -CH₃). FT-IR (KBr), cm⁻¹, 3292 (O-H stretch), 2921, 2852 (Aliphatic C-H stretch), 1707 (Ester C=O stretch), 1644 (C=N stretch of imine) 1450 (ring C=C stretch), 1342(O-H bending), 1267 (C (=O)-O stretch).

A similar protocol was employed for the synthesis of other derivatives.

4.2.10. Synthesis of DOX conjugated DEX-IM: Doxorubicin (0.029 g, 0.05mmol) and PDP-amine (5) (0.042 g, 0.11 mmol) were dissolved in anhydrous DMSO (10 mL). Sodium carbonate (0.1g) was added to the above solution and the reaction mixture was purged with nitrogen for 10 min. DEX-CHO-10 (0.25 g) was dissolved in anhydrous DMSO (80 mL) and added to it and the nitrogen purging was continued for another 15 min. The reaction mixture was heated to 50 °C for 4 hours under nitrogen atmosphere. DMSO was removed from the reaction mixture under vacuum and the product was precipitated in cold methanol. Further purification was done by dissolving in DMSO and dialysed (MWCO 3,500) against PBS (pH 7.4) for 24 h. The product was collected as bright red powder after lyophilization. The formation of DOX conjugated DEX-IM was confirmed using Gel Permeation Chromatography, where UV detector was set to 480 nm. Absorption spectroscopy method was used for the determination of the amount of doxorubicin conjugated on the DEX-IM system.

4.2.11. Preparation of DEX-vesicles and critical vesicular concentration (CVC): Dextran vesicles were prepared by adopting nanoprecipitation method. Typically, 20 mg of DEX-IMINE-PDP (DEX-IM) was dissolved in 3 mL of DMSO and 3 mL of PBS (pH 7.4) was added drop wise under moderate stirring at 25 °C. The resulting solution was extensively dialyzed against buffer solution (pH 7.4) using regenerated cellulose dialysis membrane (SPECTRA/POR, MWCO 3500) for 48 h.

The critical vesicular concentration (CVC) was determined by using hydrophobic pyrene and nile red as fluorescent probes. Concentration of pyrene and nile red was fixed as 0.6 μM and 1.0 μM respectively. In a typical experiment, required amount of dye in acetone was pipetted into 5 mL glass sample vials and then acetone was allowed to evaporate completely. DEX-IM solutions at different concentration, varying from 6.2×10^{-7} M to 3.1×10^{-3} M were added to the vial containing pyrene or nile red and equilibrate for overnight. The solutions were purged with nitrogen gas before photophysical experiments. In pyrene containing experiment, the excitation wavelength was set at 337 nm, the excitation slit at 3 nm, and the emission slit at 3 nm. The ratio of fluorescent intensity in emission spectra at 375 and 386 nm was calculated and plotted against logarithm concentration of polymer concentration. The CVC was estimated at a polymer concentration of onset of the increase in I_{375}/I_{386} ratio. In nile red experiment, excitation wavelength was set at 485 nm, the excitation slit at 2 nm, and the emission slit at 3 nm. The emission intensity at

wavelength of 626 nm was plotted versus concentration of polymer added and the deflection point was taken as critical vesicular concentration.

4.2.12. Encapsulation of hydrophilic Rhodamine-B and determination of loading efficiency: Water soluble rhodamine B was encapsulated into vesicles inner core by dialysis method. Briefly, 20 mg of DEX-IM-5 and 2 mg of rhodamine B were co-dissolved in 3 mL DMSO. This solution was stirred at 25 °C in a beaker and 3 mL of PBS (pH 7.4) solution was added drop wise into it. The resulting suspension was stirred for 12 h at 25 °C and extensively dialysed by taking in regenerated cellulose dialysis bag (SPECTRA/POR, MWCO 3,500). The dialysis was continued until rhodamine B leaching out was completely stopped from the dialysis bag. DEX-IM-5 vesicles loaded with rhodamine B was collected from the dialysis bag and diluted to 10 mL with PBS. The loaded rhodamine B was completely allowed to release by dissolving 100 µl of above stock solution in 2.9 mL DMSO solvent. The loading content of rhodamine B was calculated by measuring absorbance of above solution at 552 nm and substituting it in Beers law equation where molar absorption coefficient of rhodamine B were fixed as 1,15,000. The loading content of RhB was determined as 2.70 wt %.

4.2.13. Encapsulation of Doxorubicin.HCl and Drug loading Efficiency: Water soluble DNA intercalating Doxorubicin HCl (DOX.HCl) was loaded into the inner aqueous cavity of DEX-IM vesicles by dialysis method. Briefly, 100 mg of DEX-IM-5 and 2 mg of DOX.HCl were dissolved in 3 mL of DMSO and stirred moderately at 25 °C under dark condition. Self assembly of DEX-IM-5 was induced by slow injection of 3 mL of PBS (pH 7.4) into above solution. The mixed polymer drug solution was incubated at 25 °C under dark condition for 12h. To remove DMSO and unloaded DOX.HCl, the solution was transferred to a dialysis bag (SPECTRA/POR, MWCO 3500) and dialysed against PBS (0.01 M, pH 7.4) for 24 h with 6 times exchange of dialysis medium with fresh buffer solution. The whole procedure was carried out under dark conditions. The resulting DOX.HCl loaded DEX-IM vesicle solution was filtered through 0.45µm filters and lyophilized. The DOX.HCl loading content in vesicles was determined absorption spectroscopy method. In brief 3 mg of freeze dried DOX.HCl loaded DEX-IM powder was dissolved in 1.0 mL of DMSO and subsequently 100 µL of this solution was diluted to 3.0 mL with DMSO. The

absorbance at 480 nm was measured on UV-visible spectrophotometer and the amount DOX.HCl loaded in the vesicles was determined using the molar extinction coefficient of DOX as 11,500. Drug loading content (DLC) and drug loading efficiency (DLE) were calculated using following equations.

$$\text{DLC (\%)} = \left\{ \frac{\text{Weight of drug encapsulated in vesicles}}{\text{Weight of Drug loaded polymer}} \right\} \times 100 \% = 2.9 \text{ wt\%}$$

$$\text{DLE (\%)} = \left\{ \frac{\text{Weight of drug encapsulated in vesicles}}{\text{Weight of drug in Feed}} \right\} \times 100 \% = 58 \%$$

4.2.14. In-vitro Release studies: The drug elution from the vesicles was performed at pH 7.4 (physiological pH), pH 6.5 (tumor tissue pH), pH 6.0, pH 5.5 (endosomal pH) and pH 5.0 (lysosomal pH). Briefly, 3.0 mg of DEX-IM vesicles encapsulated with DOX.HCl or DOX conjugated vesicles in 3.0 mL buffer solution of required pH were placed in a dialysis tube (SPECTRA/POR, MWCO 8000). The tube carrying DOX loaded or conjugated DEX-IM vesicles was immersed in 100 mL buffer solutions having similar pH of reconstituted buffer taken in a beaker and whole solution was incubated at 37 °C. After suitable time interval (30 min or 60 min) 3.0 mL of dialysis medium was withdrawn and replaced with equal volume of fresh buffer. Absorbance of the each aliquot was determined at 480 nm using UV-Vis absorption spectrophotometry and amount of the DOX released were calculated using Beer's law, where molar absorption coefficient was kept as 11,500. Esterase assisted drug release was performed by adding 10 U esterase enzyme into the dialysis bag prior to the release studies.

4.2.15. Cell Viability Assay (MTT Assay): The cytotoxicity of blank DEX-IM, free DOX.HCl, DOX loaded DEX-IM vesicles was studied in WTMEFs, DLD1 and MCF-7 cells using the tetrazolium salt, 3-(4,5-dimethylthiazol-2-yl)-5-(3,4-diphenyl) tetrazolium bromide (MTT). The assay was performed using 0.5 µM DOX concentrations. 1×10^3 number of wild type mouse embryonic fibroblasts or 2×10^3 number of DLD1 or MCF-7 cells was seeded per well to fibronectin (2 µg/well) coated 96 well plate (Corning, USA) in 100 µl media and allowed to adhere for 24 hrs. DMEM with 10 % FBS (Fetal Bovine Serum) was used for MCF7 and WTMEFs cells and RPMI with 10 % FBS was used for DLD1 cells. Prior to drug treatment, medium from cells was aspirated and 25 µg DEX-IM, 0.5 µM free DOX, DEX-IM with encapsulated DOX

(final concentration 0.5 μ M DOX) were prepared in corresponding media and added accordingly. A blank control, DMEM with FBS in the absence of cells and an untreated control, cells with DMEM containing FBS, were used in each experiment. All control and treated experiment wells were in triplicate. Cells were incubated for 24 hrs without a change in medium and after 24hrs, drug containing medium was aspirated. Freshly prepared stock of MTT in sterile PBS (5 mg/mL) was diluted to 50 μ g/mL in 100 μ l DMEM with FBS and added to cells. Cells were then incubated with MTT for 4 hrs at 37 °C. Medium with MTT was then aspirated from wells and the purple formazan crystals formed as a result of reduction of MTT by mitochondrial dehydrogenase enzyme from cells were dissolved in 100 μ l of 100% DMSO (added per well). The absorbance from formazan crystals was immediately measured using microplate reader at 570 nm (Varioskan Flash) and is representative of number of viable cells per well.

Values from the triplicates for each control and treated set were noted and their means used for calculations. If one value from the triplicate had deviated substantially from the other two, it was not considered in the mean calculations. If the triplicate values were all variable the experiment was not used in the final calculations. The mean of the absorbance values for the blank control samples was subtracted from the average of the untreated control and treated samples respectively. The values thus obtained for the untreated control samples were equated to 100 % and relative percentage values for DEX-IM, free DOX.HCl, and DOX physically loaded and chemically conjugated to DEX-IM calculated accordingly. Percentage values thus obtained for all above four cases were subtracted from the untreated control (100%) to determine the percentage loss in cell numbers (relative to control). Results thus obtained from 3 experiments were compiled and differences between DOX loaded and conjugated statistically evaluated using a paired two tail T-Test.

4.2.16. Cellular Uptake of DOX by Confocal Microscopy : Wild type mouse embryonic fibroblasts (WT MEFs) were seeded at a density of 5×10^4 cells on flame dried cover slips placed in 6 well plates containing DMEM medium with 10%FBS and incubated at 37°C for 18 hours. The cells were then exposed to required concentration of Free DOX, DOX loaded DEX-IM vesicles and DOX conjugated DEX-IM-5 vesicles. (dissolved in PBS) for 4 h in a CO₂ incubator at 37°C. After incubation, drug-containing medium was aspirated from each well, cells washed twice with PBS (1mL X 2) and fixed with 3.5% paraformaldehyde solution in PBS for

15min at room temperature. The cells were washed twice with PBS (1 mL) and stained with phalloidin conjugated to Alexa 594 (Invitrogen) diluted 1:500 in 3% BSA solution in PBS. After 1 hour incubation, at room temperature in the dark the excess dye was washed from the plate and cells were again gently rinsed with PBS. These cover slips were then incubated with DAPI (0.05ug/ml) for 2 min each to stain the nucleus. The cover slips were mounted on slides using fluoromount mounting medium (Southern Biotech) and dried overnight at room temperature in the dark. The cells were imaged using a LSM710 confocal microscope using the λ 488 nm (green channel) and λ 568 nm (red channel) lasers.

4.3. Results and Discussion

4.3.1. Synthesis of pH and Enzyme responsive Dextran Amphiphiles

The synthesis of modified dextran is shown in Figure 4.5. 4-Hydroxybenzaldehyde was reacted with tert-butyl bromoacetate to produce (1). The compound (1) was hydrolyzed and the resultant carboxylic acid derivative (2) was coupled with dextran using DCC/DMAP to get aldehyde substituted dextran (DEX-CHO-x). Renewable resource 3-pentadecyl phenol (PDP) was reacted with Boc protected 2-ethanol amine (3) to make amine functionalized PDP (5). The coupling of aldehyde group in DEX-CHO-x with PDP-amine (5) produced pH responsive imine containing dextran derivative DEX-IM-x (see figure 4.5). All the intermediates and polymer derivatives were characterized by NMR and FT-IR spectroscopy.

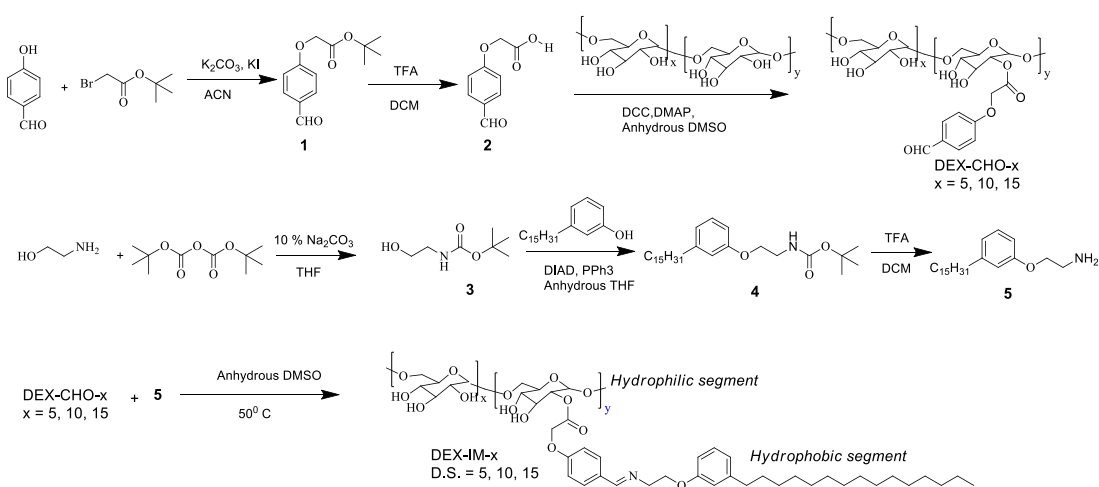


Figure 4.5. Synthesis of pH and Enzyme Responsive dextran derivatives.

The ^1H -NMR spectrum of the DEX-CHO-x (see Figure-4.6a) exhibited peaks at 9.82, 7.82 to 7.08 ppm for the aldehyde and aryl protons, respectively. The peaks from 3.00 to 5.00 ppm correspond to dextran protons. The protons of ester linkage, $\text{Ar-OCH}_2\text{-COO-DEX}$, appeared at 4.64 ppm, which was merged with the anomeric protons of dextran (see Figure 4.6a). The coupling of PDP amine with DEX-CHO-x also confirmed using ^1H NMR analysis. The appearance of HC=N protons at 8.2 ppm (see proton c in figure 4.6b) and disappearance of aldehyde proton at 9.82 ppm (see proton c in figure 4.6a) evident for the formation imine linkage in DEX-IM-x (see figure 4.6b).

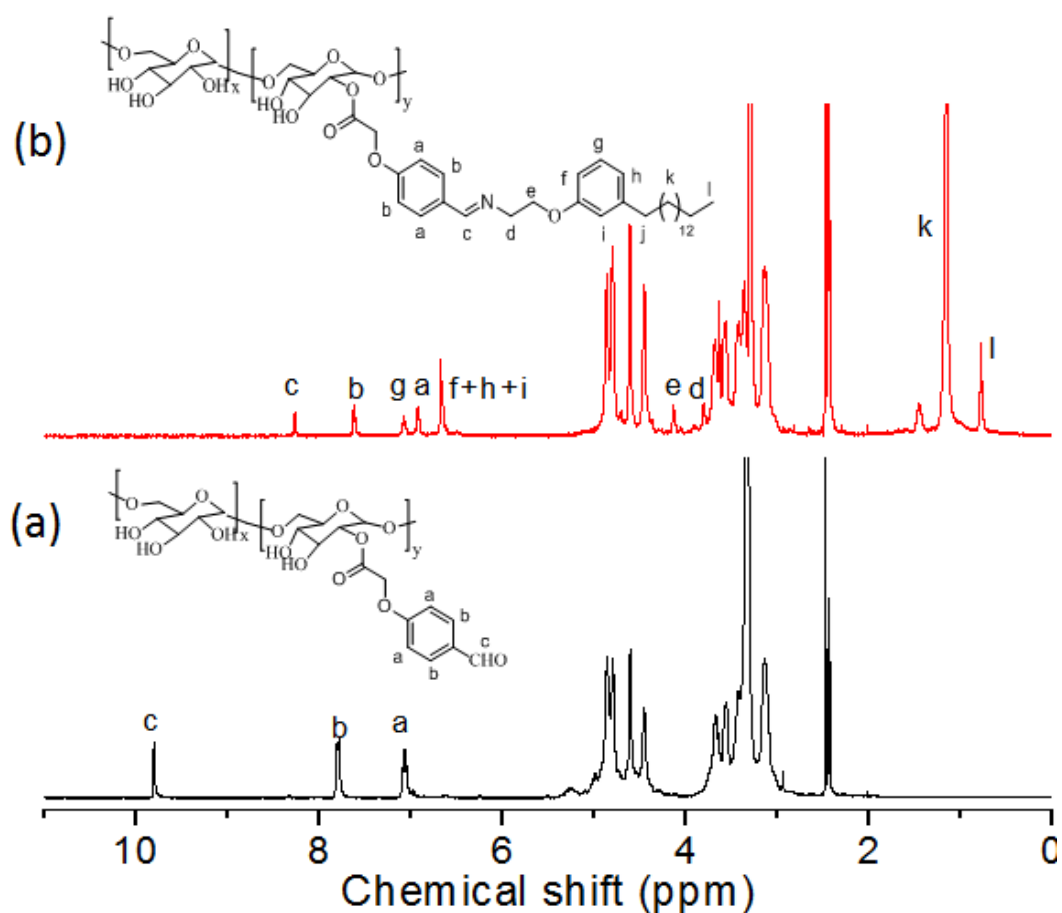


Figure 4.6. ^1H NMR spectra of (a) DEX-CHO-x and (b) DEX-IM-x in d_6 DMSO.

^{13}C -NMR spectrum of DEX-CHO showed a peak at 65.11 ppm and 168.41 ppm corresponds to ester carbon atom, $\text{Ar-OCH}_2\text{-COO}$ and $\text{Ar-OCH}_2\text{-C=O}$ respectively as expected in the formation of coupled product (See figure 4.7). All the protons and carbons in the structure of DEX-CHO-X molecule were assigned using 2D NMR-HSQC technique (See figure 4.8).

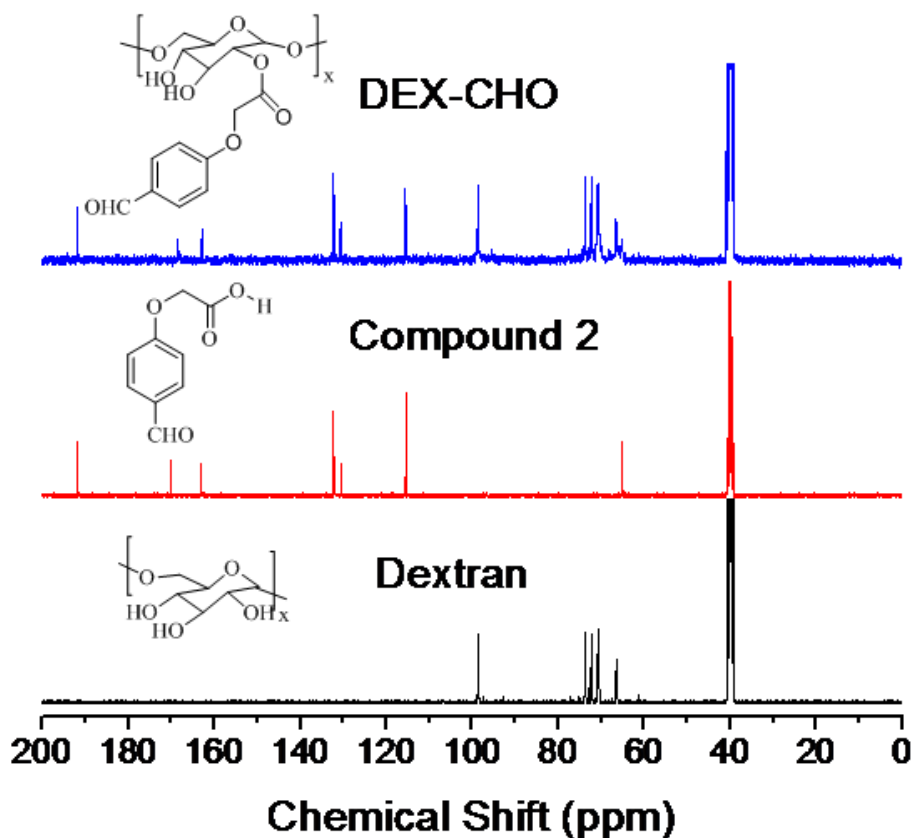


Figure 4.7. ^{13}C NMR spectra of Dextran, Compound 2 and DEX-CHO in DMSO (d_6)

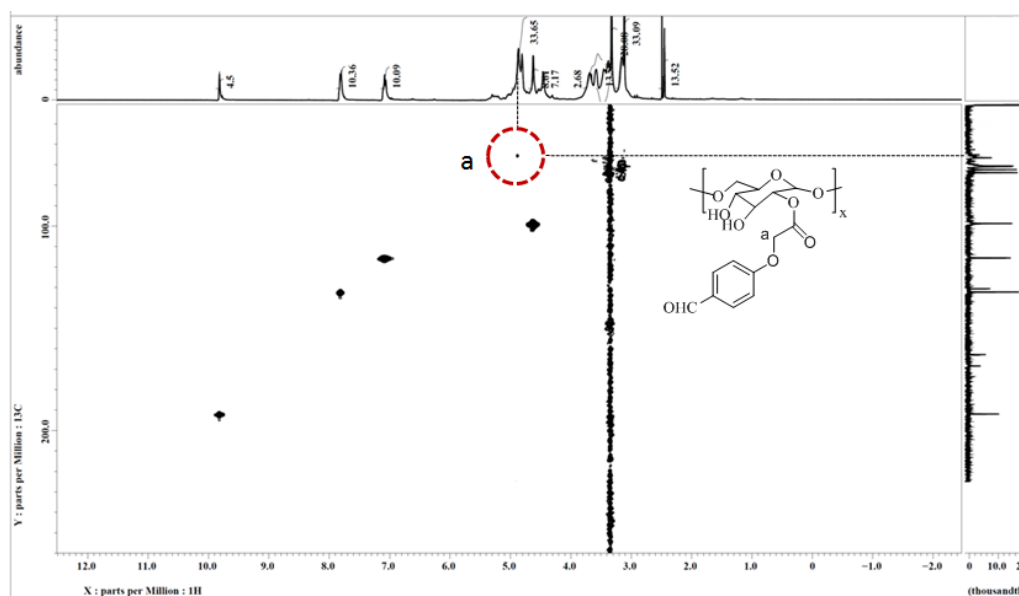


Figure 4.8. 2-D NMR-HSQC spectrum of DEX-CHO in DMSO (d_6)

The degree of substitution (DS) on dextran back bone was calculated by comparing the peak intensities of anomeric proton in dextran at 4.62 ppm with the aryl protons at 7.82 ppm.^{26,28} DS on dextran was obtained as 5.2, 10.1 and 14.7 % (for

simplicity 5, 10 and 15) for feed ratio of 0.5, 1.0 and 1.5 equivalents of 4-formyl phenyl acetic acid. DEX-IM-x with different DS was synthesized by the reaction between DEX-CHO-x and PDP amine.

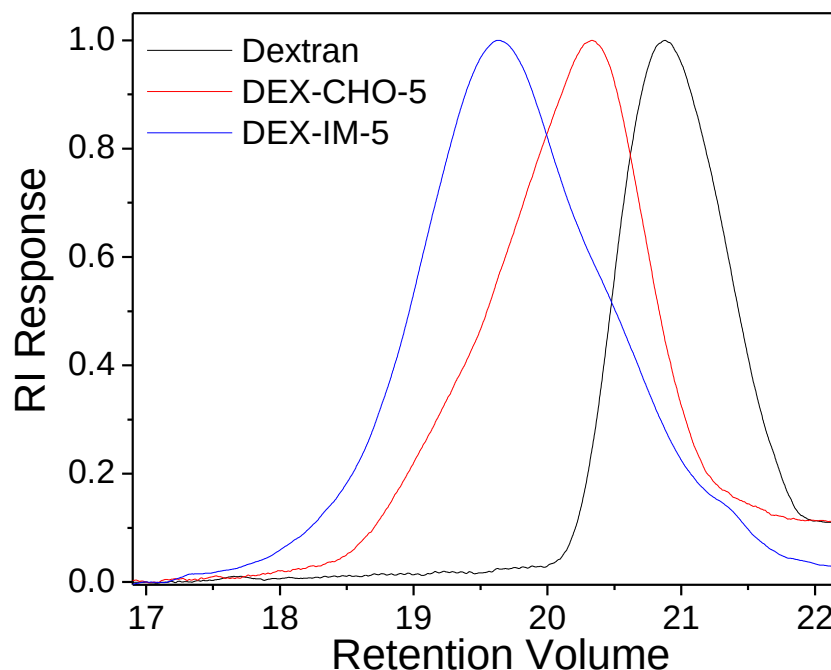


Figure 4.9. GPC chromatograms of dextran, DEX-CHO-5, and DEX-IM-5 collected using RI detector

The GPC chromatograms of the dextran, DEX-CHO-5 and DEX-IM-5 recorded using RI detector are shown in figure 4.9. All the peaks showed mono-modal distribution with respect to the homogeneous grafting on the dextran backbone. As expected, the molecular weight of the polymers increased in the sequence Dextran < DEX-CHO < DEX-IM with increase in the grafting content in the dextran backbone (see MW in table 4.1).

Table 4.1. Table depicting molecular weight of Dextran derivatives determined using GPC in DMF solvents with polystyrene standard.

Type of Polymer	Mn (g/mol)	Mw (g/mol)	PDI
Dextran	16,800	18,000	1.07
DEX-CHO-5	22,600	26,000	1.15
DEX-IM-5	38,300	50,200	1.31

FT-IR spectra of the polymers are given in figure 4.10. FT-IR spectrum of 4-formyl phenyl acetic acid showed distinct stretching band at 1714 cm^{-1} with respect to -C=O stretching frequency. Upon the formation of ester linkage with dextran -C=O stretching frequency shifted to higher wave number at 1750 cm^{-1} . The free hydroxyl groups in the dextran were also showed broad peaks at 3400 cm^{-1} . The appearance of distinct band at 1597 cm^{-1} corresponds to benzoic imine which further validates the formation of pH responsive imine linkage (See figure 4.10).

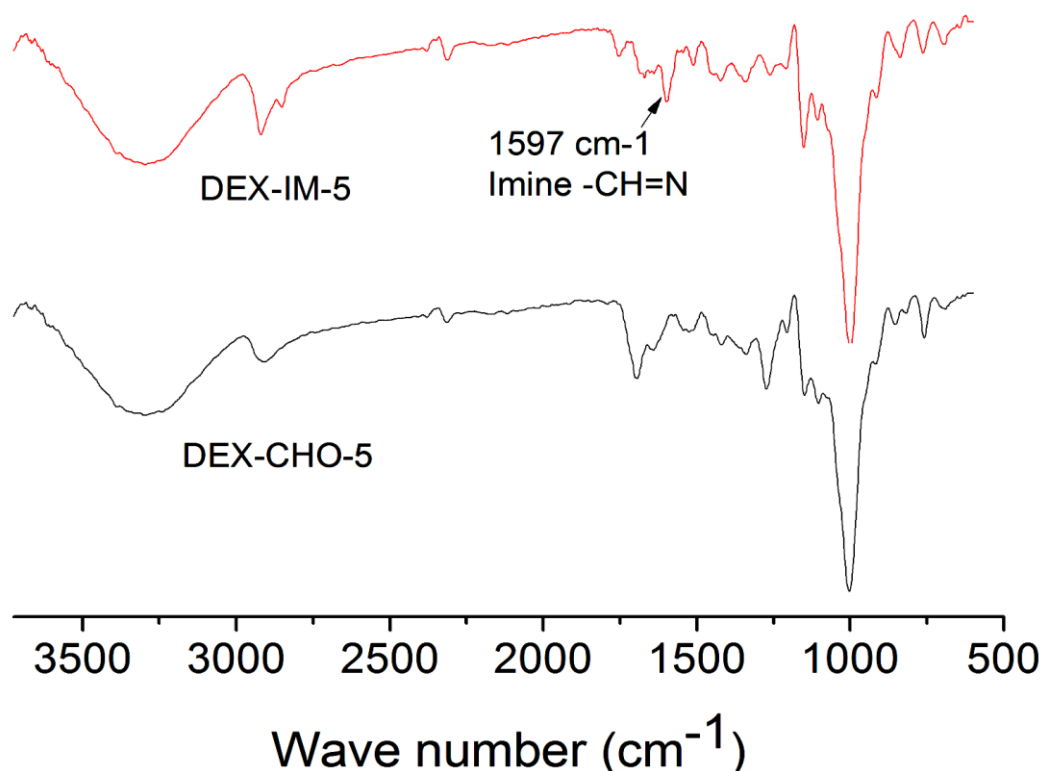


Figure 4.10. FTIR spectra of DEX-CHO-5 and DEX-IM-5

4.3.2. Self-assembly of Dual Responsive Dextran Amphiphiles

The newly synthesized pH responsive dextran derivatives (DEX-IM-5) were self-organized in PBS via solvent exchange method in which the polymer dissolved in DMSO followed by dialysis in PBS for 48h. The resultant DEX-IM-5 solution in PBS was subjected to dynamic light scattering (DLS) analysis. The DLS histograms showed mono-model distribution (see figure 4.11a for histogram and autocorrelation) with hydrodynamic diameter (D_h) $220\text{ nm} \pm 10\text{ nm}$. From this, the hydrodynamic radius ($D_h/2 = R_h$) of the polymer assembly was calculated as 110 nm . Static light scattering (SLS) analysis of this polymer assembly provided its radius of gyration

(R_g). The slope of the plot of square of scattering vector magnitude (q^2) (guinier plot, see in-set in figure 4.11a) provided the $R_g = 110$ nm. The ratio of $R_g/R_h=1$ revealed that DEX-IM-5 polymer assemblies corresponding to vesicular structures.²⁸⁻³⁰

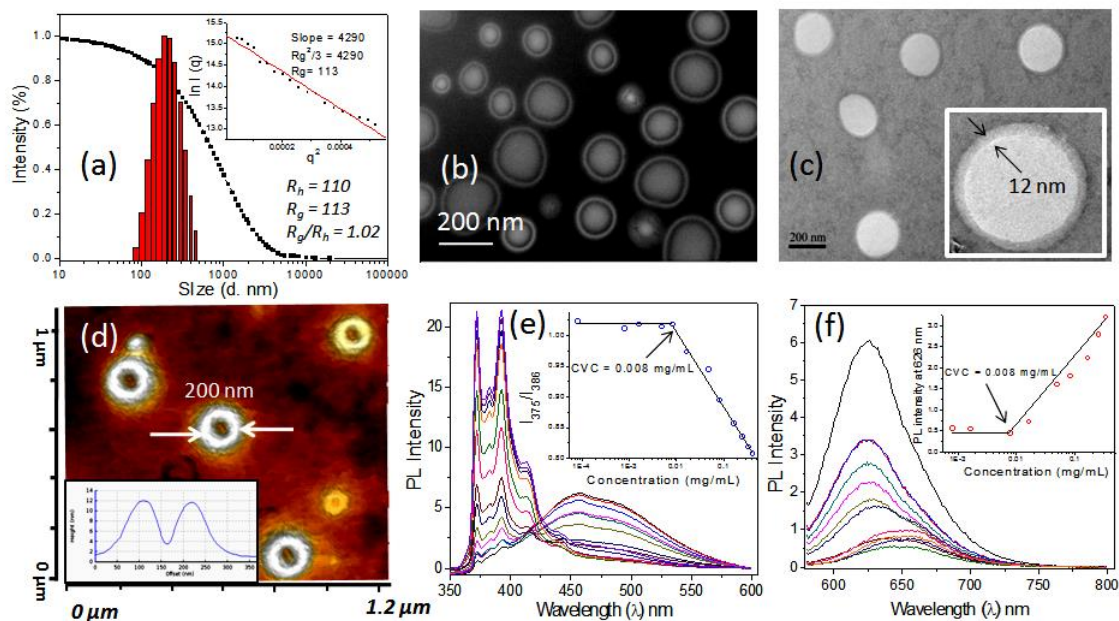


Figure 4.11. DLS histogram and SLS plot (inset) (a), FE-SEM (b), TEM image [collected without staining] (c) and AFM image (d) of DEX-IM-5. Inset in figure (c) showed the magnified vesicular structures. Cross sectional analysis of AFM were depicted as inset in (d). Emission spectra of pyrene (e) and Nile red (f) at different DEX-IM-5 concentrations. I_1/I_3 of pyrene vs the concentration of polymer and emission intensity at 626 nm vs the concentration of polymer are shown as inset in (e) and (f), respectively

Electron microscopes were used to visualize the shape and size of the DEX-IM-5 self-assemblies. The FESEM image of DEX-IM-5 polymer showed the formations of spherical objects with distinct hydrophobic layer and inner cavity with respect to the vesicular assemblies (see figure 4.11b). The average diameter of these vesicles was calculated using image J software as 200 ± 30 nm (see figure 4.11b). HR-TEM images of the DEX-IM-5 aggregates were (see figure 4.11c) further evident for the existence of spherical vesicular morphology with a well defined hydrophobic periphery. The average size of the vesicles obtained for HR-TEM images as 220 ± 25 nm which was in accordance with FE-SEM analysis. The thickness of the hydrophobic membrane was calculated for individual vesicles as 12 ± 3 nm. Atomic force microscope (AFM) analysis in the tapping mode was employed for the DEX-IM-5 sample on the mica surface. A representative AFM phase image (see in figure

4.11d) proved that the vesicles were collapsed on mica surface and as a result differential response were provided to the force of the AFM tip from the soft interior and hard peripheral region of vesicular structures. AFM image of the vesicles were observed as hollow cavity inside which was further supported by the cross sectional analysis (see Figure 4.11d, inset).^{31,32} The diameter of the vesicles observed in AFM was matched with that obtained in FE-SEM and TEM analysis. Thus the microscope images prove that the vesicular assemblies in the sample DEX-IM-5.

The critical vesicular concentration (CVCs)) of DEX-IM-x was determined using pyrene^{33,34} and Nile red³⁵ as fluorophores. For this purpose, the concentration of pyrene was fixed as 0.6 μM and Nile red 1.0 μM and the amount of DEX-IM-5 was varied from 0.5 mg/mL to 8.3×10^{-4} mg/mL (see figure 4.11e and 4.11f). In case of pyrene, the ratio (I_1/I_3) of intensities of first emission peak (375 nm, I_1) to the third emission peak (386 nm, I_3) was compared to estimate the CVC (see figure 4.11e). The encapsulation of Nile red in the hydrophobic cavity enhances the emission intensity (at 626 nm) and this parameter was used for CVC determination (see figure 4.11f). Based on this CVC of DEX-IM-5 were calculated as 0.008 mg/mL in both the experiments. Similarly CVCs of DEX-IM-10 was determined 0.016 mg/mL (see figure 4.12) which was almost double as that of DEX-IM-5. The lack of complete solubility of DEX-IM-15 in water restricted its CVCs determination. Comparatively, DEX-IM-5 was found to easily dispersible and produces stable vesicles (CVC with less amount of polymer) in water; Thus, DEX-IM-5 was chosen for loading and delivering of anticancer drug molecules in the vesicular scaffold.

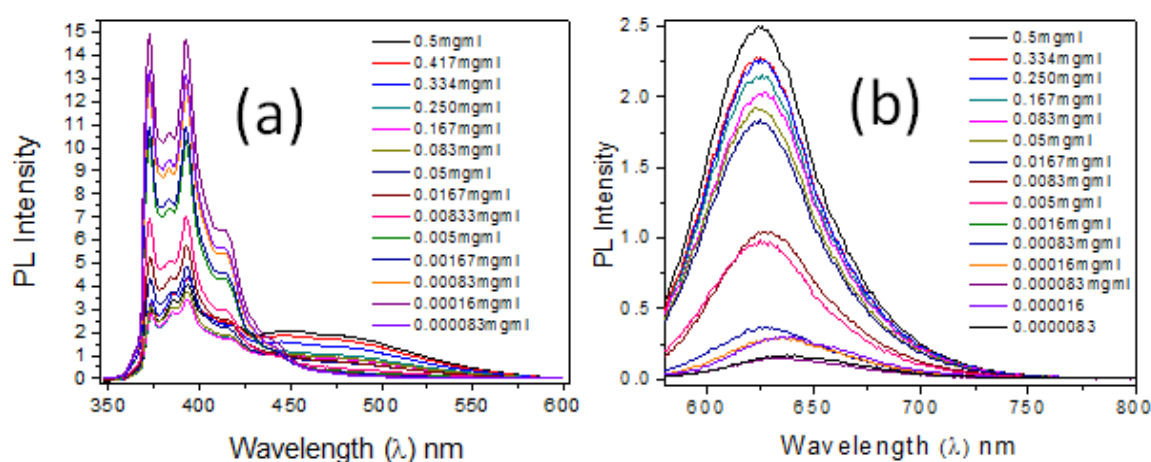


Figure 4.12. Pyrene (a) and Nile red (b) emission spectra at different concentration of DEX-IM-10 polymer.

4.3.3. Loading Capabilities of Dual Responsive Dextran Vesicles

The loading capability of hydrophilic molecules in the inner cavity of the dextran vesicle was tested using water soluble dye or drug molecules. Water soluble rhodamine B (RhB) was selectively encapsulated in the hydrophilic corona of the vesicles by dialysis method (See figure 4.13a). The dye and polymer (DEX-IM-5) co-dissolved in DMSO and dialyzed in PBS using semi-permeable dialysis membrane. The un-encapsulated RhB was removed by continuous replacement of fresh PBS and the dialysis was continued for 3-7 days. The dialyzed sample was highly fluorescent and their photographs are shown in figure 4.13a. The DLS histograms of RhB loaded vesicles (see figure 4.13b) revealed that hydrodynamic diameter of the vesicles was 220 nm which was almost similar to the unloaded vesicles (See figure 4.11a). FE-SEM analysis of RhB loaded DEX-IM-5 (see figure 4.13c) showed the existence of spherical morphology with distinct hydrophobic layer with respect to the vesicular geometry. A similar encapsulation procedure was followed to load water soluble anticancer drug DOX.HCl (Topoisomerase II inhibitor) ³⁶ in its hydrophilic cavity. The polymer to drug ratio was maintained as 5 wt% and drug loading content in the vesicles were estimated as 2.9 wt% by using absorption spectroscopy. Thus drug loading efficiency was calculated as 58 %. These values are almost close to that of stearic acid attached dextran amphiphiles reported earlier.²⁷

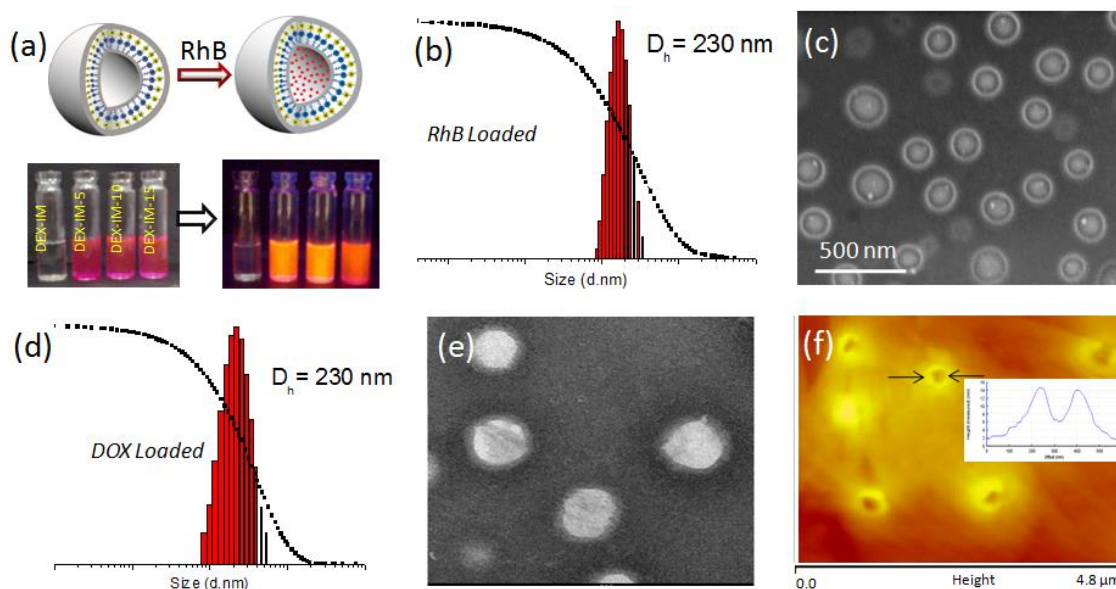


Figure 4.13. Schematic representation of rhodamine B (RhB) encapsulation to vesicle and RhB loaded vesicle solution (a), DLS histogram of RhB Loaded DEX-IM Vesicles (b), FE-SEM Image of RhB loaded vesicles (c), DLS histogram (d), HR-TEM image (e) and AFM image (f) of DOX loaded DEX-IM vesicles. Inset graph in (f) represents cross sectional analysis of vesicle in AFM image.

The morphology of DOX loaded DEX-IM-5 vesicles were characterized by both light scattering and electron microscopic methods. In figure 4.13d, the DLS histograms revealed the size diameters of DOX loaded vesicles as 230 nm which was almost similar to nascent vesicles (see Figure 4.11a) or RhB loaded vesicles (4.13b). FE-SEM analysis of DOX loaded DEX-IM-5 also proved the existence of vesicular geometry with an average diameter of 200 ± 5 nm. In figure 4.13e, the HR-TEM image confirmed the retention of spherical vesicular structure in DOX loaded DEX-IM vesicles. The average diameter of vesicles was 210 ± 10 nm which was comparable to DLS result (see figure 4.13d). The AFM analysis of the DOX loaded DEX-IM were performed in tapping mode and the result obtained substantiated the vesicle geometry with an average diameter of 200 ± 5 nm and height of 13 ± 3 nm (see figure 4.13f and cross sectional analysis in the inset). Thus, the above analysis proved that the loading of the DOX.HCl did not alter the morphology of the nascent dextran polymer and the RhB and DOX loaded samples were found to retain the vesicular structure.

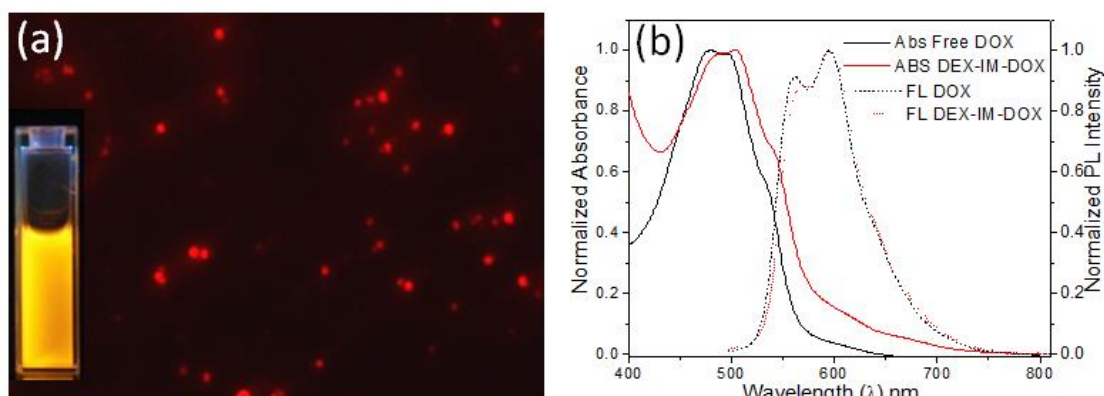


Figure 4.14. Fluorescence microscopic image of DOX loaded DEX-IM vesicle (a). Absorbance and fluorescence plot of free DOX and DOX loaded DEX-IM vesicles (b) Inset in (a) shows DOX loaded vesicle solution under hand held UV lamp.

Since DOX is a fluorescent drug, the DOX loaded vesicles were subjected to photophysical characterization as well as fluorescent microscopic imaging. FL images captured under red channel proved the existence of intact luminescent spherical structures for DOX loaded systems (See Figure 4.14a). The photographs of the vial containing the DOX loaded vesicles showed orange emission followed by the photo-excitation. Absorbance spectra of the free DOX and DOX loaded vesicles displayed almost identical λ_{max} at 480 to 484 nm (see figure 4.14b). Interestingly DOX loaded assemblies showed identical emission spectra as that of free DOX. This proves that the DOX molecule was very well preserved inside the dextran vesicles.

4.3.4. pH and Enzyme Responsive *In Vitro* Drug Release Studies

The benzylic imine linkage connecting dextran back bone with PDP unit is sensitive to acidic pH conditions. Thus, in acidic conditions, (pH 5.0 to 6.5) the imine linkage cleave into DEX-CHO-5 and PDP amine as shown in figure 4.15a. This would also expect to accompany the change in the morphology of the vesicular structure. DEX-IM-5 in d_6 -DMSO was subjected to ^1H NMR analysis after stirring with a drop of trifluoroacetic acid and the spectra is shown in figure 4.15b. DEX-IM-5 showed the imine proton $\text{HC}=\text{N}$ at 8.33 ppm which was disappeared in the TFA treated sample. A new peak at 9.90 ppm appeared in the hydrolyzed sample with respect to the regeneration of the $-\text{CHO}$ functional groups as similar to DEX-CHO-5 (see figure 4.15b).

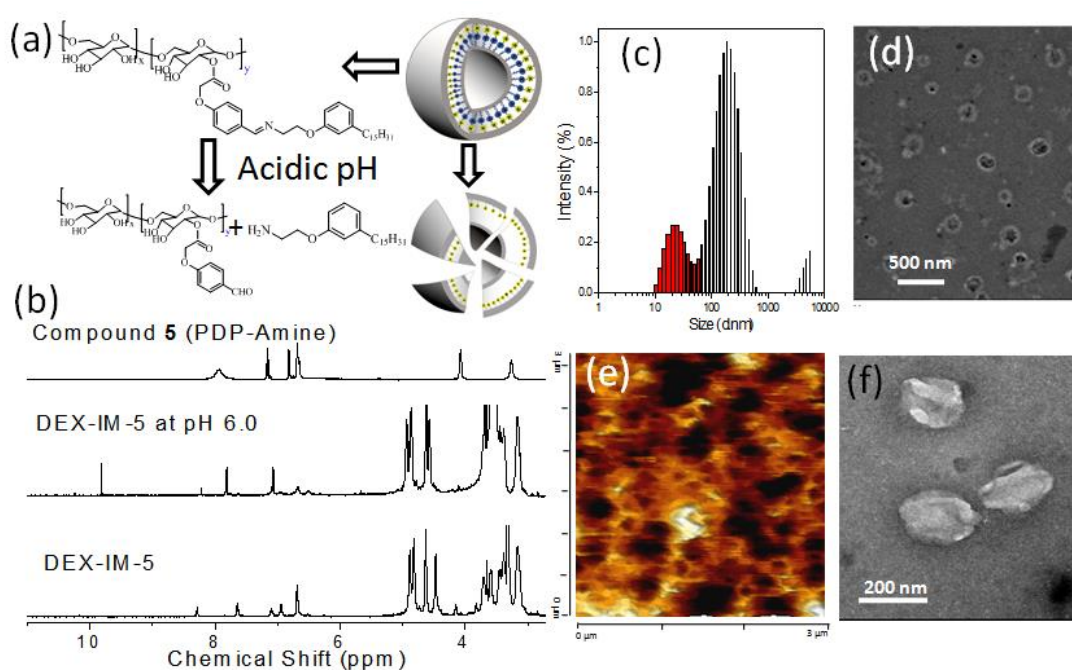


Figure 4.15. Cleavage of DEX-IM-5 under acidic condition (a). (b) NMR spectra, (c) DLS histogram, (d) FE-SEM image, (e) AFM image, and (f) TEM image of DEX-IM-5 at acidic pH (6.0) conditions.

Further, the protons in the $\text{PDP}-\text{OCH}_2\text{CH}_2\text{N}=\text{CH}-\text{Ar}-\text{dextran}$ was disappeared in the hydrolyzed sample and protons corresponding to $\text{PDP}-\text{OCH}_2\text{CH}_2\text{NH}_2$ (with respect to compound 5) were appeared at 4.09 ppm. This control experiment confirmed the cleavage of the imine under the acidic conditions. DLS histograms of the DEX-IM-5 were recorded in pH 7.4 and pH 6.0 at the different time interval. Interestingly, at pH 7.4, DEX-IM-5 retained the mono-modal distribution for a long duration (checked up to 7 days, see figure 4.16). This clearly proves the stability of

DEX-IM-5 vesicles at physiological pH conditions. On the contrary, at acidic pH conditions (pH 6.0) the uni-modal distribution of DEX-IM-5 was lost and it showed multi-modal distribution with respect to the existence of non-homogeneous aggregates. This suggests that DEX-IM-5 is prone to get cleaved under acidic conditions. With increase in time, the distribution of aggregates became more boarded and the sizes varied from 20 nm to 1.0 μm (see figure 4.15c). The pH responsive cleavage of the DEX-IM-5 was further investigated in pH = 6.0 by FE-SEM, HR-TEM and AFM (see figure 4.15d, 4.15e and 4.15f). FE-SEM image showed in figure 4.15d pointed out that the vesicles got disorganized at acidic pH unlike in the case of pH=7.4 (see image 4.11b). A similar observation was made in TEM image (See figure 4.11c and 4.15e) and AFM analysis (See figure 4.11d and 4.15f). Thus newly designed DEX-IM-5 polymer forming stable vesicular structures at physiological pH = 7.4 (see images in figure 4.11) and got cleaved in acidic pH to corresponding DEX-CHO and PDP amine unit (see images in figure 4.15).

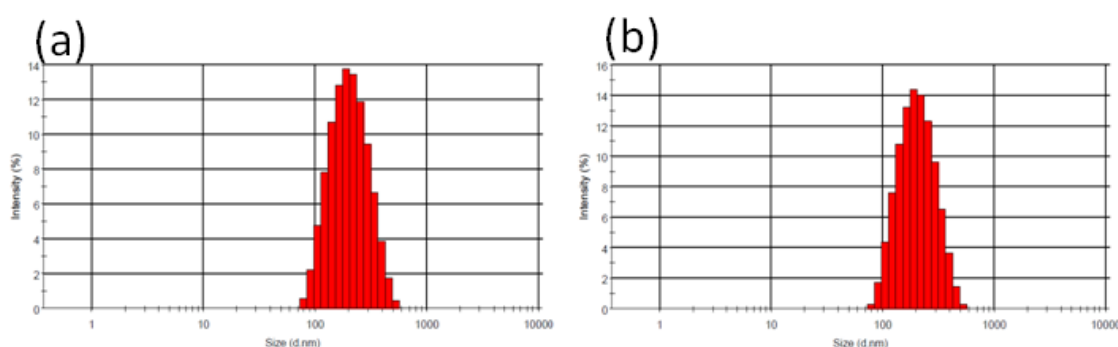


Figure 4.16. DLS histogram of DEX-IM-5 at pH 7.4 day 1 (a) and day 2 (b).

Vaupel *et al.* and others have reported that the pH of mammary carcinoma was 6.7 and in some micro areas it was dropped to about pH = 5.4.^{37, 38} So the *in-vitro* drug release studies for both DOX loaded DEX-IM-5 vesicles were performed at various pH conditions ranging from pH 7.4 to 5.0 by dialysis method. *In vitro* release studies were done in PBS buffer or PB buffer using dialysis method and the amount of the DOX released was quantified by absorbance spectroscopy up to 48 hrs. The cumulative release of DOX using pH and esterase as stimuli are shown in figures 4.17a and 4.17b. At pH = 7.4 (normal tissue pH), the vesicles underwent natural leakage to release about 25 ± 5 % of the drugs. A similar trend was observed at pH 6.5 (30 ± 5 % drug release) which is similar to the cancer tissue pH. Thus, minimum 20-30 % drug leakage was unavoidable from the vesicles under physiological pH

conditions. The release was observed to reach 80 % at pH 5.5 and close to 95 % at pH 5.0. In order to investigate the effect of esterase enzyme that cleaves the aliphatic ester linkage connecting the hydrophobic segment, two control experiments are carried out: (i) esterase stimuli at pH = 7.4 and (ii) esterase stimuli at pH = 6.0 and 5.0. The first conditions provides insight into the action of esterase enzyme alone (abundant in the lysosomes)³⁹ and whereas both pH and esterase act together in the second experiment (dual stimuli). The result showed that the presence of esterase enzyme (10 U enzymes) enhanced the DOX release in all pH conditions. At pH = 7.4, the release of the DOX was increased from 25 % (absence of enzyme, see figure 4.17a) to 70 % (in the presence of enzyme, see figure 4.17b). At pH= 6.0 and pH = 5.5, the esterase enzyme cleaved the vesicles completely and discharged the DOX from 30 % at pH 7.4 to 100 % (see Figure 4.17b).

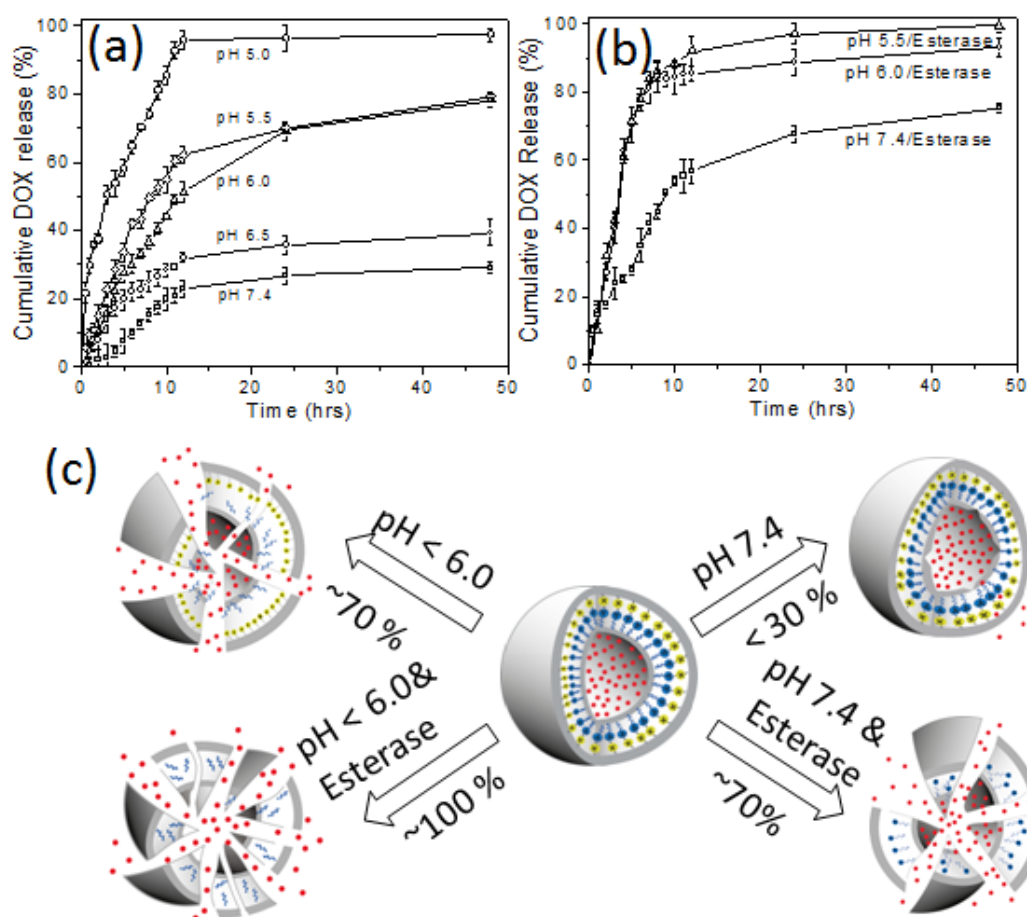


Figure 4.17. In-vitro DOX release profile from DOX physically loaded DEX-IM-5 at various pH (a), in the presence of esterase and various pH (b), Schematic representation of DOX release at various conditions (c).

To further validate the stability of the DOX loaded dextran vesicles at the extracellular conditions, it was subjected to release studies in fetal bovine serum (FBS) and the data are summarized in (annexure figure 4.1) at 37 °C. The DOX loaded vesicles were found to be more stable in FBS and only 18-21 % leaching of the drug was noticed at 37 °C. Further, the DOX loaded dextran vesicles were also subjected to DLS analysis in FBS (also FBS+PBS) for 48 h to analyze their stability (see annexure figure 4.2). The sizes of the nano-vesicles were retained as 220 nm for the period of 48 h without any change. These control experiments revealed that the dextran nano-vesicles are very good in stabilizing DOX in the internal cavity and selectively ruptured in the presence of esterase enzyme at low pH as similar to that of intracellular environment as shown in figure 4.4a. To further support the above trend, the rate constant (k) for the drug release was estimated using first order rate equation.⁴⁰ $\ln(A_t/A_0) = -kt$, where A_t and A_0 are corresponding to drug in the vesicles at time 't' and initial, respectively. The rate constants are given in the table 4.2. At pH=7.4 and pH=6.5 for DOX loaded vesicles showed the rate constants 6.5 to 8.4 μs^{-1} which was almost half compared to that of the DOX conjugated vesicles (2.4 to 4.0 μs^{-1}). At endosomal pH =6.0 to 5.5, the rate constants increased three folds (16.5 to 22.6 μs^{-1}). At very low pH = 5.0, the drug release rate was almost 8 times when compared with circulatory pH =7.4. In the presence of esterase, the rate constants were obtained higher with respect to dual action of pH and enzyme.

Table-4.2: Rate constant of DOX release from DEX-IM loaded vesicles at various conditions

Condition Employed	pH 7.4	pH 6.5	pH 6.0	pH 5.5	pH 5.0	pH 7.4 and Esterase	pH 6.0 and Esterase	pH 5.5 and Esterase
$k(\mu\text{s}^{-1})$ DOX loaded vesicle	6.5	8.4	16.5	22.6	59.5	19.1	58.8	63.0

The above *in vitro* studies proved that both DOX loaded vesicles preserved significant amount of drug under circulatory pH conditions and extracellular pH conditions, but released substantially under *in-vivo* acidic tumor conditions resembling those seen in some tumors.^{15, 37, 38} As a result this newly developed dextran vesicles were considered as potential candidates for intracellular delivery of anticancer drugs. Based on the above data, the schematic presentations for the drug release pattern for DOX loaded DEX-IM-5 vesicle and are provided in figure 4.17c.

Here the data shows that about 30 % of drug molecules leached out from the vesicles under normal physiological conditions. The drug leaked from vesicles before reaching into target site may cause unwanted side effect. Earlier literature reports proved that DOX chemically conjugated on the polymer backbone may reduce leaching from the self assembled carrier.¹⁸⁻²³ Hence efforts were put to synthesize pH responsive DOX chemically conjugated dextran amphiphiles for delivery to cancer cells.

4.3.5. Synthesis of pH responsive DOX Conjugated Dextran Amphiphiles

The free amine group in DOX and PDP amine (5) was simultaneously reacted with DEX-CHO-x to produce drug-conjugated dextran polymer DEX-IM-DOX. The synthetic scheme is schematically represented figure 4.18).

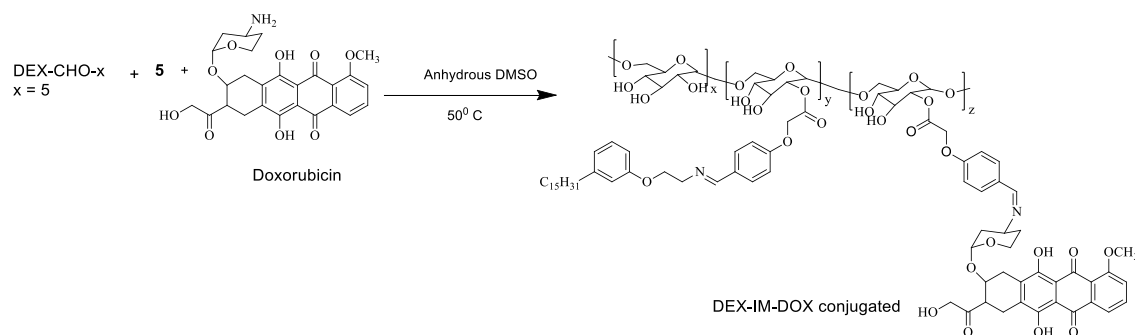


Figure 4.18. Synthesis of DOX conjugated dual responsive dextran derivative.

¹H-NMR spectra of the drug-conjugated polymer DEX-IM-DOX (see annexure figure 4.3) showed peaks with respect to the presence of DOX; however, the signals are very noisy for their estimation. In order to determine the amount of the DOX drug conjugated in the DEX-IM-DOX, solution absorbance spectra of drug-conjugated polymer was recorded in DMSO (see figure 4.19a). Based on the equation $A = \epsilon Cl$, the amount of DOX conjugated on the dextran backbone was calculated as 29.20 $\mu\text{g}/\text{mg}$ of polymer.⁴¹

The GPC chromatograms of DEX-IM-DOX recorded using UV-Vis detector (with λ of detection at 480 nm) clearly showed the mono-modal distribution for the DOX drug conjugated polymer (see figure 4.19b). On the other hand, the polymer DEX-PDP-5 (nascent polymer) did not show any signal in the UV-Vis detector (see figure 4.19b). This control GPC experiment has clearly evident for the drug conjugation on the dextran backbone.

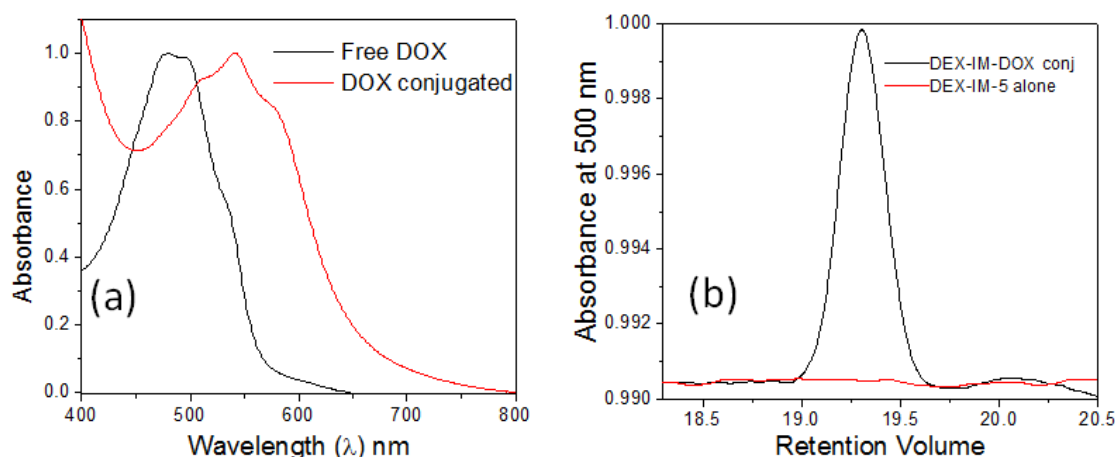


Figure 4.19. (a) Absorbance Spectra of free DOX and DOX conjugated DEX-IM-5. (b) GPC chromatograms of DEX-IM-5 and DEX-IM DOX conjugated using UV absorbance detector, detection $\lambda = 480$ nm

4.3.6. Self Assembly of DOX Conjugated Dextran Amphiphiles

DOX chemically conjugated on the dextran backbone was dissolved in DMSO solvent. PBS (pH 7.4) was added dropwise with constant stirring into above solution and the resulting suspension was dialyzed against PBS (pH 7.4) for 48 hours. The dialysed solution was subjected to DLS analysis and the histograms showed mono-model distribution indicating their uniform self-organization (see figure 4.20a). The hydrodynamic diameter of the assembly was obtained as 240 ± 5 nm (see figure 4.20a) which was almost comparable to the DOX loaded in DEX-IM-5 or nascent polymer assemblies (see figure 4.13d and 4.11a).

FE-SEM image of DOX conjugated sample appeared as spherical vesicular structure and it was further confirmed by the HR-TEM image as well (see figure 4.20b and in-set). Spherical structure and well defined hydrophobic membrane observed in HR-TEM image evident for the vesicular geometry. Fluorescent microscopic images collected under red channel proved the existence of intact luminescent spherical structures for DOX conjugated systems (figure 4.20c). The photographs of the vial containing the DOX conjugated showed matching emission as that of DOX physically loaded vesicle solution followed by the photo-excitation (see inset in figure 4.20c). Interestingly, both DOX loaded and DOX conjugated assemblies showed identical emission spectra (see figure 4.20d). On the other hand, the DOX conjugated dextran showed 30 nm red-shifted with respect to the chemical conjugation (see figure 4.19a).

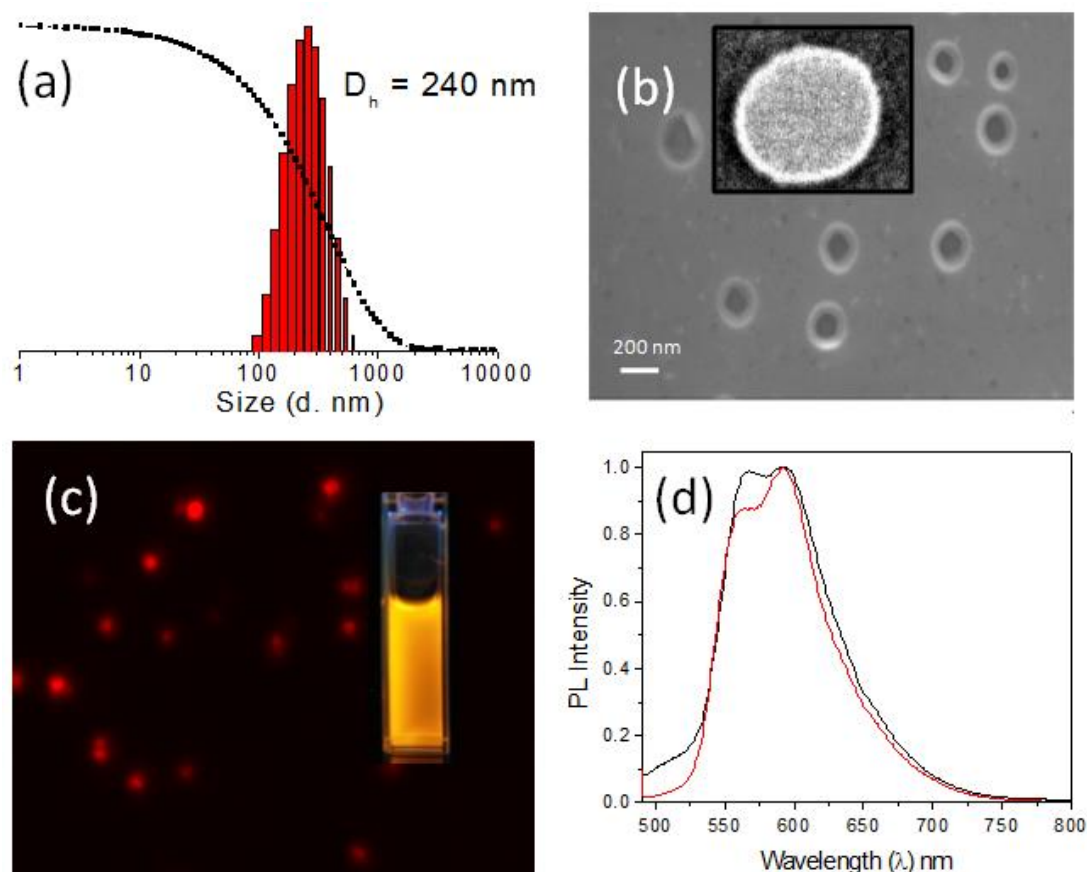


Figure 4.20. DLS histogram (a), FE SEM image and HR-TEM image (inset) (b), fluorescent microscopic image (c) and fluorescence plot (d) of DOX conjugated DEX-IM vesicles. Inset in (c) shows DOX conjugated vesicle solution under hand held UV lamp

Earlier studies showed change in the nano-carrier morphology for the polymers after anchoring of the drug on the backbone;^{24,25} however, the long renewable PDP unit in the present case behaved as structure directing hydrophobic segment to retain the self-organization and the resultant morphology. This unique behavior of PDP is attributed to the ability of the pendadecyl chain to inter-digitize to produce stable vesicular morphologies. Hence, it may be concluded both the nascent polymer DEX-IM-5, its DOX loaded form and as well its drug conjugated form retained the identical vesicular morphology with almost similar sizes. This provide new opportunity to study the drug releasing pattern as well their cellular uptake for both DOX loaded as well as DOX conjugated polysaccharide nano-carriers of identical morphology. Similar experiments were carried out to study the drug release kinetics of DOX conjugated dextran vesicles (see figure 4.21a and 4.21b). The conjugated drug showed very less natural leaching (< 10 %) under normal circulatory

conditions at pH = 7.4. Thus it is proved that linker connecting DOX to dextran backbone is stable in physiological conditions.

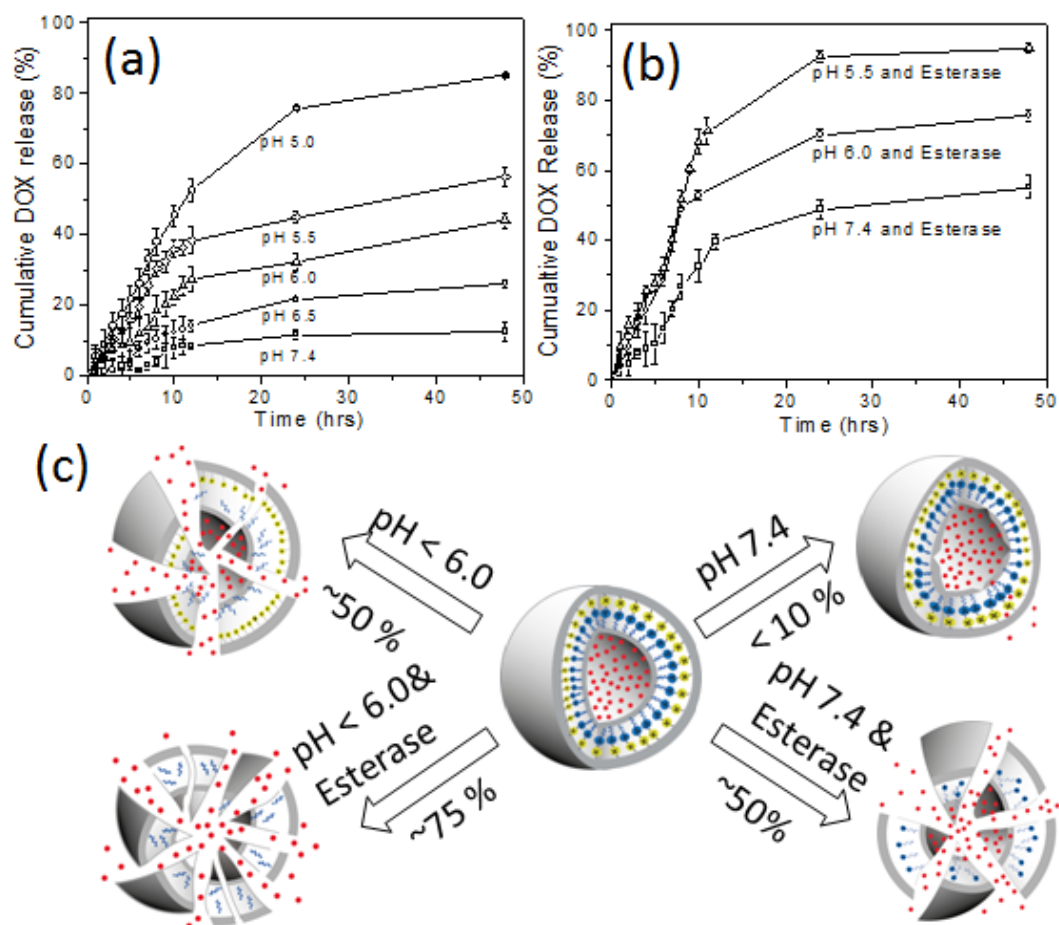


Figure 4.21. In-vitro DOX release profile from DOX conjugated DEX-IM-5 at various pH (a), in the presence of esterase and various pH (b), Schematic representation of DOX release at various conditions (c).

With decrease in pH, the drug releasing ability of the vesicles increased; however, about 30 % of the drugs was not accessible even at lower pH= 5.0 (see figure 4.21c). The esterase enzyme assisted a faster release of DOX to 55 % in pH 7.4 and to about 75 % in pH 6.0 and close to 90 % in pH 5.5 conditions.

Table-4.3: Rate constant of DOX release from DEX-IM conjugated vesicles at various conditions

Condition Employed	pH 7.4	pH 6.5	pH 6.0	pH 5.5	pH 5.0	pH 7.4 and Esterase	pH 6.0 and Esterase	pH 5.5 and Esterase
$k(\mu s^{-1})$ DOX loaded vesicle	2.4	4.0	7.7	11.9	17.3	11.7	22.0	30.7

However, the release rate was slight lower (see figure 4.21b) compared to that of the DOX loaded vesicles (see figure 4.17b). The drug conjugated vesicles also showed increased rate constants while decreasing the pH and in the presence of esterase; however, the values were just half compared to that of the DOX loaded vesicles (table 4.3). Over all, both DOX loaded and DOX conjugated dextran vesicles could be interesting candidate for drug delivery to cancer cells. Hence its cytotoxicity potential and intracellular drug delivery capabilities should be analyzed.

4.3.7. Cellular Uptake and Cytotoxicity

Cytotoxicity of the polymer vesicle (DEX-IM-5) was investigated in WTMEFs (fibroblast cells) by MTT assay method.⁴² As shown in Figure 4.22a, free polymer (DEX-IM-5) showed more than 80 % cell viability even at highest concentration of 500 $\mu\text{g/mL}$. This result confirmed the high biocompatibility of the newly designed dextran vesicles for further delivery applications. Cytotoxicity of DOX, DOX loaded DEX-IM-5 vesicles and DOX-conjugated vesicles were tested in both breast cancer MCF 7 cells and normal WTMEFs cell lines. Under identical conditions and the drug concentration was maintained as 0.289 $\mu\text{g/mL}$ (equivalent to 0.5 μM) for free DOX in PBS, DOX loaded and DOX conjugated vesicles in PBS. The cytotoxicity data are summarized for MCF 7 and WTMEFs in figures 4.22b and 4.22c, respectively.

DOX loaded in vesicles exhibited about 50 % of killing in MCF-7 cells at 0.5 μM concentrations which was about 15 % more than free DOX (see figure 4.22b). The DOX conjugated vesicle showed comparable killing as free DOX. Since the DOX loaded or conjugated nano-vesicles are capable of showing better uptake in the cancer tissues by EPR effect compared to free drug; the cytotoxicity data shown by the new scaffolds indeed very good for drug administration purposes. The comparison of the cytotoxicity among MCF7 cells and normal WTMEFs suggested that the present scaffold design showed significantly better killing in the breast cancer cells. Further, the effect of DOX-conjugated vesicles and DOX loaded vesicle could be enhanced by the surface modification of nano-carrier using targeting ligands may provide better response.

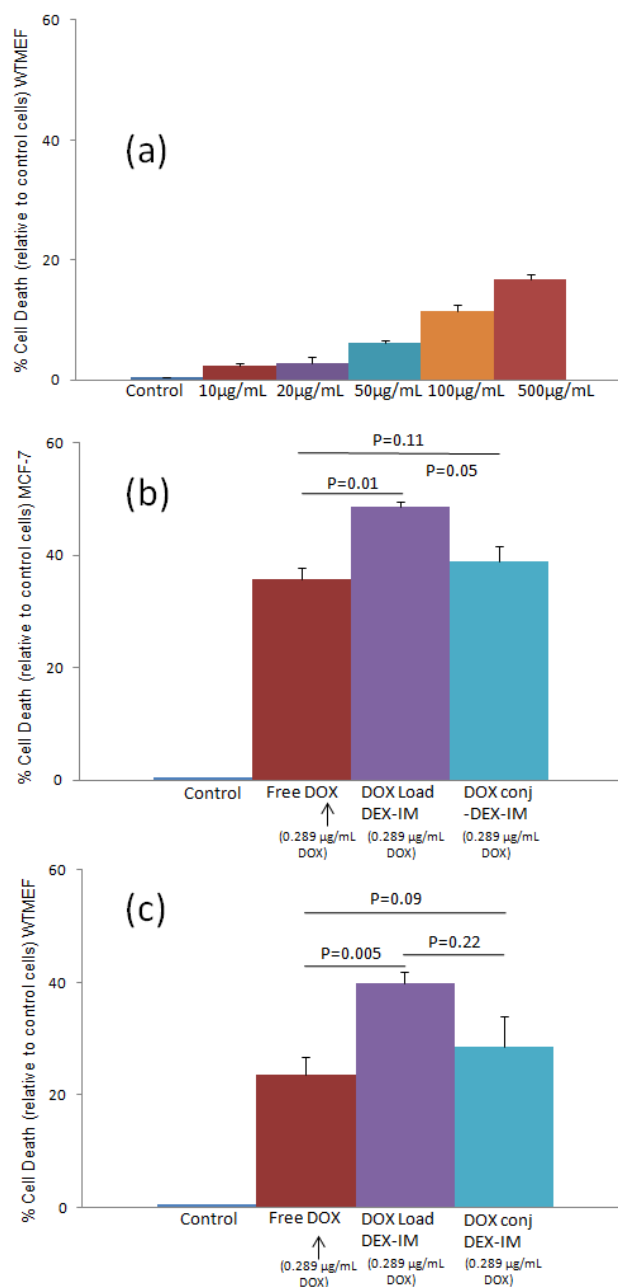


Figure 4.22: (a) Histogram depicting cytotoxicity of DEX-IM-5 vesicles in WTMEFs at various concentration. (b) Cytotoxicity of DOX, DOX loaded DEX-IM-5 and DOX conjugated DEX-IM-5 in MCF-7. (c) Cytotoxicity of DOX, DOX loaded DEX-IM-5 and DOX conjugated DEX-IM-5 in WTMEFs cells. Concentration of drug was maintained as 0.289 µg/mL (0.5 µM) in free, DOX loaded and DOX-conjugated. The graph represents mean $\pm$ SE of percentage cell death relative to untreated control in three independent experiments and statistically analysed (graph b and c) using the standard T Test.

The cellular uptake studies provide more insight into the DOX delivery efficiency of vesicles. The red fluorescence of DOX at ~ 595 nm assisted to study the uptake of free drug and drug loaded vesicles into cells.⁴³ Cells were treated with Free DOX, DOX loaded DEX-IM-5 vesicles and DOX conjugated DEX-IM-5 vesicles for

1.0 μM DOX concentration. DOX molecule is known to accumulate in the nucleus of the cells.⁴³ Thus, the nucleus was mapped in these experiments by staining with DAPI. Actin cytoskeletal network of cells were also stained with phalloidin conjugated to Alexa 488 dye (green). This staining was used to identify the morphology of the adherent spread cell. The cells were observed through blue channel (at λ 405 nm) for DAPI-nucleus and green channel (at λ 488 nm) for Phalloidin- actin cytoskeleton. The collected images were merged together and they are shown in left side of each pannel in figure 4.23. DOX fluorescence was observed through red channel (at λ = 568 nm) and these images are shown in the right side in figure 4.23. The closer inspection of the images revealed a very good overlap between the DOX and DAPI staining in the nucleus (compare left and right side in each row). Administration of free drug DOX showed a diffused staining in the cytosol and some accumulation in the nucleus (see Figure 4.23b). DOX loaded in dextran DEX-IM-5 vesicles showed an increased red fluorescence in nucleus (see Figure 4.23c). DOX conjugated vesicles showed higher uptake compared to free DOX (compare figures 4.23b and 4.23d); however, the intensity was relatively less compared to that of DOX loaded vesicles (compare figures 4.23c and 4.23d). The nuclear binding of DOX released from the vesicles is better than free DOX seems to suggest the greater availability of DOX at its site of action when in the vesicle than in free drug. This better binding of DOX showed higher fluorescence intensity while administrated DOX in the loaded form.

The difference in the uptake between the DOX loaded and DOX conjugated vesicles are attributed to the difference in their release profiles (see figure 4.17 and 4.21). The DOX loaded vesicles showed much faster release compared to the DOX conjugated vesicles; as a result, the physically loaded DOX showed enhanced intensity in cellular imaging. Based on the cellular imaging studies alone, it is rather difficult to prove the pathways under which the nano-vesicles entered and ruptured in the intracellular compartments. More experimental proofs are required in terms of organelle specific accumulation of nano-vesicles such as endosome, lysosome, etc. To address the problem, currently the research work is focused on developing pH responsive dye probes which could be either physical loaded or chemically conjugated in the dextran vesicles. Nevertheless, the detailed *in vitro* drug release studies, cytotoxicity data cellular imaging proved that the current dextran vesicles are

potential vectors for the releasing the loaded cargoes at the intracellular compartments in breast cancer cells. Further, the combined effect of esterase and pH on the cytotoxicity data would be more visible only in the *in vivo* studies at the cancer tissues.

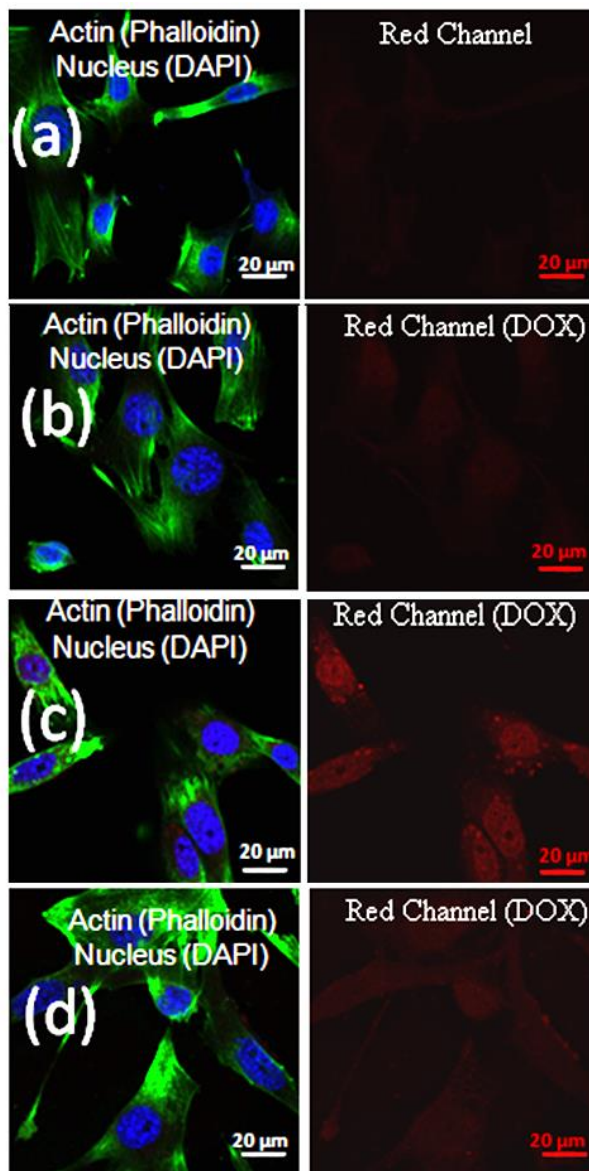


Figure 4.23: Confocal microscopic image of (a) control, (b) free DOX, (c) DOX loaded DEX-IM vesicles and (d) DOX conjugated DEX-IM treated WTMEFS cells. The nucleus was counter stained with DAPI (blue), actin cytoskeletal network in cells is stained with phalloidin (green). The cells were observed through red channel to locate DOX fluorescence (red). Scale bar in each pannel represents 20 μm .

4.4. Conclusion

In conclusion, a new polysaccharide dextran derivative is designed and developed based on renewable resource hydrophobic unit as a pH and enzyme dual

responsive nano-carrier for delivering drugs exclusively at cancer environment. The pH and enzyme responsiveness were achieved through acid labile imine linkages and the lysosomal esterase enzyme cleavable the aliphatic ester linkage (second stimuli) that connects the hydrophobic unit on the dextran backbone respectively. The modified dextran derivative self-organized as vesicular scaffold in which water soluble anticancer drug DOX.HCl was successfully encapsulated for testing in the cancer cells. The imine chemistry was further exploited to anchor the DOX at the dextran backbone which produced DOX conjugated dextran vesicles. Remarkably, the vesicular geometry of dextran scaffold was retained even after the loading and anchoring of DOX molecules. The vesicular assemblies were characterized by light scattering, electron microscopes, atomic force microscope and photophysical studies. *In vitro* studies revealed that the vesicular assemblies showed significant stability at normal physiological pH conditions. The acid labile benzylic imine linkages were cleaved under acidic pH (≤ 6.0) conditions which lead to disassemble of the dextran vesicles. Esterase enzyme (abundant in lysosomal compartment of cells) further assisted the vesicular cleavage so that 100 % of loaded drug-DOX released from vesicles under intracellular conditions. MTT assay revealed that the dextran vesicle was non toxic to cells upto 500 $\mu\text{g/mL}$. DOX loaded vesicles exhibited better cell killing efficiency than free drug and drug conjugated vesicles. The DOX loaded and conjugated vesicles exhibited significantly better cell killing efficiencies in breast cancer cells (MCF7). Though, the approach demonstrated here provide new design principle for suitably modify dextran as pH and enzyme responsive nano-carrier; this approach is not only restricted to dextran, and in principle, applicable to wide variety of other polysaccharides. Further, in the present studies only one water soluble anticancer drug DOX.HCl was explored; however, the approach may be expanded to other water soluble drugs in the inner cavity as well as water insoluble drugs in the hydrophobic layer.

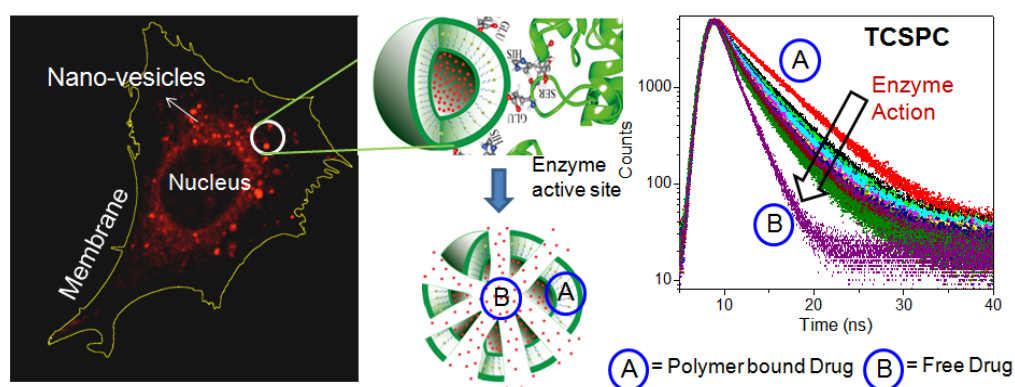
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Chapter 5

Real-time Drug Release Analysis of Enzyme Responsive and Cell Penetrable Polysaccharide Nano-vesicles



Real-time Drug Release Analysis of Cell Penetrable Polymer vesicles

Chapter 5

Real-time Drug Release Analysis of Enzyme Responsive and Cell Penetrable Polysaccharide Nano-vesicles

The accurate estimation of drug release kinetics from polymeric vehicles is an indispensable prerequisite for the developments of successful drug carriers for cancer therapy. The investigation in this chapter illustrates the development of time resolved fluorescence spectroscopic approach to resolve real-time enzymatic/ acidic pH cleavage of cell penetrable polysaccharide vesicles that are potential vectors for delivering anticancer drug molecules in the cancer treatment. The polysaccharide vesicles described in Chapter 2 and chapter 4 are loaded with fluorescent anticancer drug Doxorubicin.HCl (DOX) and fluorophore dye Rhodamine B (RhB) showed identical in vitro drug release pattern, cellular uptake and peri-nuclear accumulation in the live cell. Semi-permeable membrane dialysis method + absorbance spectroscopic technique was found to have inherent limitation in the accurate estimation of drug release. Real-time drug release protocols are established for DOX and RhB loaded polysaccharide vesicles and their responsiveness to the esterase enzyme and/or acidic pH by time correlated single photon counting (TCSPC) techniques. RhB loaded polysaccharide vesicles showed significant difference in the fluorescence life time values with respect to its response to acidic pH/ esterase enzymes in buffer. This facilitated the TCSPC approach to precisely compute the normal release (without enzyme or pH stimuli) and stimuli-induced release (with enzyme &/or acidic pH) and estimate their fractional contribution for real time drug kinetics analysis. Moreover, the ability of enzyme to cleave the polysaccharide vesicles was validated using AUTODOCK programme. The study bestowed an entirely new method to obtain authentic information about drug release parameters from polysaccharide vesicles and this new technique could be very useful for the estimation of drug release from polymer based drug delivery vehicles, in general.

5.1. Introduction

Cancer tissues or cancer related pathological inflammation are found to be over expressed by enzymes such as glutathione reductase¹ and esterases.^{2,3} A variety of synthetic and natural resource including polysaccharides based polymeric vesicles were developed for targeted drug delivery applications to exploit the cancer tissue environment for selective and targeted delivery to cancer cells.^{4,5} Nevertheless, there is no effort has been taken until now to study the real-time drug release kinetics of enzyme responsive polymer vesicles to unlock the mechanism of enzymes action on polymer backbone. Tunability of drug release rate at the site of action is also an indispensable requirement for optimizing the efficacy of vesicular carriers. Moreover, reliable methods to calculate the *in vitro* drug release kinetics are essential for establishing a good correlation between *in vitro*- *in vivo* elution properties of delivery system, which in turn define clinical effectiveness of formulation *in vivo*.⁶

Absorption spectroscopic analysis is routinely employed in combination with semi-permeable membrane dialysis for the *in vitro* drug release kinetics.⁷ Recent studies revealed that this approach had limitation due to the process by which the estimation of drug release analysis was performed.⁸ Typically, the dialysis approach involved two steps: (i) the rupture of the polymer membrane and transportation of the loaded cargoes (or drugs) to the liquid medium inside the dialysis tube and (ii) transportation of the drug molecules from the dialysis tube to the outer reservoir by crossing the barrier of the dialysis membrane. These processes are a little different from the actual forces experienced by the polymer vesicles when they are administrated in the cell lines under *in vitro* conditions or intravenous injection *in vivo*. As the consequence, most often the *in vitro* drug release kinetics data obtained from the absorbance+ dialysis method are found to be different at the cellular uptake of polymer nano-scaffolds either *in vitro* in cells or *in vivo* in animal models.⁸

Real-time analysis of drug release kinetics by in-situ methods are very good protocol to overcome some of the above limitation and correlates the drug release kinetics pattern by the spectroscopic tools to their actual action in the cell lines *in vitro* conditions. Fluoroprobe tagged polymer structures were employed as model systems to study the real-time drug release patterns using triggers such as pH and temperature.^{9,10} For example, Shabat and coworkers have reported a coumarin linker based fluorescent activation system for real time drug release studies.⁹ Cargo release

and subsequent latent fluorophore activation generated fluorescent signal, which was used for real time detection of drug release as shown in the figure 5.1.

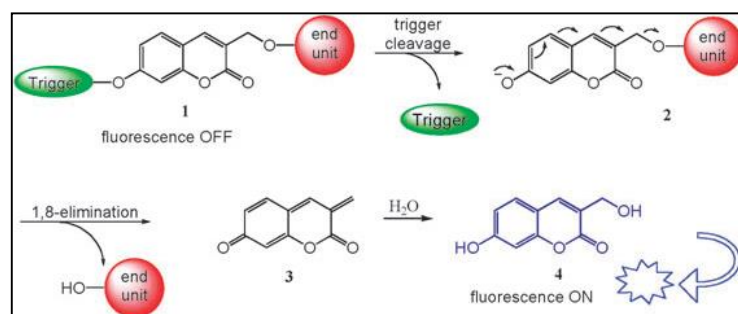


Figure 5.1. Real time drug release monitoring using coumarin linker based prodrug (adopted from Weinstein, R. et al. *Chem. Comm.* **2010**, 46 (4), 553- 555).

Viger *et al.* demonstrated the application of time resolved fluorescence technique for probing drug release kinetics of poly(L-glutamic acid) nanoparticles.¹¹ PLGA nanoparticles were encapsulated with Coumarin153 and fluorescein as hydrophobic and hydrophilic molecules respectively. Fluorescence intensity of coumarin 153 was quenched upon release from the nanoparticles: On the other hand, fluorescence intensity of fluorescein was increased upon release from particles (see figure 5.2). Multi-exponential decay along with distinctive excited state life time components showed by released fluorophore molecules in TCSPC studies was utilized to distinguish different environment in particle suspensions. The contributions of fluorophore released and loaded were separately estimated by fitting time resolved fluorescence data to determined release pattern from the nanoparticles.

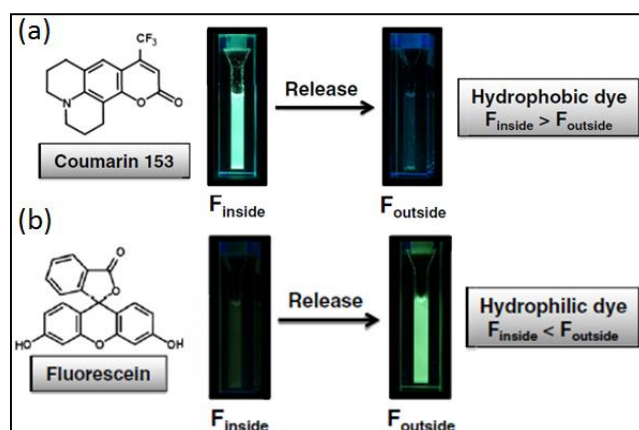


Figure 5.2. Quenching and enhancement of fluorescence upon release from nanoparticle. (Adopted from Viger et al. *J. Control. Rel.* **2013**, 171, 308-314).

Unfortunately, these studies did not provide any information on the cytotoxicity, cellular uptake mechanism or cellular up taking capability of their polymer design. Thus, the real-time drug release kinetics of the drug loaded synthetic polymer vesicles that responsive to the biological stimuli such as enzymes and also tested as potential carriers for cancer treatment would be an excellent choice to address the above problem for enhancing the efficacy of cancer treatment. Herein, the cell penetrable polysaccharide nano-vesicles described in chapter 2 and 4 were loaded with hydrophilic DOX. HCl (drug) and Rhodamine (RhB) (dye) molecules (See Figure 5.3a). Cellular uptake studies proved that both DOX and RhB loaded vesicles showed almost identical cell penetrating ability, peri-nuclear accumulation as well as responsiveness to biological stimuli like enzymes.^{12,13} Thus, developing new methods to study the real-time drug release kinetics of these enzymes responsive and cell penetrating polysaccharide vesicles would facilitate better understanding of the diagnostics as well as the therapeutics of polymer based drug delivery carriers for improved cancer treatment, in general.

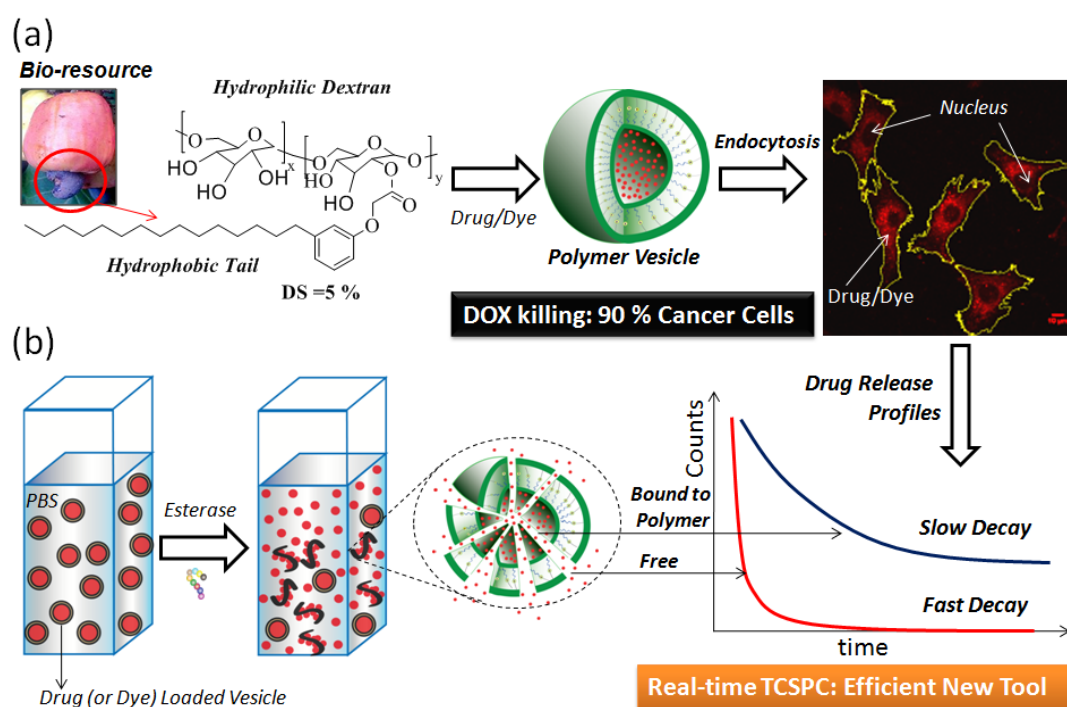


Figure 5.3. (a) Polysaccharide vesicles and its cellular entry. (b) Drug/dye loaded vesicles and its cleavage in the presence of esterase enzyme. TCSPC method distinguishes the free and polymer bound drug/dye molecules.

This chapter is emphasized to develop real-time drug release analysis for polymer based vesicles (mentioned above) that are responsive to biological enzymes

&/ or acidic pH using time resolved fluorescence spectroscopy. The pitfalls of dynamic absorbance+ dialysis technique for exact drug release patterns were successfully overcome by time resolved fluorescent (TCSPC) method (see Figure 5.3b). DOX and RhB loaded vesicles were subject for the above purpose and it was found that RhB loaded vesicular system is an excellent candidate for real-time drug kinetic analysis by TCSPC. Further, the DEX-PDP and DEX-IM-5 molecules were also subjected to docking studies with esterase enzyme active site to trace the enzyme-mediated drug release process. The overall investigation revealed that the real time fluorescence method is very powerful methodology for understanding the drug release kinetics of cell penetrable polymer vesicles that are potential vectors for cancer treatment.

5.2. Experimental Section

5.2.1. Materials

Doxorubicin.HCl (DOX), Rhodamine B (RhB) and horse liver esterase enzyme were purchased from Aldrich chemicals. Modified dextran derivatives were synthesized as described in chapter 2 and chapter 4 were used for the studies.^{12,13} Dextran conjugated with PDP via ester linkage (described in chapter 2 and 3) is abbreviated as DEX-PDP. Dextran conjugated with PDP via pH responsive imine and enzyme responsive ester linkage (described in chapter 4) is abbreviated as DEX-IM-5.

General Procedures: Encapsulation and release studies were followed as explained in chapter 2, chapter 3 and chapter 4.

5.2.2 Encapsulation of Rhodamine-B: Water soluble rhodamine B was loaded into vesicles inner core by the method described in chapter 2.¹² Briefly, 20 mg of polysaccharide derivatives (DEX-PDP-5 or DEX-IM-5) and 2 mg of rhodamine B were dispersed in 3 mL DMSO solvent. This solution was agitated in a beaker at 25 °C and 3 mL of Phosphate buffered Saline (PBS, pH 7.4) was added slowly (dropwise) into it. The resulting dye-polymer mixture was stirred again for another 12 h at 25 °C and transferred into regenerated cellulose dialysis bag (SPECTRA/POR, MWCO 3,500). The dialysis process was carried on until rhodamine B leaching out from the dialysis bag was entirely stopped. The vesicles loaded with rhodamine B

dye was recovered from the dialysis bag and diluted to 10 mL with PBS. The rhodamine B loaded in the vesicles was completely released by dissolving 100 µl of above stock solution in 2.9 mL DMSO solvent. The rhodamine B loading amount was determined by measuring absorbance of above diluted solution at 552 nm and substitute it in Beers law equation where molar absorption coefficient of rhodamine B were fixed as 1,11,500. The loading content of RhB in vesicles was determined as 2.70 wt %.

5.2.3. Encapsulation of Doxorubicin.HCl: Doxorubicin HCl (DOX.HCl) was encapsulated in vesicles by the method described in chapter 3 and chapter 4.¹³ Briefly, 100 mg of polymer (DEX-PDP-5 or DEX-IM-5) and 2 mg of DOX.HCl were co-dissolved in 3 mL of DMSO and stirred at 25 °C in a light protected condition. Self assembly of polymer was induced by slow addition of 3 mL of PBS (pH 7.4) into above solution. The resulting polymer drug solution was stirred at 25 °C under dark condition for 12h and transferred to a dialysis bag (SPECTRA/POR, MWCO 3500) and extensively dialysed in a light protected container against PBS (0.01 M, pH 7.4) for 24 h to remove DMSO and unencapsulated DOX molecules. The resulting drug loaded vesicle solution was freeze dried after passing through a 0.45µm filters. The drug loading content (DLC) in vesicles was estimated using absorption spectroscopy method. Briefly, 3 mg of lyophilized drug loaded polymer derivative was dissolved in DMSO (1 mL) and then 100 µL of this solution was made to 3.0 mL with DMSO. The absorbance of this resulting solution at 480 nm was measured on UV-visible spectrophotometer and the amount of drug loaded in the vesicles was determined using Beers Law where the molar extinction coefficient of DOX was fixed as 11,500. Drug loading content (DLC) and drug loading efficiency (DLE) were estimated using following equations.

$$\text{DLC (\%)} = \{\text{Weight of drug encapsulated in vesicles} / \text{Weight of Drug loaded polymer}\} \times 100 \% = 3.15 \text{ wt \% for DEX-PDP-5 and } 2.9 \text{ wt\% for DEX-IM-5}$$

$$\text{DLE (\%)} = \{\text{Weight of drug encapsulated in vesicles} / \text{Weight of drug in Feed}\} \times 100 \% = 63 \% \text{ for DEX-PDP-5 and } 58 \% \text{ for DEX-IM-5}$$

5.2.4. In-vitro Release studies: The drug and dye release from the vesicles was performed at pH 7.4 (physiological pH) using dialysis method as per the procedure

illustrated in chapter 2 chapter 3 and chapter 4.^{12,13} A control experiment was also performed using solution without polymer by just adding DOX.HCl (60 μ g) or RhB in 3 mL of PBS (pH 7.4). In brief, Polysaccharide vesicles encapsulated with RhB or DOX.HCl in 3.0 mL buffer solution (pH 7.4) were placed in a dialysis tube (SPECTRA/POR, MWCO 8000). Control free drug/dye solution was used for elution experiment without any previous dialysis. The tube carrying drug/dye loaded vesicles was submerged in 100 mL buffer solutions (pH 7.4) taken in a beaker and the whole solution was incubated at 37 °C. After appropriate time interval (30 min or 60 min) 3.0 mL of dialysate was recovered and restored with equal volume of fresh buffer. Absorbance of the collected aliquot was measured at 480 nm using UV-Vis absorption spectrophotometry and amount of the drug/dye eluted were calculated using Beer's law, where molar absorption coefficient of RhB and DOX was fixed as 1,15,000 and 11,500. The effect of esterase enzyme on drug/dye release was studied by mixing 10 U esterase enzyme into the vesicle solution prior to the release studies.

5.2.5. Photophysical Studies: The absorption studies were done using Perkin-Elmer Lambda 45 UV-Visible spectrophotometer. Emission spectra were recorded at room temperature using SPEX Fluorolog HORIBA JOBIN VYON fluorescence spectrophotometer equipped with a photomultiplier tube (PMT, Hamamatsu R928) for detection, a double-grating 0.22 m Spex1680 monochromator and a 450W Xe lamp as the excitation source. The excitation spectra are collected at 550 nm (for rhodamine B experiment) and 480 nm (for DOX experiment) and the emission spectra are recorded by exciting at the excitation maxima. The dye containing samples were purged with N₂ gas for at least 15-20 minutes prior to photophysical experiments.

Fluorescence lifetime of both free dye/drug and dye/drug loaded vesicles was measured using a time-correlated single photon counting (TCSPC) system (Horiba) equipped with a NanoLED excitation source of 560 nm for rhodamine B (RhB) and 450 nm for DOX.HCl with 1 MHz impulse repetition rate (Horiba) and a R928P detector (Hamamatsu Photonics, Japan). In RhB and DOX experiments, the detector was set to 585 nm and 560 nm respectively. The bandpass width was varied between 2 and 14.7 nm to attain suitable α value. A scattering solution of Ludox-40 (Sigma-Aldrich) in water was used as prompt (560 or 450 nm) to obtain the instrument response function (IRF). A total of 4094 channels were used with a time calibration of 0.110 channel/ns. The fluorescence lifetimes were estimated using DAS6 decay

analysis software (Horiba), where the goodness of fit was evaluated by fixing χ^2 values between 0.95 and 1.20.

Drug/Dye loaded into vesicles were fitted to mono-exponential as follows¹⁴:

$$A(t) = \alpha_1 \exp(-t / \tau_1)$$

Esterase enzyme treated samples were fitted to a sum of three exponentials. Lifetime components with insignificant fractional contributions ($f_i < 3\%$) were rejected and decays were judged to be bi-exponential, where the sum of the fractional contributions from free RhB and RhB loaded in vesicles ($f_1 + f_2$) was more than 95%.

$$A(t) = \alpha_1 \exp(-t / \tau_1) + \alpha_2 \exp(-t / \tau_2)$$

The average lifetime (τ_{AVE}) was computed as per the equation $\tau_{AVE} = \sum f_i \tau_i$, where f_i is the fractional contribution of the individual lifetime of each component (τ_i).^{11,14}

$$f_1 = [\alpha_1 \tau_1 / (\alpha_1 \tau_1 + \alpha_2 \tau_2)] * 100$$

$$f_2 = [\alpha_2 \tau_2 / (\alpha_1 \tau_1 + \alpha_2 \tau_2)] * 100$$

The experiment of DEX-PDP vesicles encapsulated with RhB was performed at pH 7.4 and by adding 10 U esterase enzyme, while that of DEX-IM vesicles loaded with RhB were performed under four conditions. (i) pH 6.0 (ii) pH 5.0, (iii) pH 6.0 and 10 U esterase and (iv) pH 5.0 and 10 U esterase.

5.2.6. Docking Protocol: To understand the interaction of dextran-PDP (DEX-PDP) and DEX-IM vesicles to esterase enzyme, docking of monomer and trimer units of DEX-PDP and DEX-IM to human liver carboxyl esterase 1 was performed. The crystal structure of human liver carboxyl esterase 1 (PDB ID: 3K9B)¹⁵ was taken as the receptor and catalytic triad composed of Serine, Histidine and Glutamine in the interface between the subunits of the enzyme was taken as the active site. The quantum chemically optimized geometry of monomer and trimer units of DEX-PDP and DEX-IM-5 were taken as the ligands. The optimization was done using Hartree-Fock theory with basis set 6-31G* using Gaussian 03¹⁶ software. The restrained electrostatic potential (RESP)¹⁷ charges on the atoms of the optimized structures were calculated using Antechamber module of AMBERTools¹⁸. For the enzyme, the charges on the atoms were calculated using AMBER99SB force field¹⁹ available in AMBER11²⁰ software. During docking, the quantum chemically derived charges on the atoms of ligands and the AMBER99SB charges on the receptor were kept unchanged. The docking was performed using AutoDock software.^{21,22} The receptor

was kept rigid and the ligands were kept flexible during docking. The grid was generated on active site of human liver carboxyl esterase 1 with grid points 126, 74 and 80 on X, Y and Z directions respectively with a grid spacing 0.192 Å. Genetic algorithm was used as the search method for the docking process.

5.3. Results and Discussion

5.3.1. DOX and RhB Loaded DEX-PDP Vesicles

Dextran amphiphiles (DEX-PDP) was synthesised by coupling with plant derived hydrophobic pentadecyl phenol (PDP) on its backbone via aliphatic ester linkage as illustrated in chapter 2 (See Figure 5.3a).¹³ The modified dextran derivatives self-assembled in water to produce nano-vesicular morphologies and these vesicles were characterized by electron microscope, atomic force microscopes, dynamic and static light scattering and the details are described in chapter 2. The present investigation is devoted to study the drug releasing capabilities of these vesicles for water soluble fluorescent anticancer drug doxorubicin.HCl (DOX) and water soluble fluorophore dye Rhodamine B (RhB). Under identical conditions, DOX and RhB were loaded in the dextran vesicles as described in chapter 2 and chapter 3, respectively (See figure 5.4a).

Photophysical characteristics of the DOX and RhB loaded vesicles are shown in figure 5.4b to 5.4g. DOX or RhB loaded vesicles showed identical absorbance and emission maxima for free as well as encapsulated drug in vesicular scaffold (see figures 5.4b to 5.4e). This indicates that steady state absorbance and emission spectroscopic methods are not capable of distinguishing between the free drug in solution and drug encapsulated in vesicles. Time resolved fluorescent decay profiles of DOX and RhB in their free state and loaded form were recorded by TCSPC techniques. TCSPC decay profiles of the free DOX and DOX in vesicles are given in the figure 5.4f. The decay profiles of the DOX followed single exponential decay with decay rate constant (τ_1) of 1.1 ns in its free form²³⁻²⁶ and 2.0 ns for loaded in the vesicles respectively. The decay rate constants revealed that the drug molecules have almost identical life time for both free and loaded in the vesicles with a difference of 0.9 ns. This difference in the DOX life time is not very useful for the differentiation of free and polymer bound drug. Remarkably, a significant difference was observed in the TCSPC life time of free RhB and RhB loaded in the vesicles. TCSPC decay

profiles of the RhB (see figure 5.4g) followed single exponential decay with $\tau_1 = 1.74$ ns for free²⁷ and 4.90 ns for loaded in the vesicles, respectively. Further, the decay profiles also showed substantial difference for free RhB and RhB in the vesicles. Thus, this large difference in the time resolved fluorescent decay process could be used as a tool to understand the real time release kinetics of the drug or dye from polysaccharide vesicles.

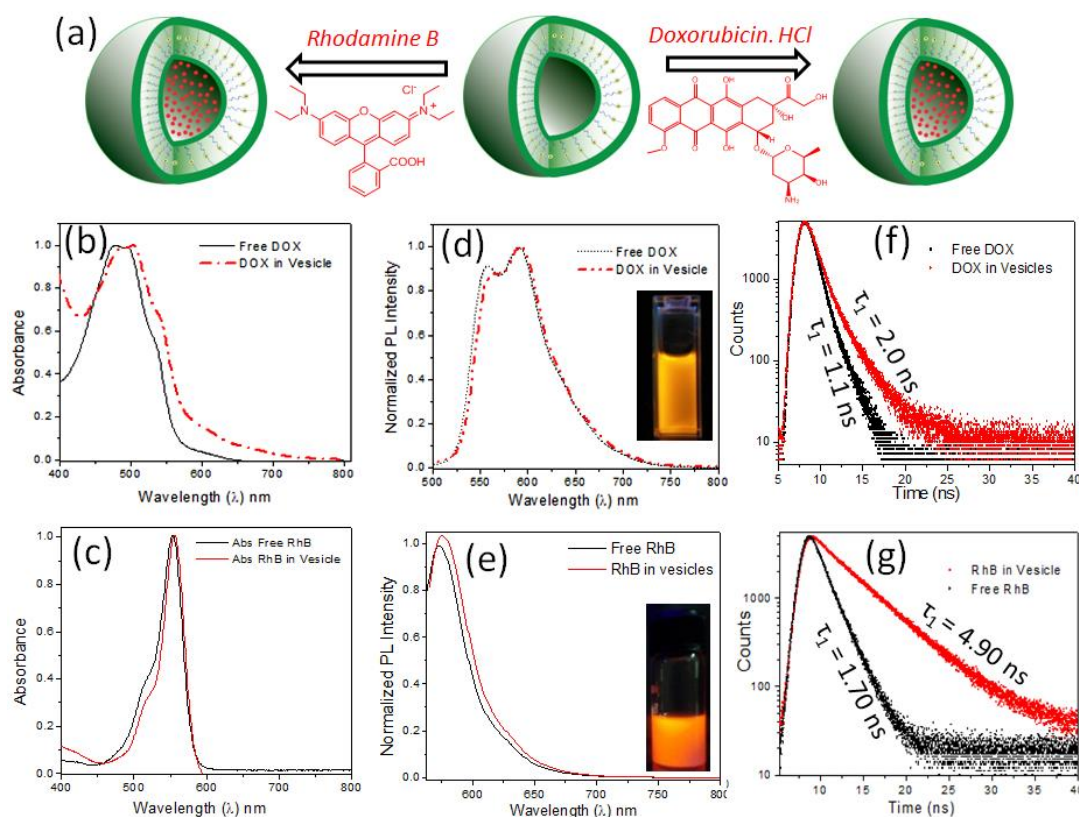


Figure 5.4. Schematic representation of DOX and RhB loading into vesicles (a) Absorbance spectra (b) of free DOX and DOX loaded in vesicles and (c) of free RhB and RhB loaded vesicles. Emission Spectra (d) of free DOX and DOX loaded in vesicles and (e) of free RhB and RhB in vesicles. fluorescence life time plot (f) of free DOX and DOX loaded in vesicles and (g) of free RhB and RhB in vesicles. Inset in (d) and (e) shows DOX and RhB loaded vesicle solution under hand held UV light respectively

Two control experiments were carried out to confirm the TCSPC decay profiles prior to the real time analysis. First, the quantum yields of the free DOX and free RhB compared to their loaded form in the vesicular scaffolds were analyzed to rule out the basis of ϕ in their difference of τ_1 value. It was found that both the free drug and loaded drug (DOX or RhB) displayed identical quantum yield. Secondly, dynamic dialysis method was employed to determine drug release pattern from the dextran vesicles loaded with RhB and DOX. The dialysis tube carrying vesicular

solutions and typical drug release pattern from dextran vesicles under normal physiological conditions (at pH = 7.4) and in the presence of esterase enzyme (at pH = 7.4) was shown in figure 5.5a and Figure 5.5b. Under normal condition about 30 ± 5 % release was observed from dextran vesicles. The ester linkage used for bonding the hydrophilic and hydrophobic segments of the amphiphile is prone to get cleaved by esterase enzyme. The level of esterase enzyme in the liver microsome and cytosols were reported by Xu et al as 9.5 to 30.5 U. So in these studies 10 U of enzymes were added and it was observed that DOX/RhB release enhanced to 70 ± 5 % in 48 hours.²⁸ The vesicular leakage in the absence of enzyme and rupture under enzyme condition is schematically shown in Figure 5.5c.

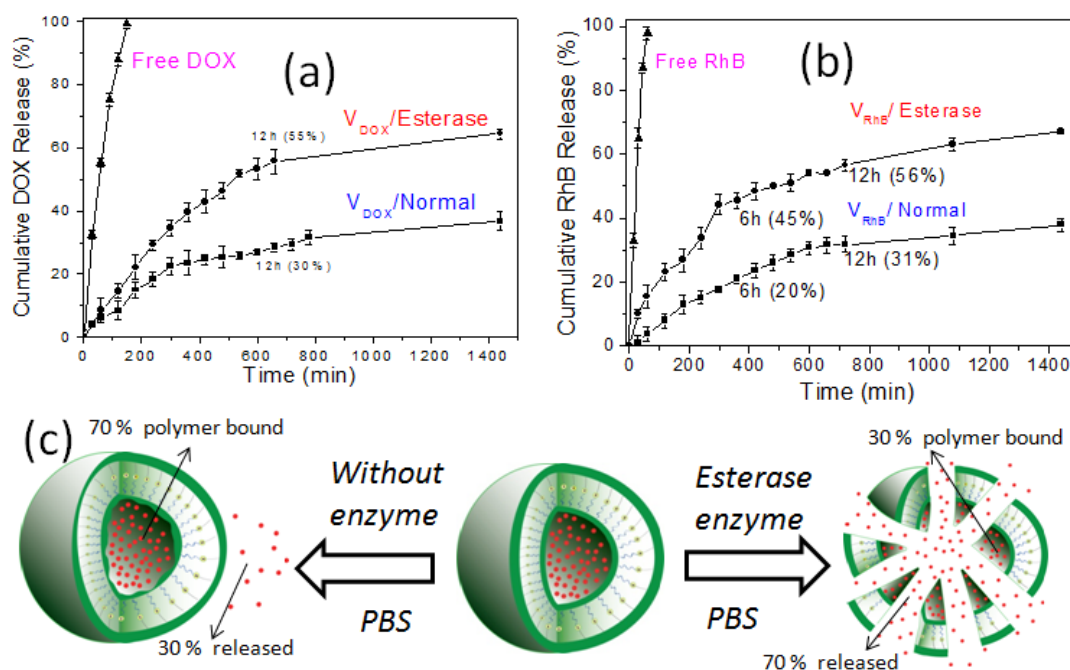


Figure 5.5. In-vitro release profile of DOX loaded vesicles (a) and RhB loaded vesicles (b). Schematic representation (c) of RhB/DOX released from dextran vesicles under normal and enzyme condition.

Under normal condition (without enzyme) and in the presence of enzyme, a part of drug/dye was bound to polymer: 70 % in the absence of enzyme and 30 % in the presence of enzyme as shown in Figure 5.5a and 5.5b. It means that the *in-vitro* studies using enzyme content equal to that present intracellularly (10 U), was not able to rupture the vesicles completely and release the drug/dye bound to polymer. This effect may be due to two reasons. (i) Esterase enzyme is not getting access to all ester bonds present in the polymer backbone and is incapable of disorganizing vesicles completely or (ii) dialysis method used for release studies have inherent limitations.

Previous studies using 30 U (3 times higher than normal) enzyme showed that the whole loaded content (100 %) of vesicles was released in less than 24 hours. These results omit the question of enzyme's ineffectiveness in vesicular cleavage.

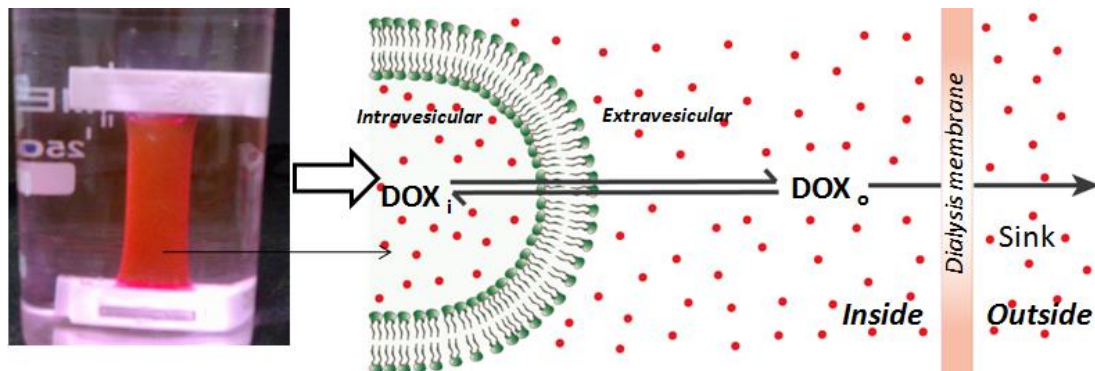


Figure 5.6. Dynamic dialysis of RhB loaded vesicle solution and schematic representation of drug/ dye transport from intravesicular to extravesicular environment and subsequent flow across dialysis membrane.

Anderson and coworkers showed the release rate determined by dialysis method do not reflect actual drug release rate from vesicles (liposomes).¹⁷ This may be due to the dual barrier properties of dialysis method, where drug released from vesicles (first barrier) has to cross dialysis membrane (second barrier) before reaching into receiver compartment (See Figure 5.6). Factors such as driving force for drug movement through dialysis membrane and properties of dialysis membrane were not considered in drug release rate determination. A few other groups also experienced this issue of inherent barrier properties of dialysis membrane and drug partitioning between dispersed phases during drug transport processes.^{7,8} Moreover, driving force for drug transport across dialysis membrane may be reduced due to the reversible drug binding with vesicular carriers through hydrophilic or hydrophobic interactions (See Figure 5.6).

In order to conquer the possible sources of errors in the data output from dynamic dialysis for the estimation of DOX release from dextran vesicles, a technique should be identified for real time monitoring of drug released from vesicles and drug loaded in vesicles. The two components viz. free dye/drug molecules and dye/drug bound to polymer have to be differentiated. Interestingly, DOX and RhB encapsulated dextran vesicles exhibited identical structural geometry, cell penetration and release capacities under *in-vitro* conditions. Both the vesicles penetrated equally into cells and resulted in a perinuclear localization. DNA intercalating topoisomerase II DOX

drug molecule permeated and accumulated more into nucleus. These studies proves that the physical and chemical properties of dextran vesicles did not vary upon change in type of molecules encapsulated.

5.3.2. Real-time Drug Release by TCSPC Method

Since DOX fluorophore has limitation to show sensitivity in TCSPC analysis, RhB loaded dextran vesicle could be used to understand its intracellular releases profiles. The vesicles endocytosed undergoes rupturing in the presence of esterase enzyme abundant in lysosomes as shown in Figure 5.7. Typically, endocytosis of nanocarrier starts with endosome formation by membrane invagination followed by delivery of cargoes into various vesicular structures and end up in lysosomes.²⁹ Degradative enzymes in lysosomes rupture the carriers and release the loaded cargoes. It is essential to distinguish drug released into cytoplasm or drug bound to polymer in order to estimate intracellular drug delivery capacity of vesicles.

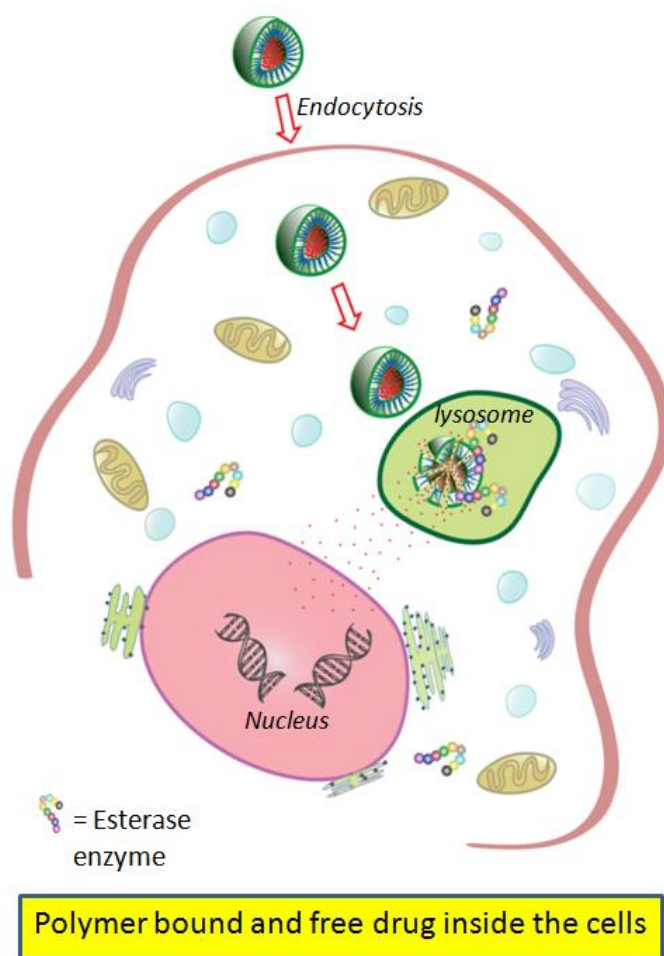


Figure 5.7. Schematic representation of endocytosis of vesicles and release of drug molecules under intracellular conditions.

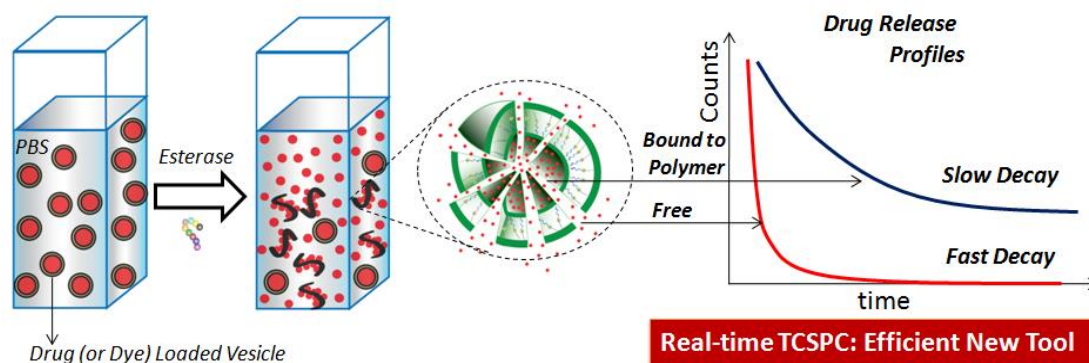


Figure 5.8. Drug/dye loaded vesicles in cuvette and its cleavage in the presence of esterase enzyme. TCSPC method distinguishes the free and polymer bound drug/dye molecules.

Here, the time resolved fluorescence offer important information about the cargo environment. Photoexcited RhB molecules return to ground state by transferring energy into neighboring RhB or solvent molecules. But when the dye molecules were present in a confined space, possibility for faster relaxation was hindered. This will lead to an increase in the life time of the molecule. The significant difference of life time of RhB in free and encapsulated form can be utilized for studying the release rate from vesicles (See figure 5.8).

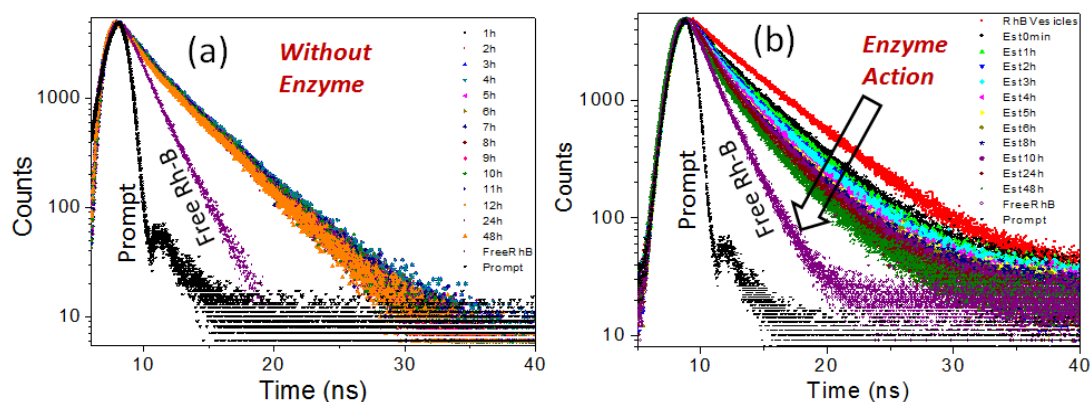


Figure 5.9. Fluorescence life time plot (a) of RhB in dextran vesicle along with prompt (black symbol) under normal condition (without enzyme) and fluorescence life time plot (c) of RhB in dextran vesicle along with prompt (black symbol) under enzyme condition.

The absorbance of RhB loaded vesicular solutions was adjusted to 0.2 O.D. using PBS (10 mM, pH 7.4) buffer and used for TCSPC analysis. The decay profile obtained was fitted with DAS6 decay analysis software. The free RhB in solution and the stock solution of RhB loaded in vesicles were fitted to mono exponential decay. This means that only one component is present in the free dye/dye loaded in vesicles

solutions. The life time of RhB in the above vesicular solution was estimated as 4.90 ns using TCSPC method (See Figure 5.4g). Time resolved fluorescence of RhB loaded vesicles were collected under physiological conditions in PBS (pH 7.4) for 48 hours time period (See Figure 5.9a and table 5.1). No appreciable difference in decay profiles was observed indicated the stability of vesicles under physiological conditions. As explained earlier, esterase enzyme can hydrolyze the aliphatic ester linkage connecting dextran and PDP subunits, leads to dissociation of vesicles and subsequent leakage of loaded content.

Table 5.1. Table depicting life time and fractional contribution of RhB in dextran vesicles and RhB released into PBS under normal conditions (without enzyme)

Description ^a	χ^2	τ_1 (ns) & Fractional contributions (%) Polymer + RhB ^b	τ_2 (ns) & Fractional contributions (%) Free RhB ^c
Rhodamine B (RhB)	1.038		
DEX-PDP-RhB	1.106		
DEX-PDP-RhB-30m	1.044	4.89 (99.49)	1.76 (0.521)
DEX-PDP-RhB-1h	1.130	4.87 (99.27)	1.74 (0.711)
DEX-PDP-RhB-2h	1.042	4.85 (98.91)	1.76 (1.011)
DEX-PDP-RhB-3h	1.133	4.79 (98.82)	1.72 (1.113)
DEX-PDP-RhB-4h	1.082	4.75 (98.61)	1.71 (1.345)
DEX-PDP-RhB-5h	1.080	4.74 (97.41)	1.74 (2.554)
DEX-PDP-RhB-6h	1.120	4.73 (96.81)	1.72 (2.884)
DEX-PDP-RhB-7h	1.160	4.69 (95.63)	1.71 (3.123)
DEX-PDP-RhB-8h	1.140	4.69 (95.10)	1.70 (4.544)
DEX-PDP-RhB-10h	1.180	4.65 (94.67)	1.70 (4.875)
DEX-PDP-RhB-24h	1.065	4.62 (93.65)	1.75 (6.339)
DEX-PDP-RhB-48h	1.120	4.58 (91.09)	1.70 (7.862)

^a means description of sample name and time of TCSPC measurement. ^b means life time of RhB bound to polymer and corresponding fractional contribution given in bracket. ^c means life time of RhB released to polymer and corresponding fractional contribution given in bracket

Table 5.2. Table depicting life time and fractional contribution of RhB in dextran vesicles and RhB released into PBS under esterase enzyme conditions:

Description ^a	χ^2	τ_1 (ns) & Fractional contributions (%) Polymer + RhB ^b	τ_2 (ns) & Fractional contributions (%) Free RhB ^c
Rhodamine B (RhB)	1.093		
DEX-PDP-RhB	1.166		
DEX-PDP-RhB-30m	1.074	4.607 (88.49)	1.786 (11.63)
DEX-PDP-RhB-1h	1.100	4.497 (84.76)	1.764 (18.89)
DEX-PDP-RhB-2h	1.072	4.457 (78.46)	1.796 (22.93)
DEX-PDP-RhB-3h	1.100	4.35 (75.71)	1.71 (24.34)
DEX-PDP-RhB-4h	1.080	4.27 (72.05)	1.761 (28.76)
DEX-PDP-RhB-5h	1.090	4.24 (67.27)	1.738 (32.96)
DEX-PDP-RhB-6h	1.084	4.15 (64.71)	1.751 (34.77)
DEX-PDP-RhB-7h	1.152	4.23 (63.44)	1.700 (37.30)
DEX-PDP-RhB-8h	1.145	4.091 (61.99)	1.758 (38.49)
DEX-PDP-RhB-10h	1.151	4.055 (60.07)	1.709 (40.40)
DEX-PDP-RhB-24h	1.075	4.002 (52.75)	1.753 (47.46)
DEX-PDP-RhB-48h	1.150	3.882 (49.88)	1.71 (51.85)

^a means description of sample name and time of TCSPC measurement. ^b means life time of RhB bound to polymer and corresponding fractional contribution given in bracket as red colour. ^c means life time of RhB released to polymer and corresponding fractional contribution given in bracket as blue colour.

The rate of RhB release directly depends on the extent to which the enzyme acts on the dextran amphiphiles. Esterase enzyme (10 U) was added to above vesicle solution and decay profiles were collected at different time intervals. Decay profile collected from the enzyme treated vesicular solution was fitted to sum of three exponentials. Fractional contribution of both released (f1) and vesicle encapsulated dye molecule (f2) was obtained by this fitting method. The third component having fractional contribution less than 3 % was considered to be insignificant since $f_1 + f_2$ was > 95 %. Therefore, the time resolved luminescent decay profiles can be used as tool to make out free RhB and RhB loaded in vesicles during the release process. All

this data were plotted together in which enzyme treated samples exhibited a slow transition towards free RhB profile (See figure 5.9b). The fitting of decay profiles aided to estimate the fractional contribution of released free RhB and RhB still located inside vesicular cavity at time 't' (See table 5.2).

The component having life time close to 4.90 ns was judged to be the RhB molecules located inside vesicular hydrophilic cavity (polymer bound). Life time close to 1.70 ns was that of free RhB released from vesicles. The fractional contribution of vesicle encapsulated RhB was 100 % at time $t = 0$ min. Interestingly, the fractional contribution of RhB released when vesicles incubated under normal condition in PBS (pH 7.4), 37 °C and in the absence of enzyme was reached to < 10 % in 48 hrs time period (See Figure 5.10a) On the contrary, dynamic dialysis method overestimated this normal release to about 30 % in 48 hours. The excess mechanical force exerted during dynamic dialysis process may contribute to this reduced stability of vesicles. Interestingly, the life time values of RhB in vesicles and free form did not alter during release process (See Figure 5.10b).

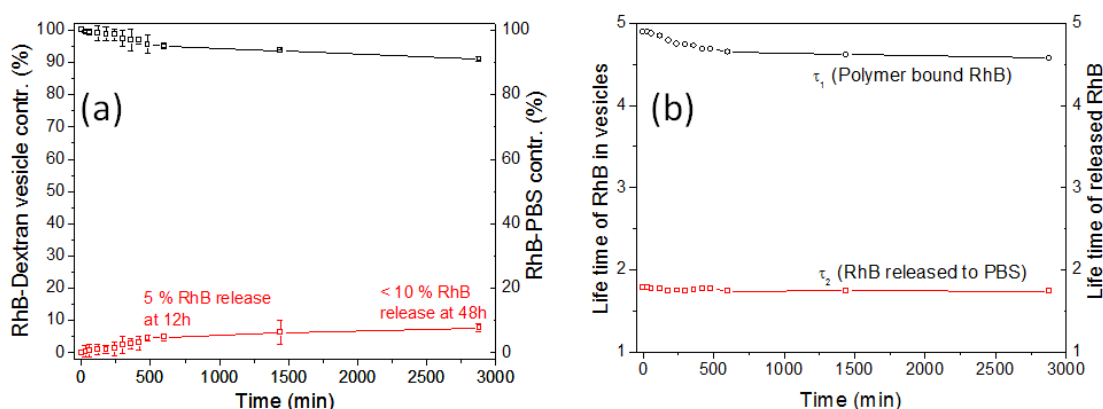


Figure 5.10. Fractional contribution of RhB released and restrained in vesicles Vs time plot (a), life time change of RhB released and confined in vesicles Vs time plot (b). under normal conditions (without enzyme), fractional contribution of RhB released and restrained in vesicles Vs time plot (c) and life time of RhB released and confined in vesicles Vs time plot (d) under enzyme conditions

The fractional contribution of RhB retained in vesicles was reduced upon addition of enzyme and arrived at 35 % in 48 hours time period. The plot of fractional contribution of both the components versus time (See figure 5.11a) provided actual release profiles from dextran vesicles. The life time value of this component was changed from 4.90 at time $t = 0$ min to 3.88 at time $t = 48$ hours (see figure 6d). On the other hand, contribution of free RhB was escalated from 0 % to 58 % in 48 hours. The life time of RhB eluted into solution retained within the range of 1.78 to

1.71 ns, which was identical to that of reported values of RhB in water (See figure 5.11b).

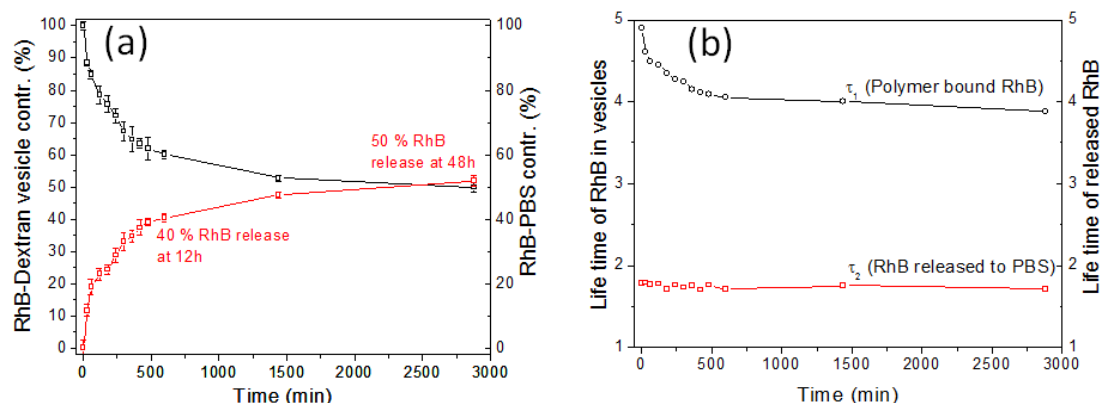


Figure 5.11. Fractional contribution of RhB released and restrained in vesicles Vs time plot (a) and life time of RhB released and confined in vesicles Vs time plot (b) under enzyme conditions

Hence, compared to dynamic dialysis method, real time monitoring using TCSPC technique displayed about $20 \pm 5\%$ difference under normal conditions (without enzyme) and $20 \pm 1\%$ under enzymatic conditions. The actual drug concentration present in the interior hydrophilic cavities of vesicles and rate of drug release across the vesicular wall are the main factors to be considered during drug elution from vesicles in systemic circulation (*in vivo* conditions) as well as in real time analysis using TCSPC technique. But in dialysis method, properties of dialysis membrane and drug partitioning between vesicle and dialysis membrane also affect the final release rate calculation. The physical disturbance occur due to additional mechanical force exerted during dialysis process hasten the transport of drug/dye across the wall. The study confirmed that the stability assessed by real time analysis using TCSPC method can be used for comparing *in-vitro* drug analysis.

5.3.3. DOX and RhB Loaded DEX-IM-5 Vesicles

DOX and RhB were loaded into pH and enzyme dual responsive DEX-IM-5 vesicles and its details were given in chapter 4 (figure 4.15). Light scattering and microscopic image data proved that the vesicular geometry of DEX-IM-5 amphiphiles was not altered with the encapsulated molecules. Similar to DEX-PDP vesicles, the normal absorbance and emission methods could not identify any distinctness between free DOX or RhB and DOX or RhB loaded in DEX-IM-5 vesicles. The absorbance spectra of DOX loaded DEX-IM-5 vesicles showed 4 nm red shift compared to free

DOX (See figure 5.12a). Similarly, RhB in DEX-IM-5 vesicles exhibited 3 nm red shift compared to free RhB in PBS (see figure 5.12b). On the other hand, emission spectra did not display any appreciable difference between RhB and DOX in loaded and free form (see figure 5.12c and 5.12d). Even though drug exist in a confined medium in DEX-IM-5 vesicles as in the case of DEX-PDP vesicles, the absorbance and emission techniques were not able to distinct the change in physical environment. Time resolved luminescent decay profiles of the DOX were studied in the free and in the loaded form in DEX-IM-5 vesicles. TCSPC decay profiles of the free DOX and DOX in vesicles are given in the figure 5.12e. The decay rate constants τ_1 for DOX in free form and in vesicles were obtained as 1.1 and 1.3 ns, respectively. The decay rate constants revealed that the drug molecules have identical life time in free and loaded form in the vesicles and are not suitable for differentiating these two species. Analogous to DEX-PDP vesicles, DEX-IM-5 vesicles also showed a significant difference in the TCSPC life time of free RhB in PBS or water and RhB in vesicles. Life time of free RhB was calculated as 1.74 ns and that of RhB in DEX-IM-5 vesicles was 4.82 ns (see figure 5.12f). Thus the time resolved fluorescence decay profile could be used to quantify the release from pH and enzyme responsive DEX-IM-5 vesicles.

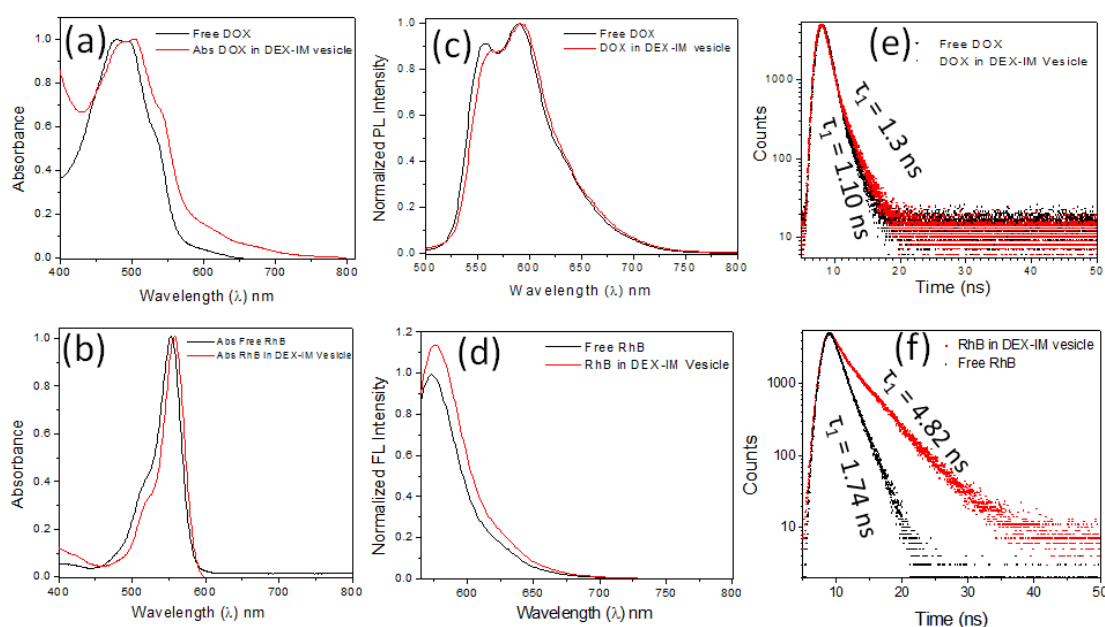


Figure 5.12. Absorbance spectra (a) of free DOX and DOX loaded in DEX-IM-5 vesicles and (b) of free RhB and RhB loaded DEX-IM vesicles. Emission Spectra (c) of free DOX and DOX loaded in DEX-IM vesicles and (d) of free RhB and RhB in vesicles. fluorescence life time plot (e) of free DOX and DOX loaded in DEX-IM vesicles and (f) of free RhB and RhB in vesicles.

5.3.4. Real-time Drug Release Studies from DEX-IM Vesicles by TCSPC Method

Drug release studies under acidic pH conditions and in the presence of esterase enzyme were performed by adopting similar procedure as illustrated in the case of DEX-PDP vesicles. The absorbance of RhB loaded DEX-IM-5 vesicular solutions was adjusted to 0.2 O.D. using phosphate buffer (PB, pH 6.0 or pH 5.0) and used for TCSPC analysis directly or by adding 10 U esterase enzyme as explained in experimental sections. The decay profile collected at pH 6.0 (see figure 5.13a), pH 5.0 (see figure 5.13b), pH 7.4 and esterase (see figure 5.13c) and pH 6.0 and esterase (see figure 5.13d) was fitted with DAS6 decay analysis software.

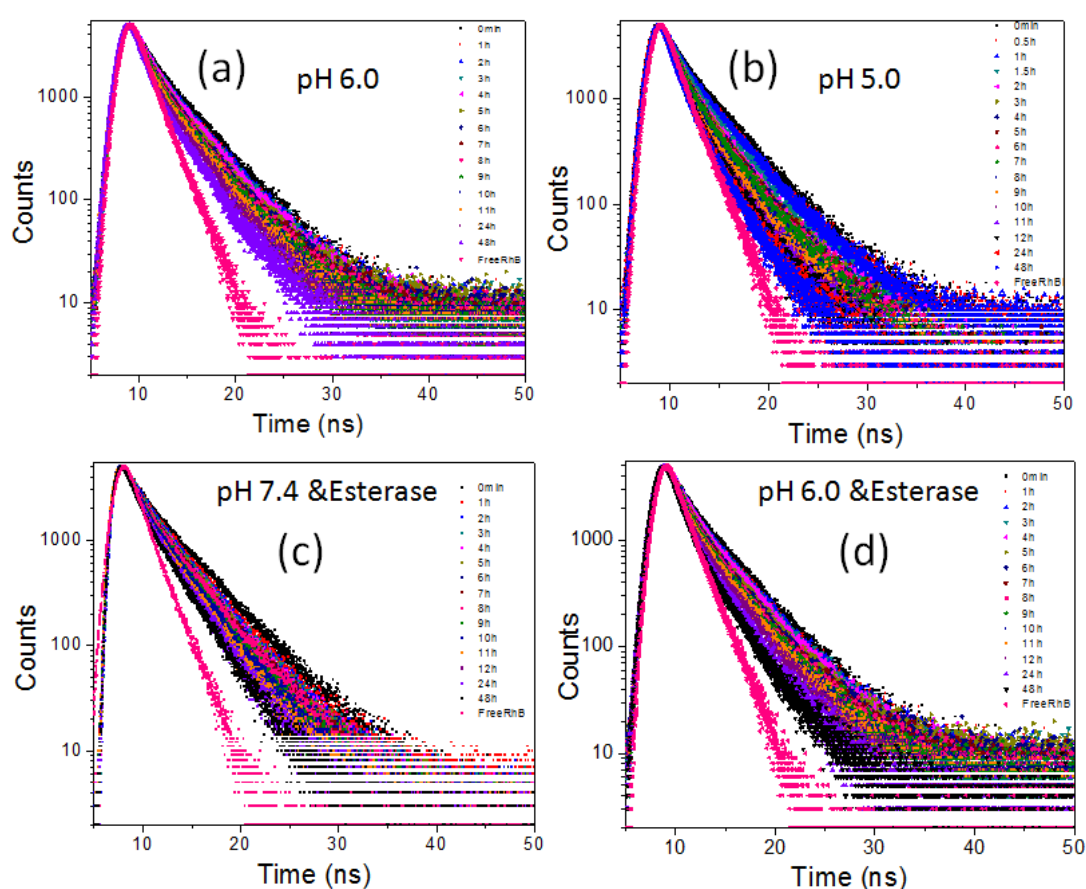


Figure 5.13. Fluorescence life time plot of RhB in DEX-IM vesicles (a) at pH = 6.0 (b) pH = 5.0, (c) at pH 7.4 and esterase enzyme and (d) pH 6.0 and esterase enzyme.

As explained earlier, acidic pH can hydrolyze imine linkage connecting PDP and dextran and subsequent leakage of loaded RhB content. Therefore the time resolved fluorescence of RhB loaded vesicles were collected under acidic pH conditions relevant to cancer pH (pH 6.0) and intracellular lysosomal pH (pH 5.0) (see figure 5.13a and 5.13b). Fractional contribution of RhB released (f1) and

polymer bound RhB molecule (f2) was obtained by fitting method as described in DEX-PDP vesicles (see figure 5.10 and 5.11) (See annexure for tables).

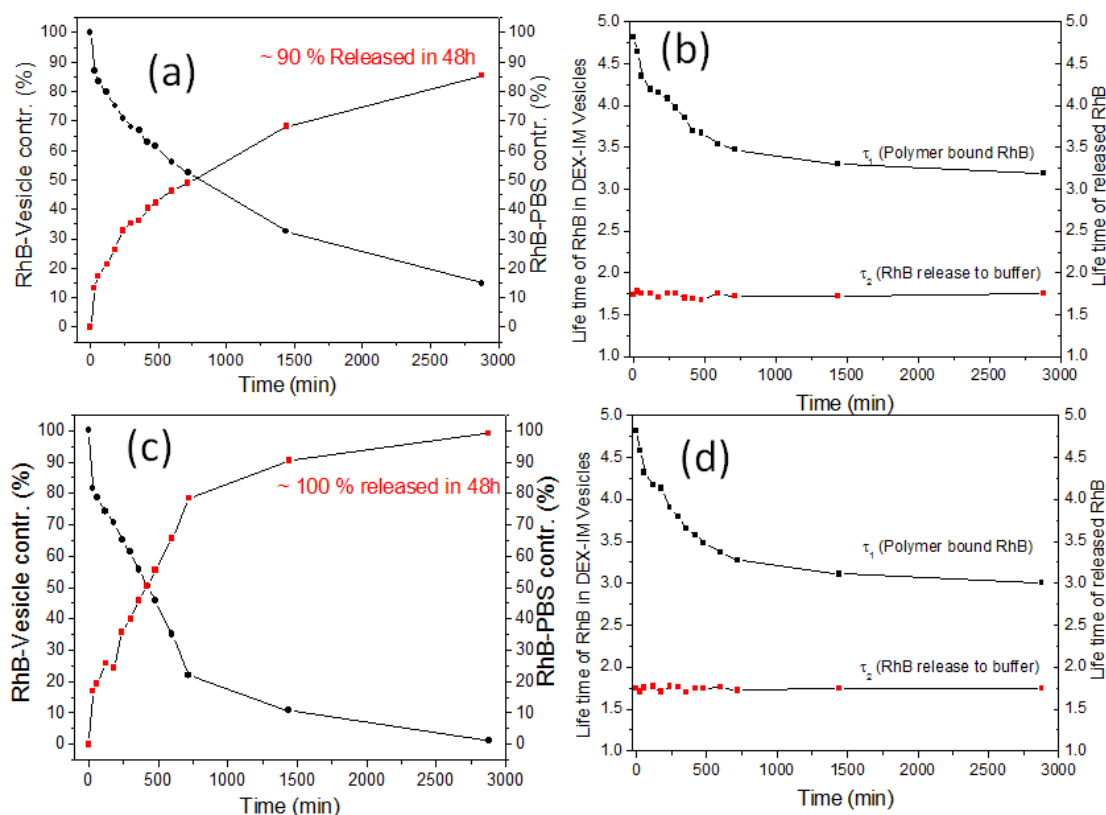


Figure 5.14. Fractional contribution of RhB released and retained in vesicles Vs time plot (a) and life time of RhB released and confined in vesicles Vs time plot (b) under pH 6.0 conditions. Fractional contribution of RhB released and retained in vesicles Vs time plot (c) and life time of RhB released and confined in vesicles Vs time plot (d) under pH 5.0 conditions.

At pH 6.0, the component having life time close to 4.82 ns was decreased with time and reached to 90 % (see figure 5.14a and 5.14b). It means that the RhB molecules located inside vesicular hydrophilic cavity (polymer bound) eluted out and the fractional contributions of vesicles loaded RhB was declined. Remarkably, this value was almost 20 % more than that predicted by dynamic dialysis method (see figure 4.19). Life time of RhB loaded in the vesicles also reduced to 3.2 ns from 4.82 ns (see figure 5.14b). A similar trend was observed in the case of pH 5.0 (see figure 5.14c and 5.14d). At this higher acidic pH more rapid release of RhB was observed from DEX-IM-5 vesicles and the release was reached to almost 100 % in 48 hours time period. Life time of RhB loaded in the vesicles drastically reduced to 3.0 ns from 4.82 ns (see figure 5.14d). However, life time of released RhB was retained in the range of 1.68 ns to 1.78 ns as observed in the case of DEX-PDP vesicles.

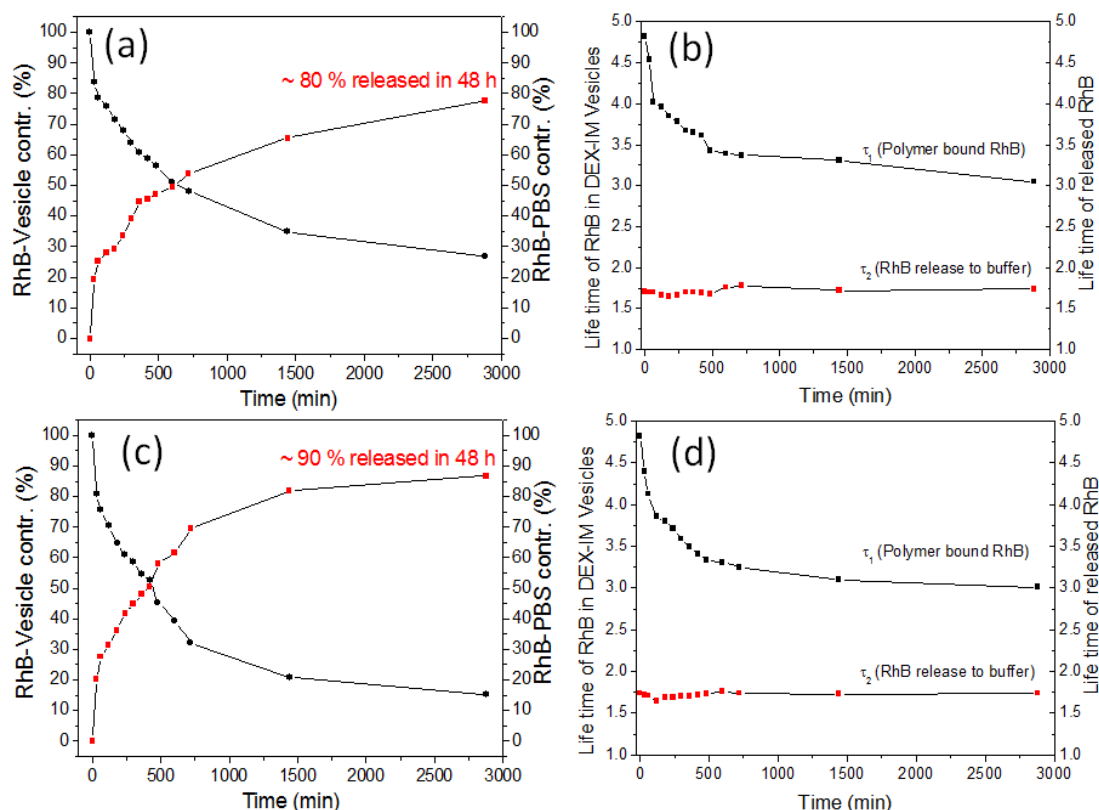


Figure 5.15. Fractional contribution of RhB released and retained in vesicles Vs time plot (a) and life time of RhB released and confined in vesicles Vs time plot (b) under pH 7.4, and esterase enzyme conditions. Fractional contribution of RhB released and retained in vesicles Vs time plot (c) and life time of RhB released and confined in vesicles Vs time plot (b) under pH 6.0 and esterase enzyme conditions.

Esterase enzyme along with acidic pH enhances the release rate from the DEX-IM-5 vesicles (see figure 4.19). This result was more accurately proved using TCSPC analysis, where esterase enzyme was added to pH 7.4 PBS incubated DEX-IM-5 vesicle solutions (see figure 5.15a and 5.15b) and pH 6.0 PB incubated DEX-IM-5 vesicle solutions (see figure 5.15c and 5.15d). Esterase enzyme at pH 7.4 itself augmented the release rate from DEX-IM vesicles and about 80 % of RhB was released in 48 hours (see figure 5.15a). Life time value of polymer bound RhB was reduced from 4.82 ns to 3.02 ns (see figure 5.15b). On the other hand acidic pH (pH 6.0) together with esterase enzyme further improves the release rate from DEX-IM-5 vesicles to about 90 % in 48 hours (see figure 5.15c). In this case life time of RhB loaded in vesicles was reduced to 3.02 ns from 4.82 ns (see figure 5.15d). As expected, life time of RhB eluted in to buffer solutions was stayed in the range of 1.68 ns to 1.76 ns (see annexure for tables). Thus, the time resolved luminescent decay profiles can be used as tool to make out free RhB and RhB loaded in vesicles during the release process.

5.3.5. Docking studies: Esterase enzyme action on DEX-PDP and DEX-IM vesicles

The *in-vitro* drug release studies using dynamic dialysis and TCSPC method showed that the release of RhB from the DEX-PDP and DEX-IM-5 vesicles was enhanced in the presence of esterase enzyme. So to understand this rapid dissociation of drug from vesicles in detail, docking of monomer and trimer units of DEX-PDP and DEX-IM-5 vesicles to the catalytic site of human liver carboxyl esterase-1 was performed. The studies on esterase enzyme proved that its active site constitutes a catalytic triad composed of three amino acids - Serine, Histidine and Glutamine, which is responsible for hydrolysis of esters (See Figure 5.16a and 5.16b). As a first step, carbonyl carbon of the ester portion of the substrate gotten attacked by the nucleophilic Serine hydroxyl group. This nucleophilic attack breaks the π -bond and forms a tetrahedral intermediate, which is stabilized by the oxanion hole of the enzyme. Later on, cleavage of intermediate is happened to form alcohol and acid which was removed from the active site by diffusion^{30,31} (See Figure 5.16c for mechanism of cleavage illustrated using DEX-PDP as an example).

The aim of the docking study was to understand the affinity and preference of monomer and trimer units of DEX-PDP and DEX-IM-5 vesicles towards the amino acids in the catalytic triad of the esterase enzyme. The optimization of monomer and trimer units of DEX-PDP and DEX-IM molecules were performed using Gaussian 03 software (Hartree-Fock theory using 6-31G* basis set)¹⁶ and the restrained electrostatic potential (RESP)¹⁷ charges on the atoms of the optimized structures were estimated using Antechamber module of AMBERTools¹⁸ (See Figure 5.16d for optimized structures). As the initial step in the hydrolysis process was the nucleophilic attack by the hydroxyl group of Serine, the extend of cleavage of dextran-PDP vesicles could be correlated to the position and orientation of the carbonyl group of the vesicles with respect to the hydroxyl group of Serine (See Figure 5.16c for mechanism of DEX-PDP ester bond cleavage associated with catalytic triad. A similar mechanism can be predicted for DEX-IM-5).

The docked configurations of monomer units of DEX-PDP vesicles showed that they are positioned well inside the catalytic triad (as shown in Fig. 5.17a) with appreciable binding energies (see table 5.3). The binding energy of DEX-PDP monomer was lower and suggested that it forms much favorable interaction with the active site of the enzyme. Also, the docked configurations confirmed that the carbonyl

group of DEX-PDP monomer was closer to the serine hydroxyl group. The catalytic cleavage of the ester group in monomer unit of DEX-PDP may be faster because oxygen of the carbonyl group faces downwards exposing the carbonyl carbon more towards the serine hydroxyl group (as shown in Fig. 5.17a) for easy nucleophilic attack.

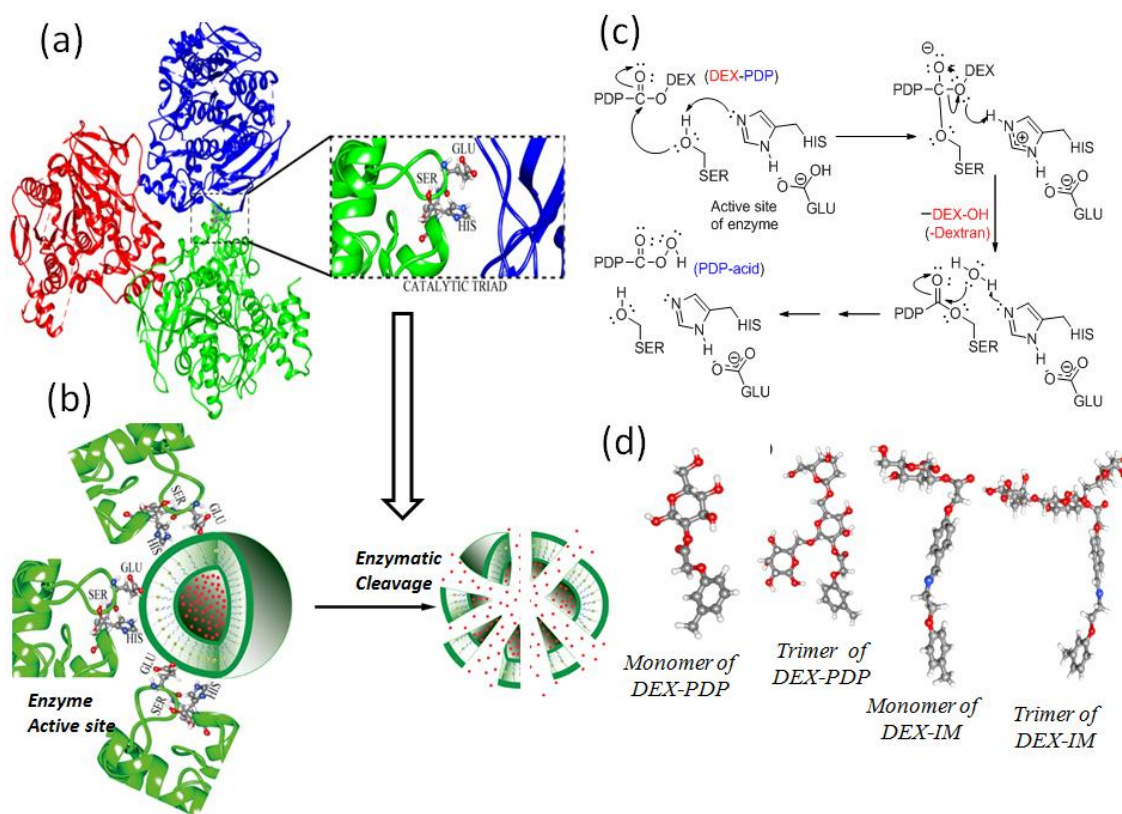


Figure 5.16. (a) The ribbon representation of Human liver carboxyl esterase-1(PDB ID: 3K9B), (b) schematic representation of catalytic triad binding to vesicles and vesicle cleavage, (c) Mechanism of action of Ser-His-Asp catalytic triad on DEX-PDP molecule to get dextran and PDP acid molecules and (d) the optimized structures of monomer of DEX-PDP, trimer of DEX-PDP, monomer of DEX-IM and trimer of DEX-IM.

To understand the effect of polymer chain, the docking was extended from monomer to trimer units of DEX-PDP vesicles. The docking results are summarized in the table 5.3 and the corresponding docked configurations are shown in Figure 5.17b. The interaction between serine hydroxyl group and ligand carbonyl group was shown and marked by the yellow oval shapes. Here also, the binding energy of DEX-PDP trimer was more favorable and the positive binding energy was due to the presence of large number of rotatable bonds in comparison with the corresponding monomers (See table 5.3). The analysis of the docked configurations demonstrated

that the DEX-PDP trimer was more prone to nucleophilic attack by serine hydroxyl group because of the close vicinity and favorable orientation of the carbonyl group (Fig. 5.17b).

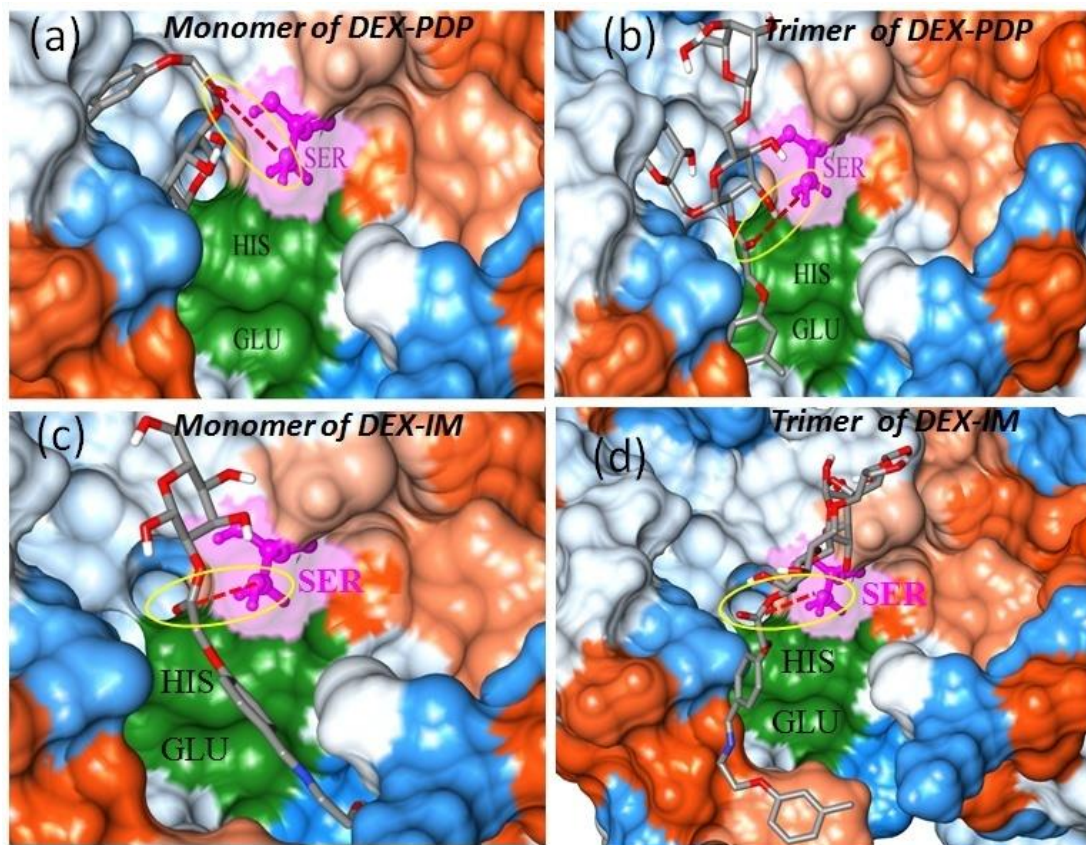


Figure 5.17. The docked configurations of (a) monomer of DEX-PDP (b) trimer of DEX-PDP (c) monomer of DEX-IM and (d) trimer of DEX-IM

A similar observation was made in the case of monomer and trimer unit of DEX-IM-5 vesicles (see figure 5.17c and 5.17d). The docked conformation of monomer unit of DEX-IM-5 system showed that they were situated inside the catalytic triad (see figure 5.17c) of esterase enzyme. Moreover, the negative binding energy values of DEX-IM-5 monomer indicated superior binding and favorable interaction with active site of the enzyme (see table 5.3). The docked configurations further established that the serine hydroxyl group of enzyme is adjacent to carbonyl group of DEX-IM-5 monomer (see yellow circles in figure 5.17). This close proximity enhances catalytic cleavage of the ester group in the DEX-IM-5 molecules. The docking studies were extended to trimer unit of DEX-IM-5 and the corresponding docked conformations were shown in figure 5.17d. The close vicinity of serine hydroxyl group of enzyme and carbonyl group of DEX-IM-5 supports rapid

hydrolytic cleavage action of esterase. Thus the docking studies explained the experimental observation of rapid hydrolysis of DEX-PDP and DEX-IM-5 vesicles by esterase enzyme in terms of favorable binding energy, position and orientation of carbonyl group with respect to serine hydroxyl group for the initial nucleophilic attack during ester hydrolysis.

Table 5.3: Table depicts different energy parameters obtained after docking of DEX-PDP and DEX-IM-5 on Human liver carboxyl esterase-1.

Energy (Kcal/mol)	DEX-PDP Monomer	DEX-PDP Trimer	DEX-IM-5 Monomer	DEX-IM-5 Trimer
Binding Energy	+0.4	14.2	-0.11	3.08
Internal Energy	-2.1	6.9	-4.58	-4.98
Vander Walls Desolvation Energy	-2.1	6.9	-4.58	-4.98
Electrostatic Energy	-1.3	-1.5	-0.36	-1.9
Tortional Energy	3.0	6.3	4.47	8.05
Unbound Energy	-3.0	0.9	-3.41	-8.66

To sum up, a novel technique based on fluorescent decay profile and life time of rhodamine B fluorescent dye molecule was described to determine the enzymatic release kinetics from polysaccharide vesicles. The limitations of conventional steady state absorbance techniques+ dialysis method in distinguishing species such as RhB-PBS (released) and RhB-carrier (polymer bound) was effectively prevailed over using real time analysis method. The rapid degradation kinetics of vesicles in the presence of enzyme stimuli was precisely calculated explained using TCSPC method and docking studies. The method is not only restricted to RhB, but in general, it may be applicable to wide range of probes such as proteins and genes to evaluate their delivery systems.

5.4. Conclusion

In conclusion, a new method based on fluorescent life time of RhB fluorophore was developed to study the drug release kinetics from enzyme &/or pH responsive polysaccharide vesicles. Amphiphilic dextran polysaccharide derivatives were encapsulated with RhB and DOX molecules and these luminescent vesicles provided new opportunity to study the real time drug release profiles in cell penetrable polymer carrier in cancer therapy. The pitfalls of widely adopted dynamic dialysis+ absorbance method in drug release kinetics were successfully overcome using time resolved fluorescent method. Unlike dialysis approach, TCSPC method provided important information regarding the superior stability of dextran vesicles under normal systemic circulatory conditions. The real-time drug release kinetics based on fluorescence life time and their fractional contribution was found to be more accurate by 20-25 % compared to the dialysis method. The basis for enhanced hydrolysis rate in the presence of enzyme was investigated using docking studies. The specific positioning of the ester linked amphiphilic unit in the active site of enzyme and the binding energy data supported the ability of enzyme to attack on vesicles. The present investigation provided a new insight into the accurate release rate determination from enzyme responsive polysaccharide vesicles that are very good drug delivery vehicles for breast and colon cancers. Though, the approach here demonstrated only to RhB and DOX loaded polysaccharide vesicles, in principle, this technique could be adapted for large number of other polymers as well as drug molecules. Further, polysaccharide vesicles loaded with RhB is very good biological probe for fluorescent life imaging microscopes for quantifying or studying the cellular uptake mechanistic pathways and live cell imaging. Currently, the future research work is focused on these directions to utilize the new polysaccharide probe for studying cancer as well as making new therapeutics for better cancer treatment.

5.5. References

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Summary and Future Directions

The thesis entitled “***Tailor-made Polysaccharide Vesicles for Drug Delivery to Cancer Cells***” deals with design and development of amphiphilic molecules from polysaccharide dextran conjugated with renewable resource Cashew Nut Shell liquid (CNSL) based pentadecyl phenol (PDP) and cardanol for drug delivery applications. The thesis is focused to develop highly organized vesicular geometry from polysaccharide structure for encapsulating hydrophilic and hydrophobic drug molecules in its aqueous core and membrane respectively. The chemical structures were suitably designed in order to bring responsiveness to vesicles against stimuli such as enzymes (esterase) and pH for intracellular drug delivery. Efforts were also put to develop a new method based on time co-related spectroscopy for *in-vitro* drug release evaluation from polysaccharide vesicles.

The roles of the hydrophobic segments on the molecular self-organization of dextran backbone into vesicles or nanoparticles were investigated by conjugating with 3-pentadecyl phenol (PDP), cardanol and commercially available stearic acid (SA). Single crystal structures were resolved and their data revealed that the renewable hydrophobic tails of PDP inter-digitized to produce lamellar structure for the formation of dextran vesicular assemblies. The vesicular scaffolds were found to be very unique dual loading carriers for hydrophilic and hydrophobic guest molecules. This concept was explored initially using encapsulation of Rhodamine-B (water soluble dye) and water insoluble anticancer drug camptothecin (CPT). Esterase enzyme acts as stimuli for disassociating the polymer scaffold for faster release of drug molecules. The confocal microscopic images of the RhB loaded vesicles and CPT loaded vesicles evidence for the cellular uptake abilities of dextran vesicles and their selective perinuclear localization in cells. The drug was found to be twice efficient in killing the cells when loaded in the vesicular scaffold compared to CPT alone.

The role of the caveolar endocytic pathway in vesicles endocytosis were evaluated using caveolae containing (Cav-1 ^{+/+} MEFs) and lacking (Cav-1 ^{-/-} MEFs, breast cancer cells, MCF7 and colon cancer cells, DLD1) cells. The study using rhodamine B loaded dextran vesicles proved the uptake of these polymer scaffolds is interestingly promoted by the absence of caveolae. Thus more amount of drugs accumulated in cancer cells than normal cells. The multiple drug loading capabilities of these vesicles were further studied using anticancer drugs DOX (DNA intercalating

agent, topoisomerases II inhibitor) and CPT (topoisomerases I inhibitor). The sequential deliveries as well as synergistic killing of cancer cells were evaluated using these drug combinations. Dual drug loaded vesicles were also compared to drug cocktails with individual drugs in separate nanoparticles (at comparable molar ratios) to further assess the significance of dual loading in cancer cell killing. Results from these experiments have revealed that newly developed polysaccharide vesicles loaded with DOX and CPT drugs as potential candidates for improved cancer cell killing.

The concept of dual responsive polysaccharide vesicles were brought by synthesizing dextran conjugated PDP via imine chemical linkage (pH stimuli) and the aliphatic ester bond (esterase enzyme stimuli). Importantly, DOX drug chemically conjugated and physically loaded polysaccharide scaffolds were made with identical size and shape (vesicular morphology) and drug release kinetics. This allowed to understand the roles of core-loaded and layer-anchored doxorubicin (DOX) vesicular carriers in breast cancer cells. *In-vitro* drug release studies proved that the polysaccharide vesicles deliver almost 100 % of the anticancer drugs at low pH = 6.0 to 5.0 (both at the cancer tissue as well as intracellular compartments) and under enzymatic cleavage (intracellular by lysosomal enzyme- esterase). DOX loaded vesicular scaffolds (as well as anchored) were tested for cellular uptake and cytotoxicity in MCF7 cells. The chemically conjugated drug showed slow drug release pattern with 30 % killing effect; thus, it could be very useful for long circulation of drugs in the *in vivo* conditions.

. The reliable methods to calculate the *in vitro* drug release kinetics are necessary for establishing a good correlation between drug release capabilities and clinical effectiveness of formulation of synthetic polymer vesicles. Real-time analysis of drug release kinetics in-situ methods are very good protocol to overcome some of the above limitation and correlate the drug release kinetics pattern by the spectroscopic tools to their actual action in the cell lines *in vitro* conditions. However, there is no effort has been taken until now to study the real-time drug release kinetics of these enzyme responsive polymer vesicles to unlock the mechanism of these enzymes action on polymer backbone in the literature. The pitfalls of widely adopted dynamic dialysis+ absorbance method in drug release kinetics were successfully overcome using time resolved fluorescent method. Esterase enzyme responsive DOX and RhB loaded dextran vesicles were used to develop real-time drug release

protocols. Real time analysis predicted enzyme responsive release kinetics with 20-25 % much less compared to the absorbance+dialysis method that routinely employed in the literature. The ability of enzyme to cleave the polysaccharide vesicles was also proved using docking studies.

Future Directions

This thesis work brought together the concept of renewable resource polysaccharide vesicles for loading and delivering anticancer drug molecules. However, one of the main problems associated with current design is the natural leakage of physically loaded drug molecules from vesicles under physiological conditions. About 30 to 40 % of drug molecules were eluted from vesicles before reaching to target site. One method to reduce this natural leakage is to stitch vesicle membrane using stimuli responsive chemical linkages (see figure 6.1a). Therefore ongoing projects concentrated on developing carboxyl group functionalized dextran-PDP molecules and subsequent vesicle membrane cross linking using cisplatin anticancer drug molecules. The design provides not only polysaccharide conjugated cisplatin prodrug but also cross linked vesicles for loading and delivery other anticancer drugs (see figure 6.1a). Moreover, *in-vivo* studies using animal models provides more information about the clinical usefulness of both drug loaded and drug conjugated vesicles.

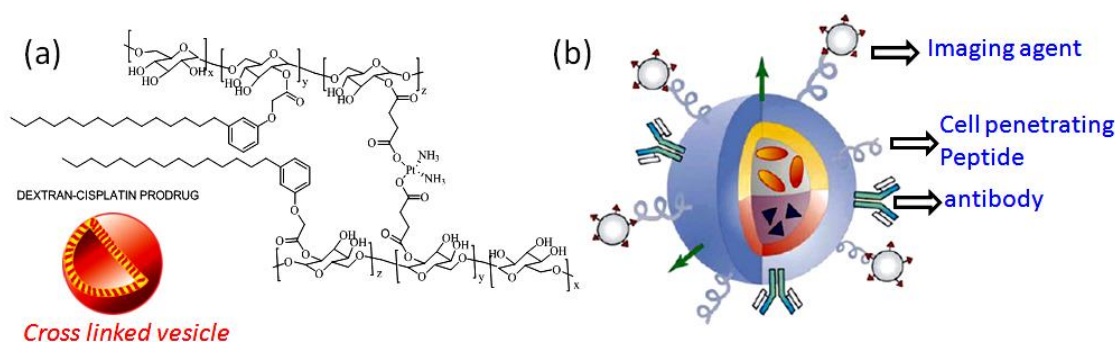


Figure 6.1. Future directions of thesis work: (a) development of cross linked vesicle by stitching DEX-PDP molecule with cisplatin anticancer drug. (b) Schematic representation of vesicle attached with imaging agent, cell penetrating peptide and targeting antibody.

Ideally, the next generation polymer vesicles should combine key features such as biodegradability, biocompatibility, stimuli (pH, temperature, reduction, enzyme etc.) responsiveness along with attachment of functional particles such as

imaging probes, antibody to target and reactive functionality (see figure 6.1b). For instance, conjugation of diagnostic agent (*e.g.* magnetic nanoparticles, quantum dots) brings theranostic properties to drug loaded/conjugated polymer vesicles. Similarly, attachment of cell penetrating peptides (*e.g.* poly Arg peptide TAT) along with targeting antibody (*e.g.* Alemtuzumab for lymphocytic lymphoma, Rituximab for Hodgkin lymphoma, Trastuzumab for breast cancer, Cetuximab for colorectal tumor, Panimumab for Metastatic colorectal tumor) enhances the intracellular delivery capabilities of vesicles as well as reduces the unwanted side effects associated with non specific delivery. Therefore future research work focuses on the merging the aspects of functionalization and attachment of targeting ligand to dextran-PDP (DEX-PDP) by maintaining its vesicular geometry.

List of Publications

Publications in International Journals:

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1. **Pramod, P. S.**; Takamura, K.; Chaphekar, S.; Balasubramanian, N.; Jayakannan, M. Dextran Vesicular Carriers for Dual Encapsulation of Hydrophilic and Hydrophobic Molecules and Delivery into Cells. *Biomacromolecules*, **2012**, 13, 3627-3640. (Appeared as spot light article).
2. **Pramod, P. S.**; Shah, R.; Chaphekar, S.; Balasubramanian, N.; Jayakannan, M. Polysaccharide nano-vesicular multidrug carriers for synergistic killing of cancer cells. *Nanoscale*, **2014**, 6, 11841-11855.
3. **Pramod, P. S.**; Shah, R.; Jayakannan, M. Dual Stimuli Polysaccharide Vesicles for Chemically Conjugated and Physically Encapsulated Doxorubicin Delivery in Breast Cancer Cells. *Nanoscale*, **2015**, 7, 6636-6652.
4. **Pramod, P. S.**; Deshpande, N. U.; Jayakannan, M. Real-time Drug Release Analysis of Enzyme Responsive and Cell Penetrable Polysaccharide Nano-vesicles. *J. Phys. Chem B*, **2015**, Just accepted, DOI 0.1021/acs.jpcc.5b05795.
5. Sridhar, U.; **Pramod, P. S.**; Jayakannan, M. Creation of dextrin vesicles and their loading-delivering capabilities. *RSC Adv.* **2013**, 3 (44), 21237-21241. (not included in thesis chapter).
6. Suranar, B.; **Pramod, P.S.**; Jayakannan, M.; *ZAAC*, **2014**, 640, 1119-1126. (Dedicated to C.N.R Rao: not included in thesis chapter).

Publications in International Conference Proceedings:

1. Pramod, P.S.; Balasubramanian, N.; Jayakannan, M. Dextran Unilamellar Vesicles for Camptothecin loading and delivery, *INTER IISER CHEMISTRY MEET*, Trivandrum, India, Dec. 10-12, **2011**. (Selected for best Poster Award).
2. Pramod, P.S.; Balasubramanian, N.; Jayakannan, M. Polysaccharide Vesicular Nanocarrier for Encapsulation and Delivery of Molecules into Cell, *PolyTech – 2012: International Conference on Advanced Polymeric Materials and Nanotechnology*, Pune, India, December, 15-17, **2012**.

3. Pramod, P.S.; Balasubramanian, N.; Jayakannan, M. Polysaccharide Vesicular Nano carrier for Encapsulation and Delivery of Anticancer Drugs, *FAPS-MACRO 2013*, Bangalore, India, October, 21-24, **2013**.
4. Pramod, P.S.; Balasubramanian, N.; Jayakannan, M Polysaccharide Vesicular Nano carrier for Encapsulation and Delivery of Multiple Anticancer Drugs, *Bilateral Indo-US workshop on Bioinspired supra molecular self assembly*, Trivandrum, India, December 15-17, **2013 (Selected for best Poster Award)**.