Synthesis and Characterization of pH Responsive Methoxy Polyethylene Glycol - Polypropylene Fumarate Polymeric Micelles for Controlled Drug Release

A DISSERTATION SUBMITTED

BY S. GIRIJA

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS

FOR THE AWARD OF

MASTER OF PHILOSOPHY

SREE CHITRA TIRUNAL INSTITUTE FOR MEDICAL SCIENCE AND TECHNOLOGY

THIRUVANANTHAPURAM

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DECLARATION

I, **Girija S.**, hereby certify that I had personally carried out the entire work on depicted in the thesis entitled, "**Synthesis and Characterization of pH Responsive Methoxy Polyethylene Glycol -Polypropylene Fumarate Polymeric Micelles for Controlled Drug Release**" for the degree of Master of Philosophy in Biomedical Technology Wing. Other sources of support and information have been acknowledged in the text. I also declare that the content of this dissertation has not previously submitted for the award of any degree or diploma up to this date.

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CERTIFICATE

This is to certify that Ms. **Girija S.** from polymer division of this institute, submitted her thesis entitled "**Synthesis and Characterization of pH Responsive Methoxy Polyethylene Glycol - Polypropylene Fumarate Polymeric Micelles for Controlled Drug Release**" is bonafide work done by her in partial fulfillment for the degree of master in philosophy. No part of this embodied matter was submitted for any award of degree or diploma prior to this date.

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ABBREVIATIONS

PEG	Poly(ethylene glycol)
PPF	Carboxyl terminated polypropylene fumarate
MTT	(3-(4,5–dimethylthiazol-2-yl)–2,5–diphenyltetrazolium bromide)
HPMA	N-2 hydroxy propyl methacrylamide
DEF	Dimethyl fumarate
PDC	Polymer drug conjugate
EPR	Enhanced permeation effect
COX	Cyclooxygenase
PKa	Acid dissociation constant
FDA	Food drug administration
PBS	Phosphate buffer saline
TEMED	Tetramethylenediamine
THF	Tetrahydro furan
Ar	Aromatic
DMAEMA	N,N–dimethylaminoethyl methacrylate
HPLC	High pressure liquid chromatography
CMC	Critical micelle concentration
PLLA	Poly L Lactic acid
DLS	Dynamic light scattering

SEM	Scanning electron microscope
IC	Immunoconjugates
FBS	Fetal bovine calf serum
DEAEMA	N,N–diethylaminoethyl methacrylate
DMSO	Dimethyl sulphoxide
GPC	Gel permeation chromatography
PEAA	Poly ethyl acrylic acid
PBAAc	Poly butyl acrylic acid
PPAAc	Polyproyl acrylic acid

Chapter 1

INTRODUCTION

Background

Drug delivery is a method through which a pharmaceutical compound is delivered for a safer therapeutic effect. Based on a drug's metabolism and toxicity, the design and route of administration is often decided. In conventional drug therapy, the drug is delivered as a single bolus dose and this is associated with many unintended systemic side-effects. Hence, delivery systems with controlled and targeted drug release capabilities are greatly needed to minimize systemic toxicity and to increase therapeutic efficacy. This is relevant to pathologically chronic conditions such as cancer, rheumatoid arthritis, inflammatory bowel diseases, hypersensitivity reactions etc. These conditions would greatly benefit if the drug is released in controlled and sustained manner in response to the surrounding pathological milieu to reduce systemic toxicity. Hence, newer technologies in the area of controlled drug delivery are clearly needed to deliver drugs safely at the targeted site to achieve maximal therapeutic effect.

Research on drug delivery is progressing for the past 60 years. It started in the early 1950's with controlled oral delivery and transdermal delivery systems. The mechanism of

delivery was primarily through osmosis, ion exchange, diffusion and dissolution.[1] Oral drug intake was also very frequent during these times. Drug absorption depended on many factors such as mixing of drug within the digestive fluid, gastro-intestinal pH, and different rates of absorption in different sites of the digestive system; all of which led to poor systemic circulation of drug in the body. This limitation was overcome by the 1980s through the development of zero order and first order drug release kinetics from drug carriers such as polymer drug conjugates, liposome, and micelles. But zero order release kinetics failed to stabilize the concentration of the released drug in the blood and first order kinetics always maintained a constant drug concentration in blood, both of which are not ideal for drug therapy. Later, smart polymers, which delivered the drug in response to the environmental stimulus, with tissue-targeting were developed. These newer drug delivery systems showed promise for lowering systemic toxicities. These novel methodologies paved the way for the delivery of biological molecules like DNA through gene delivery and macromolecules such as proteins through protein delivery via liposomes (mimics lipid bilayer of cell [2]) and micelles. Further progresses have established quantum dots, carbon nano-tubes, amino acid and protein-based drug carriers for safer therapeutic effect. However, for clinical application of these novel drug delivery systems, long-term *in vivo* safety profiles need to be well-established in the future. Nevertheless, these developments show great promise for targeted drug delivery.

Drug carriers:

Drug carriers are used for safe, targeted and effective delivery of encapsulated drug. Carriers are designed based on drug application and route of delivery. They are classified as micro- and nano- carriers. Nanocarriers possess numerous advantages over micro carriers, as micro carriers are larger in size and get easily cleared out by the reticuloendothelial system. Furthermore, micron size particles do not possess an effective enhanced permeation

retention [EPR] effect. On the other hand, nanocarriers have special properties such as small size, higher surface to volume ratio, enhanced EPR effect which significantly increases targeting efficiency compared to micro carriers [3][4]. Due to the above reasons, drug carriers in the nano form are commonly preferred for delivery of therapeutics such as liposome, micelles, and metal nano-particles, etc.

Liposome

Major vesicular carriers include liposome and micelles which are designed using natural and synthetic polymers. Liposomes are bilayered lipid structures which are capable of carrying both hydrophobic drugs (inner core) and hydrophilic drugs (between the inner and outer layers) simultaneously[5]. However, drug loading capacity in liposomes is low and oral delivery of liposome is plagued by stability issues in the gastrointestinal tract [6]. Injectable delivery of liposomes also results in poor controlled release [7].

Micelles

Micelles are colloidal dispersions with hydrophobic core and hydrophilic shell and are amphiphilic in nature. Micelles are formed by repulsive and attractive forces. As they contain hydrophilic groups, the steric hindrance pushes apart the hydrophobic groups which attract to each other due to van der Waals force to avoid an aqueous environment. The stability of micelle is based on the strong hydrophobic block in the amphiphilic molecule. As the concentration of amphiphilic molecule increases hydrophobic end starts to aggregate and micelle formation takes place. The minimal concentration required for micelle formation is termed as critical micelle concentration (CMC). Micelles system has many advantages over other drug carriers as preparation of micelles is easy and they possess high drug loading capabilities. This in turn increases drug bioavailability and hence they stay in the body for longer periods of time [8].

Polymeric micelles

Polymer drug conjugate (PDC) was termed by Helmut Ringsdrof in 1975[9]. Ringsdrof's model polymer drug conjugates were designed with biocompatible polymer with solubilizer to improve stealth properties and for solubility of hydrophobic drugs. [10] The covalently bounded drug molecule along with cell surface targeting moieties reach the biological target site and avoid other secondary systemic toxicities. This approach clearly improved drug pharmacokinetics. Various polymeric designs such as linear polymers, dendrimers, micelles are conjugated with drugs and has been used for many biological applications. Normally, block copolymers like diblock, multiblock and graft copolymers are used to construct an amphiphilic polymer. Based on the block copolymer composition, the shape of the micelle is predicted, for example spherical structure of rods or lamellae [11]. Increased chain length of hydrophilic block improves stealth properties whereas increased chain length of hydrophobic block decreases CMC [12]. Furthermore, chain length of hydrophobic blocks leads to rapid self assembly and supports the stability of micelles. In this regard, CMC of block polymers, in the range of 10^{-6} M, which is lower than that of common surfactants [13] have been reported.

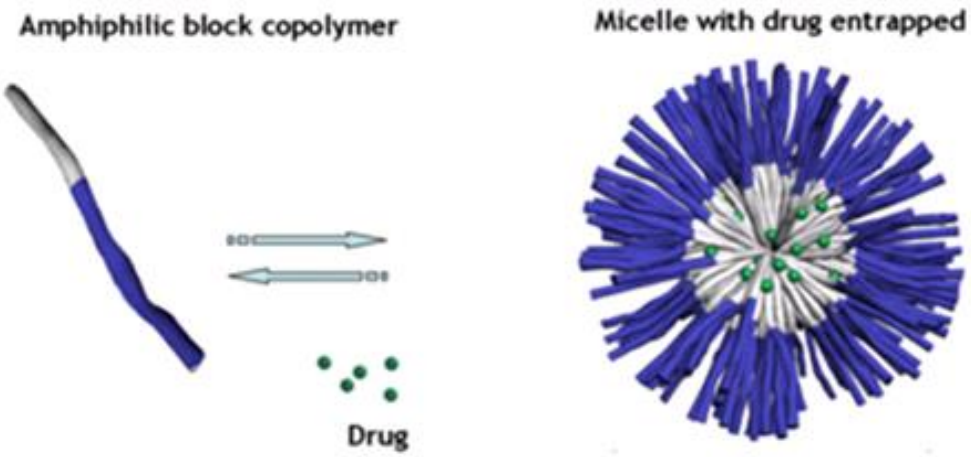


Figure 1: Block copolymeric micelle

Polymers in drug delivery:

Biodegradable polyesters such as poly (lactic acid), poly(Ɛ-caprolactone), poly(glycolic acid), δ- valerolactone, and poly(amino acids) such as glutamic acid, histidine, lysine, aspartic acid, and polyethers such as polyethylene glycol (PEG) and copolymers of PEG are commonly used as drug delivery systems. Usually polyesters are preferred for its solubility and degradation characteristics. Drug loading efficiency of polyesters depends on the hydrogen bond interaction and crystallinity of polymer as shown in case of polylactones [12]. For example, polyanhydrides have been used for drug delivery applications as it undergoes surface erosion for sustained drug release *in vivo*. Furthermore, anhydride linkage between hydrophobic polymer ends and drug conjugates in micelles aid in controlled release characteristics as anhydride bond are susceptible to hydrolysis. Polyanhydrides with disulfide bonds have also been used in the preparation of micelle with redox responsive characteristics [14]. Polyvinyl polymers also provide a healthy hydrophobic core for micelles but the drawback is that it's not biodegradable [15]. Some examples of polymers used commonly for biomedical applications are mentioned below.

PEGylated carriers: PEG is a linear polymer with a wide range of biomedical applications as it has good biocompatibility [12]. PEGylated molecules increase hydrophilicity which prevents the adhesion of opsonins to circulating nanoparticles thereby improving stealth characteristics. For example, in PEGylated gold nano particles, surface plasmon resonance is varied by using PEG of different chain lengths. Increases in PEG chain length has been shown to reduce the negative surface charge [16] and increase circulation time of gold nanoparticles. On the other hand, low molecular weight PEG is associated with very low circulation *in vivo* [17].

Polypropylene fumarate (PPF)

PPF is a biodegradable polyester that has been used as tissue engineering scaffolds and preparation of hydrogels as it possesses unsaturated carbon double bonds which favors good cross linking capability. However, PPF is highly viscous and its high hydrophobic nature makes it difficult to handle for many biomedical applications. The high viscosity of PPF has been overcome by modification in cross linking with di-ethyl fumarate (DEF) which has been applied for bone tissue applications [18]. Photo-crosslinked PPF has also been used as scaffolds for both hard tissue and soft tissue applications which promises good biocompatibility and biodegradability [19].

Characteristics of drug delivery

Effect of Size

Size in the nano-scale represents the diameter of the spherical particle which is same in all orthogonal directions. Decrease in size results in increase of surface energy which reduces the degree of crystallinity thereby improving solubility. Nanostructures have increased surface area/volume that is responsible for greater biological interaction which may increase the therapeutic efficacy or toxicity to the system [20]. Size also deals with dissolution rate and saturation solubility. Reduction in size increases the saturation solubility and dissolution rate [21] of nanocarriers. These carriers have many biological barriers to cross such as barriers affecting absorption from the site of administration, vascular barriers and the phagocytic system. The cell membrane itself acts as barrier for more than 1 kDa nanoparticles as they are cleared by the phagocytic system. In contrast, 20 nm particles can easily extravasate into blood vessels. Enhanced permeation and retention effect of nanoparticles increase with decreased size and has been employed for passive targeting of tumor tissue. This is possible due to neovascularisation, leaky and incomplete tumor

vasculature [22]. Particle size influences the route of delivery and therapeutic efficiency like listed below.

Table 1: Influence of size on nanoparticles

Endovenous passage	Particles ranges between 0-2µm can pass through capillaries
Solubility conditions	Decrease in size of the particles increase the solubility preferably in nano size (within 1µm) [23]
Glomerular clearance	Nanoparticles below 5nm are easily cleared by kidneys
Entrapment by phagocytes	Particles in micro size are easily trapped by macrophages [24].
Oral assimilation	Less than 1 µm rapidly absorbed in gut [25]
Mucous invasion	Peglayeted nano particles around 500nm easily can penetrate the mucus layers [26]
Lymphatic system	less than 30nm particles are easily drain from lymphatic system and moves in circulation [27].
Passive Targeting	60 nm gold nano particles promises more accumulation on tumor site [28]

Micelles chemistry

Material chemistry of micelle is also very important in addition to size. In PDC, polymer imparts new property to drug. For example, degradation of polymer after drug release should not be toxic to the biological system. Polymer conjugates should be stable in storage and circulating condition. Importantly, the polymer should have a monodispersity index as this affects the pharmacokinetics of the drug. Biodegradability is another important key factor for selecting a polymer for drug delivery. The monomers should not be toxic. Furthermore, high crystallinity and molecular weight of the polymer influences the rate of polymer degradation which in turn controls the rate of drug release into the surrounding milieu. Polymer molecular weight also influences the size and drug entrapment efficiency. The hydrophobic polymer segment in a micelle influences a positive interaction with hydrophobic drugs thereby increasing drug loading capacity [29].

Surface charge and characteristics

Surface charge of micelles is important as it promotes micellar stability and prevents micellar aggregation in an aqueous environment. Cationic micelles have very low circulation time compared to anionic ones as the lipid membrane in cells are negatively charge which easily attracts them towards the cell membrane [30]. These are some of the important characteristics of micelles as drug carriers. Combination of carrier properties and controlled delivery system may improve therapeutic system. The common surface modification of nanoparticles are shown in the table below.

Table 2: Surface modification of nanoparticles. [31]

Property	Surface modification	Observations
Stabilization	Electrostatic stabilizer or steric stabilizer	Decreased agglomeration, increased shelf life
Stealth property	PEGlaytion	Decreased opsonization and phagocytosis by MPS
Mucoadhesion	Thiolation	Increased adsorption of particles to the mucus
Mucus penetration	Dense covalent coating with low molecular weight protein	Increased penetration rate through the mucus
Lymphatic distribution	Surface coating with poloxamer- 407	Uncoat or less dense coat increases lymphatic residence time, and higher density coating results in high blood levels[27]
Safety	Charge detection	Cationic charges are thought to be responsible for toxicity; surface coating with nonionic stabilizers decreases the toxicity
Specificity	Antibody tag	Can be used to enhance the permeability or to achieve active targeting
Blood brain barrier	Coating with polysorbate 80 transferrein, transferrein receptor monoclonal antibodies	Increased localization of drug in brain

Cell uptake

Targeting micelles to diseased site increases therapeutic efficacy of drug and avoids systemic toxicity. Micelles are targeted both actively and passively. Mostly, passive targeting is preferred as active targeting involves the complex tethering steps with immunoconjugates (ICs). ICs are carriers with monoclonal antibodies for targeted delivery of micelles [32]. They were first designed for targeted delivery of drugs to myeloid leukemia [33]. Micelles tagged with monoclonal antibody helps in targeting the drug to the particular site of action. The targeted micelle helps overcome the problem of systemic toxicity by surrounding the cancer cell [34]. For localized delivery, IC and the stability of immune antibodies play a crucial role in biological systems. In passive targeting, nanoparticles enter the cell through specific pathways such as endocytosis and phagocytosis and non specific pathways like pinocytosis.

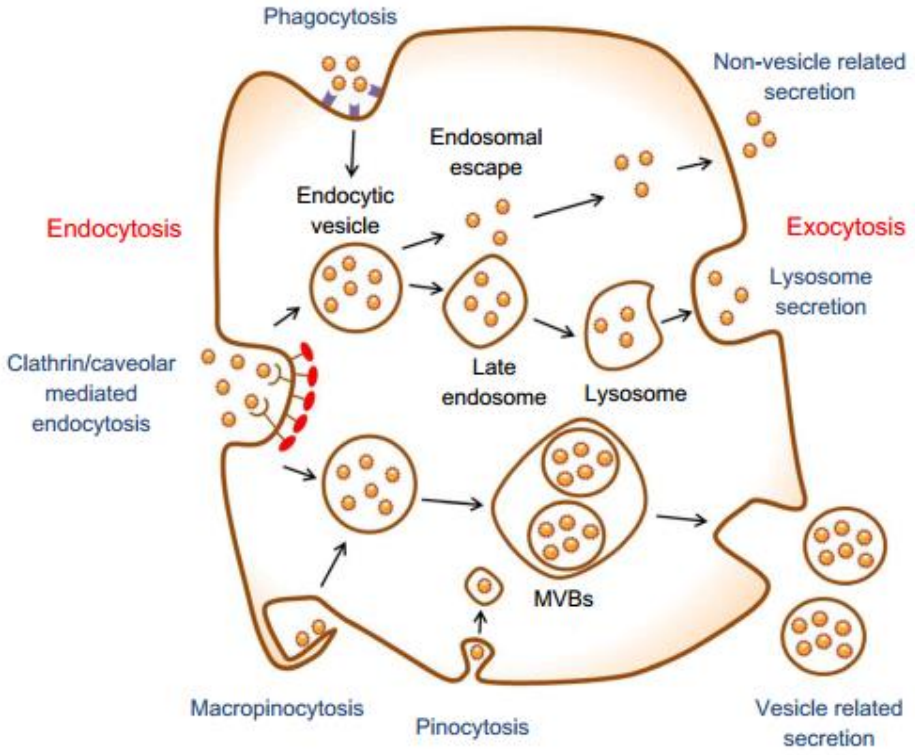


Figure 2: Mechanism of cell uptake

Endocytosis is classified into receptor mediated processes such as calthrin, caveolin, mannose, complement and scavenging mediated process. These are energy dependent processes carried out by immune cells like macrophages, phagocytes etc. In pinocytosis cells take up particles while internalizing fluids. More than 500 nm are readily internalized by phagocytes through endocytosis and 10 – 50 nm range particles are taken up by pinocytosis. Other important factors like size, shape and surface chemistry affect cellular uptake which has been previously discussed.

Smart drug delivery systems

Smart drug delivery carriers are designed with stimulus sensitive polymers which exhibit large physical or chemical changes triggered by a small environmental change. This methodology is relevant for diagnosis and therapeutics. Here the polymers are responsive to temperature, light, pH, ionic factors or redox system, and correspondingly exhibit changes in physical properties, size, shape, color, sol-gel transition or conductivity. Here the drugs are delivered in a controlled manner at predefined time intervals.

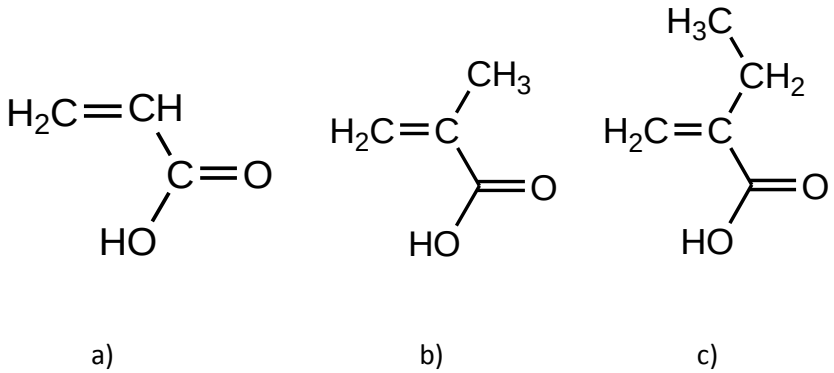
Particularly, pH sensitive delivery system is very relevant to pathological conditions such as cancer and inflammatory disorders. In cancer, production of lactic acid and hydrolysis of ATP in hypoxic regions decrease the pH in tumors tissue [35]. In arthritis condition, production of arachidonic acid, and various acidic enzymes, cytokines increases the acidity at the site of inflammation. In these clinical scenarios, pH based drug delivery systems are being considered, to avoid conventional systemic effects.

pH sensitive polymers

pH sensitive polymers have ionizable acidic or basic functional groups with PKa value close to the desired pH. Based on ionic groups pH sensitive polymers are classified into two categories, poly acidic polymers and polybasic polymers.

Polyacidic polymers

Polymers with ionizable acidic groups show response with increase in pH due to deprotonation and their electrostatic repulsion affects the physical state of the polymer. Polymers with acidic groups swell at alkaline pH. Some examples are listed below



a) b) c)

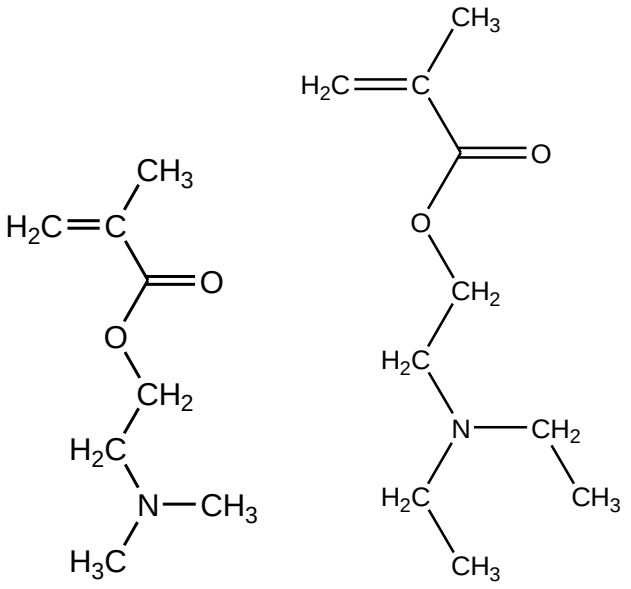
Figure 3: Poly acidic polymers (a) poly(acrylic acid) (b) poly(methacrylic acid) (c) poly(2-ethylacrylic acid)

The membrane destabilizing activity was analyzed in the above polyanions. The increase in one methyl unit (CH_2) changes the PKa value and affects the pH at which the polymer switches from hydrophilic to hydrophobic. PKa value of PEAA is 6.3 - 6.7 , PPAAc is 7.2 and PBAAc is 7.4. [36] which limits the use of PBAAc as its PKa is close to neutral pH. Carriers for delivering drugs to specific cellular compartments help in gene and peptide delivery and prevents from lysosome degradation. PEAAc was fabricated as cell membrane disrupting polymer for endosome drug delivery which has been used in gene delivery. PEAAc showed hemolytic activity of the red blood cell at pH 6.7 - 5 and PPAAc at 6.1 but

not in physiological pH 7 [37]. Percentage of hydrophobic monomer in polymer increases the pka value. For example, incorporation of hydrophobic polymer methylmethacrylate to polyacrylic acid at various ratios increases the pH which is necessary to dissolve [38] the copolymer for enteric coating of pharmaceutical products for oral administration.

Polybasic polymers

Here polymers have basic functional groups like amines which change their physical state in acidic condition due to protonation. Polycations such as *N,N'*-dimethylaminoethyl methacrylate, *N,N'*-diethylaminoethyl methacrylate, polyvinyl amine are used as cross linkers for micelle preparation and to deliver the drug in acidic condition.



DMAEMA DEAEMA

Figure 4: Polybasic polymers, a) N,N-Dimethylaminoethyl Methacrylate b) N,N-Diethylaminoethyl Methacrylate

For example, pullulan modified with carboxymethylation and amidation brings in a hydrazide group. This hydrozone bond is pH sensitive which delivers doxorubicin at acidic

pH [39]. PLLA-b-PEG-b-polyHis-biotin micelles have been shown to deliver drugs at endosomal pH [40].

Common drugs used for drug delivery

Efficacy of many conventional drugs has been improved by loading in polymeric micelles like doxorubicin, paclitaxal, cisplatin, and ibuprofen etc. Doxorubicin is well known drug for cancer therapy like breast cancer, bone sarcoma, lymphatic tumor, neuro blastoma etc, as its halts DNA replication and transcription process [41]. But conventional formulation of doxorubicin leads to hematopoietic suppression and cardiotoxicity[42]. PK1 (FCE 28068) is a polymer drug conjugate of doxorubicin with N-(2-hydroxypropyl)methacrylamide (HPMA) copolymer that promises longer circulation time[43], higher accumulation in tumor site[44] with reduced cardiotoxicity [45]. Paclitaxal resists the microtubules movements thereby arresting cell cycle leading to apoptosis [46]. However, paclitaxal has complex structure with aromatic rings and is very hydrophobic in nature. NK 105 is formulation of diblock copolymer PEG and poly aspartate with paclitaxal that provides hydrophobic core [47]. Xyotax is paclitaxal formulation of comb like linear polymer of glutamic acid which supports the slow release of drug [48].

Cisplatin is a coordination compound containing inorganic platinum which crosslinks with DNA to induce apoptosis. This was approved by FDA for ovarian and lung cancer [49]. However, patients are prone to nephrotoxicity gastrointestinal toxicities and other systemic toxicity. NC-6004 is micellar form of cisplatin with PEG and polyglutamic acid both serving as amphiphilic molecules. This micellar form of cisplatin showed longer circulation time with reduced nephrotoxicity and is released only in a chloride stimulus environment. Other viral drugs, such as anti-herptic agents like acyclovir and valaciclovir, are stabilized using a PEGylated micelle in acidic pH [50]. These drugs are released only by enzymatic

digestion thereby improving virus resistance and toxicity. These are some of the drugs in micelle form that overcomes conventional systemic toxicities.

Inflammation

Inflammation is a protective response of the body against foreign particles, infection and injury. This biological response is carried out by immune cells like macrophages, mast cells, phagocytes etc., mediated by several signaling factors such as cytokines, lymphokines, and interleukins. Inflammation is a good sign of tissue repair. But in some pathological conditions, like arthritis and some neurodegenerative diseases, inflammation acts as a destructive force damaging tissues and worsening normal condition. In chronic conditions one of the primary factors for inflammation is increased concentration of prostaglandins produced by isoforms of bi-functional homodimers cyclooxygenase 1-2 from arachidoninic acid. Cox1 derived prostaglandins are responsible for housekeeping function in normal tissues. Prostagladins derived from COX2 are reported to be dominant in inflammation like allergic and colitis and other arthritis condition [51]. Thus prostaglandins play's dual role as boon and bane in biological system. Many traditional non-steroidal anti-inflammatory drugs such as asprin, ibuprofen, naproxen, diclofenac inhibit COX1 and COX2 leading to stomach ulcers. Intake of COX2 inhibitors alone, like rofecoxib and valdecoxib, has been reported to induce heart problems. Thus traditional COX inhibitor with controlled delivery is significant in chronic inflammation condition.

Ibuprofen

Ibuprofen is a propionic acid derivative and a member of non-steroidal anti-inflammatory hydrophobic drug well known for anti-inflammatory, anti pyritic and analgesic properties. Ibuprofen is a traditional COX inhibitor which inhibits both COX-1 and COX-2. Ibuprofen tablets are supplied as 200 to 800 mg three times a day, and is almost insoluble in water

with a PKa of 5.3[52]. This drug has a short biological half life of about 1.8 – 2 hours inside the body, so frequent doses are needed to achieve longer therapeutic effect [53,54]. Over dose of Ibuprofen affects the gastrointestinal tract, kidney and coagulation system. It causes gastric intestinal bleeding, increases peptic ulcer, renal failure and epistaxis [55]. So to avoid all these side effects the dose should be minimized and controlled and localized delivery system should be designed. Here polymeric micelle loaded with Ibuprofen is analyzed for anti-inflammatory function in invitro condition.

Chapter 2

HYPOTHESIS AND OBJECTIVES OF THE STUDY

Conventional drug therapy is associated with systemic toxicity. Here the objectives were to design a pH sensitive smart polymeric micelle for control drug release and delivery. It is well known that many chronic conditions, including inflammatory disorders and cancer, are associated with a pathologically mild acidic milieu. Our aim was to design a drug tethered polymeric micelle with mPEG-PPF, maleic anhydride and ibuprofen through condensation reaction. The hydrophobic PPF corona was then crosslinked with basic crosslinker DMEAMA which protonates and swell's in acidic pH thereby hydrolyzing the anhydride bond and releasing the tethered drug. PEG is widely used as biocompatible hydrophiphillic polymer in medical application [12]. Polyproylene fumarate is biocompatible hydrophobic polyester used for many tissue applications [56]. We hypothesized that PPF will provide a strong hydrophobic core during micelle formation and its double bonds will offer use for cross linking between chains. Till now PPF has not been used for micelle preparation, so in this study PPF forms the hydrophobic core of the micelle. Maleic anhydride, which provides an anhydride linkage between mPEG-PPF and drug, is biocompatible [64]. The anhydride linkage is susceptible to hydrolysis and will therefore provide a controlled and sustained

drug release over time. Finally, ibuprofen is a commercially available antipyretic and anti-inflammatory drug that acts as a COX-2 inhibitor.

Main objectives of the study

- Synthesis of carboxyl-terminated polypropylene fumarate (PPF)
- Synthesis of polymer drug conjugates (PDC)
- Physicochemical characterization of synthesized PDC
- Preparation and characterization of micelles
- Drug release studies
- Cytotoxicity of synthesized PDCs

Chapter 3

MATERIALS AND METHODS

Materials

Maleic anhydride, acetone, methanol, acetonitrile, diethyl ether, disodium orthophosphate, sodium dihydrogen phosphate and tetrahydrofuran were purchased from Merck. Morpholine, sodium acetate, methoxy-polyethylene glycol (m-PEG), ammonium persulphate, sodium bicarbonate and ibuprofen were got from sigma. MTT (3-(4,5–dimethylthiazol-2-yl)–2,5–diphenyltetrazolium bromide), dimethyl sulphoxide (DMSO), tetramethylenediamine (TEMED) and N,N–dimethylaminoethyl methacrylate (DMAEMA) were bought form Hi Media. Toluene, sodium carbonate, acetic acid and 1,2 propane diol, were from SD fine chemicals. Dulbecco's modified eagle's medium (DMEM), trypsin and fetal bovine serum (FBS) were bought form Gibco life technology. HeLa cells were purchased from NCCS, Pune.

Synthesis of carboxyl terminated polypropylene fumarate (PPF)

CT PPF was synthesized by using maleic anhydride, 1,2 propylene glycol, morpholine, sodium acetate in molar ratio of 0.2498, 0.1200, 0.0057, 0.0060 through condensation reaction. The above contents were refluxed at 160'C for 2 h under nitrogen atmosphere.

After refluxing, the contents were vacuum condensed for 5 min at 180 ˚C to get a white pale yellow solution. The obtained polymer solution was dissolved completely in acetone and separated in a separating funnel with 25% methanol. A milky white layer was formed and over time golden yellow droplets of PPF polymer slowly started settling below. The extracted PPF was once again washed with 25% methanol to obtain pure polymer.

Conjugation of methoxy polyethylene glycol – PPF (mPEG –PPF)

Polymer conjugation was done through a simple condensation reaction. mPEG 5000 or mPEG 2000 and PPF with a molar ratio of 0.0042 and 0.0053 was taken in a 250 ml round bottom flask and was refluxed under nitrogen atmosphere for 2 h at 160 ˚C. The contents were then vacuum condensed for 5 min. The obtained golden brown viscous polymer was purified with ice cold diethyl ether to obtain a white precipitate. The precipitated polymer was then filtered, dried and stored in a nitrogen capped bottle at room temperature.

Polymer drug conjugate (PDC) synthesis

mPEG-PPF conjugate synthesized in previous reaction was conjugated with maleic anhydride and ibuprofen. Briefly, mPEG-PPF, maleic anhydride and ibuprofen at an molar ratio of 0.0012, 0.0015, 0.0008 was weighed and suspended in 25 ml of toluene. The contents were refluxed overnight at 135 ˚C under nitrogen atmosphere. The obtained golden yellow polymer solution was purified with diethyl ether and the white precipitated polymer was stored in a nitrogen capped bottle at room temperature.

Polymer Characterizations:

Gel permeation chromatography (GPC)

GPC is powerful chromatographic technique for analyzing molecular weight of polymers. Analysis basically depends on the interaction between stationary phase which are the beads packed in the column, and mobile phase in which the polymer samples are dissolved. Elution of particles is based on pore size of the stationary phase and hydrodynamic volume of the sample. In general, low molecular weight polymers have longer retention times compared to high molecular weight polymers.

Molecular weights of purified polymer comacromers and PDCs were determined by GPC using THF as mobile phase with polystyrene beads as standards.

Fourier transform Infrared spectroscopy (FTIR)

FTIR spectroscopy is infrared radiation based vibration spectroscopy mainly preferred for material analysis. The dipole molecules in material absorb infrared radiation and undergo vibrations like stretching, bending, wagging, and scissoring. These vibrational frequencies are converted into readable spectra.

Functional groups of the synthesized comacromers were determined using FTIR (JASCO'S proprietary spectra Manager ^{TM} II crossplatform software), by sandwiching samples between two potassium bromide pellets and scanned in the range from 4000 – 400 cm^{-1} at a resolution of 4 cm^{-1}.

Nuclear magnetic resonance (NMR)

Nuclear magnetic spectroscopy is a physical phenomenon and is based on difference in nuclei spin of atoms subjected to a magnetic field. The nuclei spin of proton differs

according to the nature of the bonding molecules. The free protons exhibit radio frequencies in lower magnetic strength than covalently bound protons.

The structure of PPF and mPEG-PPF comacromers, ibuprofen and PDC were obtained by proton H^1NMR (Bruker 600 MHz). Polymer samples were dissolved in chloroform–d6 and ibuprofen was dissolved in dimethyl sulfoxide-d6 solvent.

Preparation of micelles

Briefly, 60 mg of PDC was dispersed in 15ml of 0.2 M sodium carbonate buffer at pH 9.2 and filtered through a 0.2 micron filter. It was then stirred for 20 minutes at 400 rpm following which 5% or 10% DMAEMA cross linker was added and continuously stirred for another 20 minutes at 160 rpm. Then 2 M of 130 µl APS and 6 M 40 µl of TEMED were added and the contents were stirred for another 45 minutes at 80 rpm.

Characterization of micelles

Dynamic light scattering (DLS)

Particle size was determined by dynamic light scattering form Malvern instruments. This instrument measures the particle size based on the Brownian movement. Small particles have faster Brownian movements which scatter light more than larger particles. The polydispersity of particles was analyzed based on cumlant method [63]. The size distribution of the micelles in 0.2 M sodium carbonate buffer solution at pH 9 and sodium acetate buffer pH 5 was measured via dynamic light scattering method (Malvern instruments) after 3 h incubation in buffer.

Critical micelle concentration (CMC)

Critical micelle concentration was determined using pyrene as fluorophore. Different concentrations of PDC and mPEG-PPF micelles were prepared range from 0.25 ng/ml to 1 mg/ml in 1 µM solution of pyrene and incubated overnight. Then, PDC and mPEG-PPF micelle solutions were excited at 338 nm in fluorometer (cary Eclipse) and the emission was scanned between 350 to 450 nm. The fluorescence intensities of the peaks at 374 nm (I_1) and 384 (I_3) were calculated from the spectra, and the ratio of I_1 / I_3 ratio [57], vs. concentration was used for CMC determination.

Scanning Electron Microscopy (SEM)

Scanning electron microscopy reveals the surface morphology of the sample by scanning through high electron beam under high vacuum pressure. Crosslinked micelle solution prepared in sodium carbonate buffer at pH 9 was diluted four times in distilled water and incubated for 4Hrs. Few drops of this micelle solution was then air dried in a cover slip at room temperature for 30 min and SEM (ESEM, FEI, Quanta 200, USA) images were taken.

In vitro ibuprofen release from drug tethered micelles

Briefly, 25 % DMEMA crosslinked micelles prepared in sodium carbonate buffer of pH 9.5 was suspended in buffers with pH – 9.5, 7.5 and 5.3, respectively. The samples were incubated for 3 h and then centrifuged at 20000 g for 20 min for the particles to pellet out. The supernatant was collected for HPLC analysis.

HPLC is an analytical chromatographic technique for both qualitative and quantitative analyses. Here the mobile phase is passed through stationary phase in high pressure with specialized pumps and the eluted samples are analyzed through a UV detector. For HPLC, 35% acetonitrile in 0.1 M sodium acetate solution was used as a mobile phase [58]. Different

concentrations of ibuprofen dissolved in PBS were used to calibrate a standard curve. The amount of ibuprofen released from micelles were then determined using the plotted standard curve.

Cytotoxicity of micelles

Cytotoxicity studies of prepared micelles were studied using a MTT assay kit. HeLa cells loaded at the density of 1×10^5 in 96 well plate was cultured in DMEM medium (DMEM supplemented with 10% FBS, 1X antibiotic-antimycotic solution and sodium bicarbonate). After attaining 80% cell confluency, different concentration of micelles prepared in culture medium were added to cells and incubated over night in a 37 ˚C CO_2 incubator. Then, MTT solution (20 µl, 5 mg/ml) and 80 µl of serum free medium were added and the plate was incubated for an additional 6 h. The formazan crystals formed were then dissolved in 150 µl of DMSO and the absorbance of 100 µl of supernatant was read in a UV/Vis plate reader (Varian, Cary 50 USA) at 595 nm.

Cell uptake

mPEG-PPF-5000 at a concentration of 4 mg/ml was dispersed in PBS solution containing 2 µg/ml of fluorescein and continuously stirred for 20 min in the dark. The fluorescein encapsulated micelles were then dialyzed in PBS for 2 days. The dialyzed fluorescein-loaded micelle solution at a concentration of 2 mg/ml was added to cells cultured in a 24-well tissue culture plate and incubated for 4 h. After the incubation period, the wells were rinsed with PBS three times at 10 min intervals. The cells were observed under an upright fluorescent microscope and images were taken at 20X magnification.

Chapter 4

Results and Discussion

Synthesis of polymers

The synthesis of PPF, mPEG-PPF and PDC were performed via a simple polycondensation reaction. Carboxyl terminated PPF was obtained by increasing the ratio of maleic anhydride to 1, 2 propylene glycol followed by condensation reaction at 180'C. PPF was obtained as a golden yellow color after purification with 25% methanol. mPEG-PPF and PDC obtained through condensation reaction appeared as white puffy powder after purification. The synthesis scheme is shown in Figure 5. The obtained comacromers were characterized by FTIR, ^{1}H NMR, and GPC.

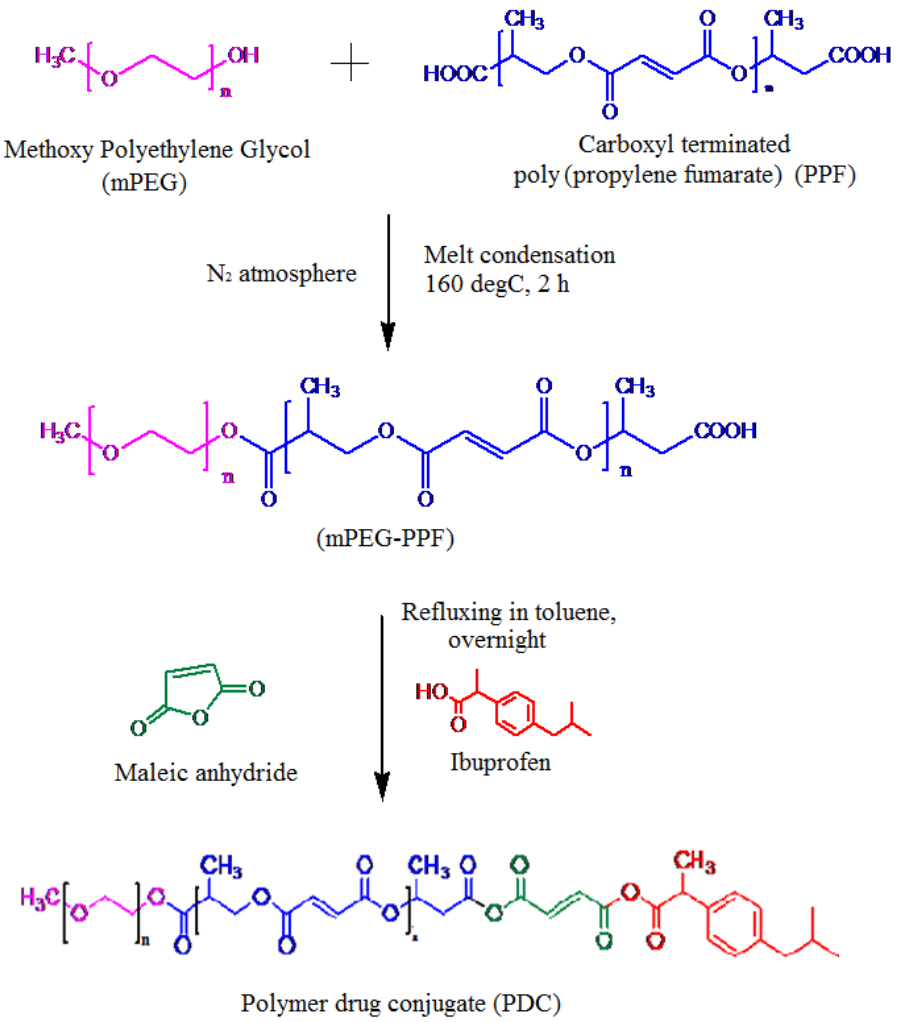


Figure 5: Reaction scheme of synthesis of Polymer drug conjugate (PDC)

Characterization

Fourier Transform Infrared Spectroscopy (FTIR)

The FTIR spectra of synthesized polymers confirmed presence of various functional groups. C-H bending of trans unsaturated C=C bond of fumarate [59] for PPF, which is exploited for covalent crosslinking, was seen at 1643 cm^{-1}. Biodegradable ester bonds of hydrophobic PPF are confirmed by strong characteristic stretching frequencies of carbonyl groups C = O at 1725cm^{-1} and C-O at 1101 cm^{-1}.

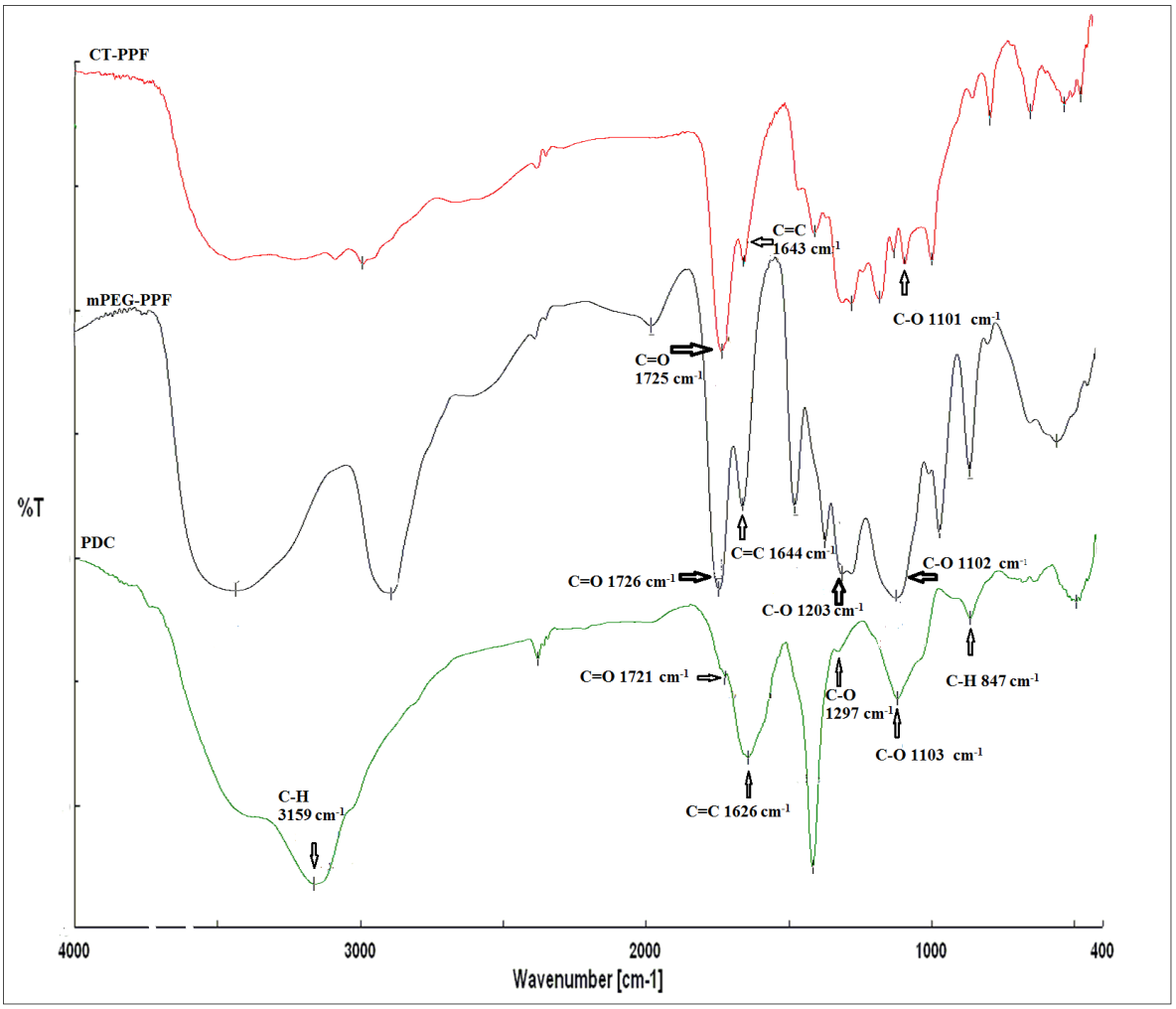


Figure 6: FTIR spectra of PDC

Poly ether group, in both mPEG-PPF and PDC, was confirmed by a peak at 1203 cm^{-1} and at 1297 cm^{-1}. In PDC, aromatic C-H of ibuprofen was confirmed by peak at 3159 cm^{-1} and 847 cm^{-1} along with ester bonds of PPF with strong peak at 1721 cm^{-1} and polyether bond of mPEG with 1297 cm^{-1}. Anhydride peak were confirmed by weak C=O stretching at 1780 cm^{-1} and 1820 cm^{-1} respectively. These functional groups observed in PDC confirms that monomers are condensed into a linear comacromer.

Nuclear magnetic resonance (NMR)

Further characterization by ^{1}H NMR reveals the structure of polymer drug conjugates and supports the FTIR data shown above. Fumarate bond (C=C) of PPF, responsible for crosslinking, was observed at 6.8 ppm. The CH_3 group of amphiphilic mPEG was found at

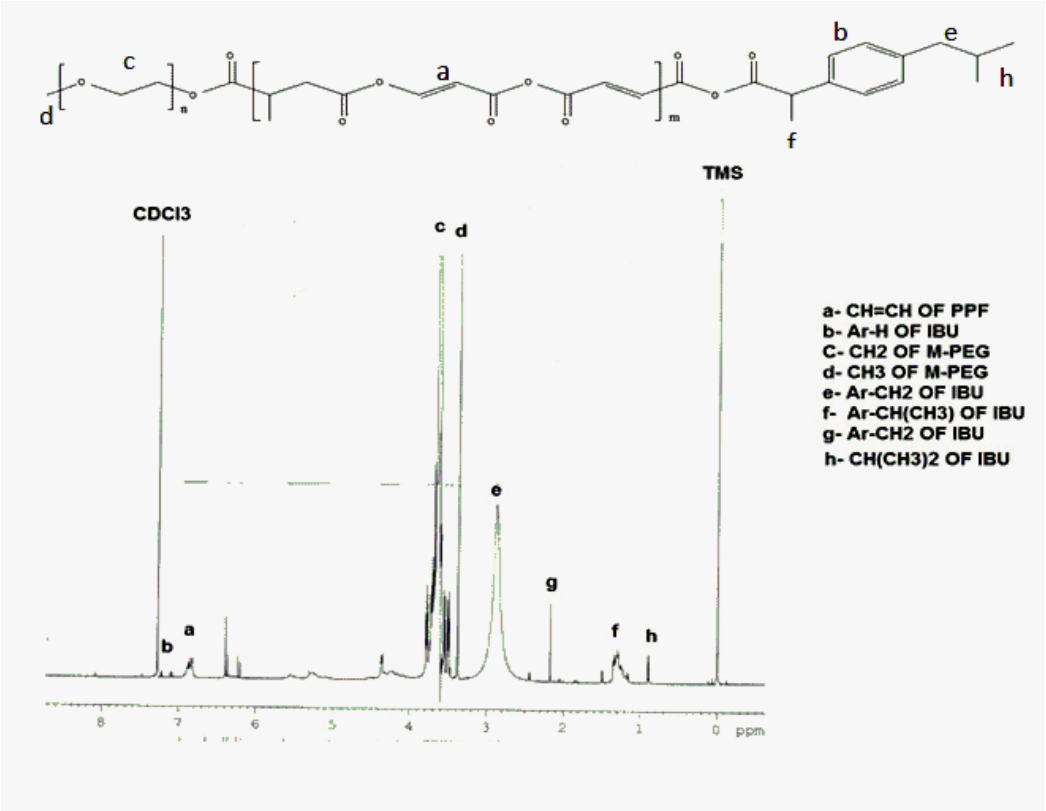


Figure 7: ^{1}H NMR data of Polymer drug conjugate (PDC)

3.3 ppm. Aromaticity of Ar - CH_2 of Ibuprofen peak was confirmed at 2.9 ppm which confirms that the drug is tethered to the comacromer. These peaks confirm the successful synthesis of PDC.

Gel permeation chromatography (GPC)

Molecular weight of obtained comacromers was analyzed in a GPC column with THF as solvent and polystyrene beads as standard. GPC confirms that monomers have been condensed to comacromers. Differences in molecular weight of comacromers of mPEG-PPF

and PDC ensured that drug and maleic anhydride are tethered to comacromers of mPEG-PPF and formed PDC. The molecular weights of synthesized polymers by GPC are listed below.

Table 3: Molecular weight of comacromer

Polymers	**Mw**	**Mn**	**PDI**
PPF	2381	-	-
mPEG-PPF	7351	4068	1.88
PDC	7712	4338	1.77

Preparation and characterization of micelles

Micelles are prepared by the dispersion of PDC in sodium carbonate buffer solution at pH 9. The strong hydrophobic PPF-ibuprofen segment forms the micelle core and the amphiphilic mPEG forms the micelle shell. Micelles are cross linked via DMAEMA by free radical polymerization with APS and TEMED which makes them pH responsive. Micelle formation and its size distribution were confirmed by SEM and DLS.

Scanning electron microscope (SEM)

SEM visually confirms the formation of micelles from dispersed polymers in water. Corona like structure was visualized with grayish inner core and a puffy outer shell in the fractured micelles. SEM confirmed the sizes of micelles around 400 nm - 500 nm and this finding was confirmed with DLS too.

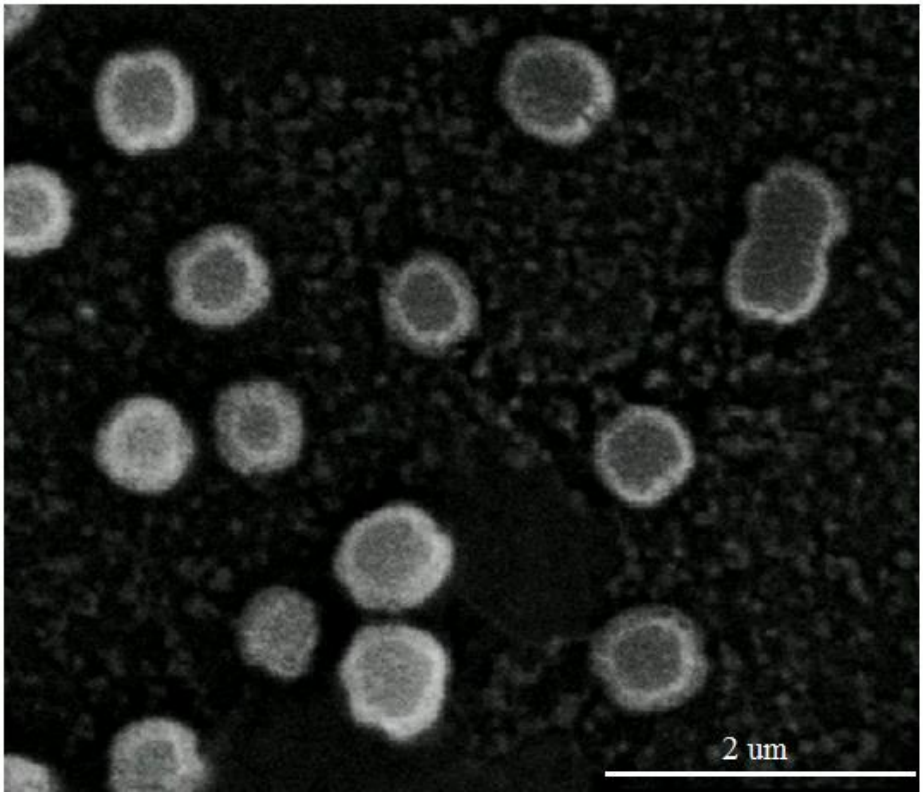


Figure 8: SEM images of micelles at pH 9

Dynamic light scattering (DLS)

The hydrodynamic size of PDC 5000 was around 545 ± 88.15 nm with polydispersity of 0.510 ± 0.051 after three hours incubation in pH 9. In pH 5, size was reduced around 490 ± 69.69 nm with PDI 0.262 ± 0.088 this shows that the micelles were swollen due to protonation of crosslinker in acidic pH thereby releasing the tethered drug. On the other hand, uncrosslinked PDC exhibited smaller size of 238 ± 110.1 nm with PDI of 0.458 ± 0.05 at pH 9. mPEG(5000)-PPF was also analyzed with crossslinking, in alkaline pH 9 size was 184.6 ± 15.20 nm with PDI 0.233 ± 0.017 and in acidic pH 5 size was increased to 264 ± 14.10 nm with PDI 0.065 ± 0.072 this shows swelling of micelle in acidic pH thus confirms the prontanation of DMAEMA and without cross linking, size of mPEG(5000)-PPF was 231.6 ± 35.39 and PDI 0.684 ± 0.009 remained similar in both pH. In current system, the

cross linking shows increase in size and thus confirms the micellar system is pH responsive suitable for smart drug delivery system.

	Size in nm (pH 9)	Size in nm (pH 5)
Crosslinked PDC 5000	545 ± 88.15	490 ± 69.69
Crosslinked mPEG(5000)-PPF	184.6 ± 15.20	264 ± 14.10
Uncrosslinked PDC 5000	238 ± 110.1	--

Critical micelle concentration (CMC)

Hydrophobic probe pyrene was used to measure the CMC, as pyrene accumulates and fluoresces only in a hydrophobic core. In aqueous solution, pyrene solubility was below 3 µM [60] so different concentration of copolymer and conjugates were dissolved in 1 µM of pyrene solution. Comacromer and PDC solutions with pyrene were excited at 338nm and the emission scan between 350 nm – 420 nm was recorded. The ratio of fluorescence intensities I_1/I_3 was calculated with I_1 = 374 nm and I_3 = 384 nm. The ratio of I_1/I_3 gave the polarity of microenvironment of pyrene [57] and was calculated using Origin software [60]. The CMC of PDC (5000) was 17.5 µg/ml, and its comacromer mPEG(5000)-PPF was 50 µg/ml. In PDC (2000) system was 22.3 µg/ml and its comacromer mPEG-PPF (2000) was 26 µg/ml. Polymeric drug conjugates system showed low CMC value then comacromers mPEG-PPF because of strong hydrophobicity of ibuprofen. Thus low value of CMC indicates the formation of stable micelles, even in lower concentration of amphiphilic comacromers. And current system may form stable micelle in blood and body fluids [14].

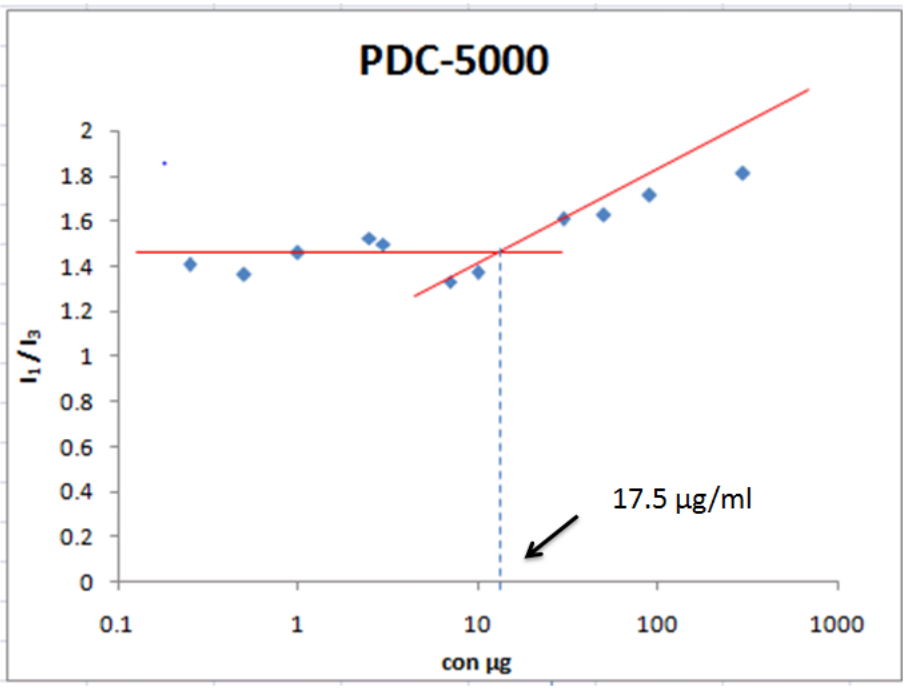


Figure 9a: CMC of PDC 5000

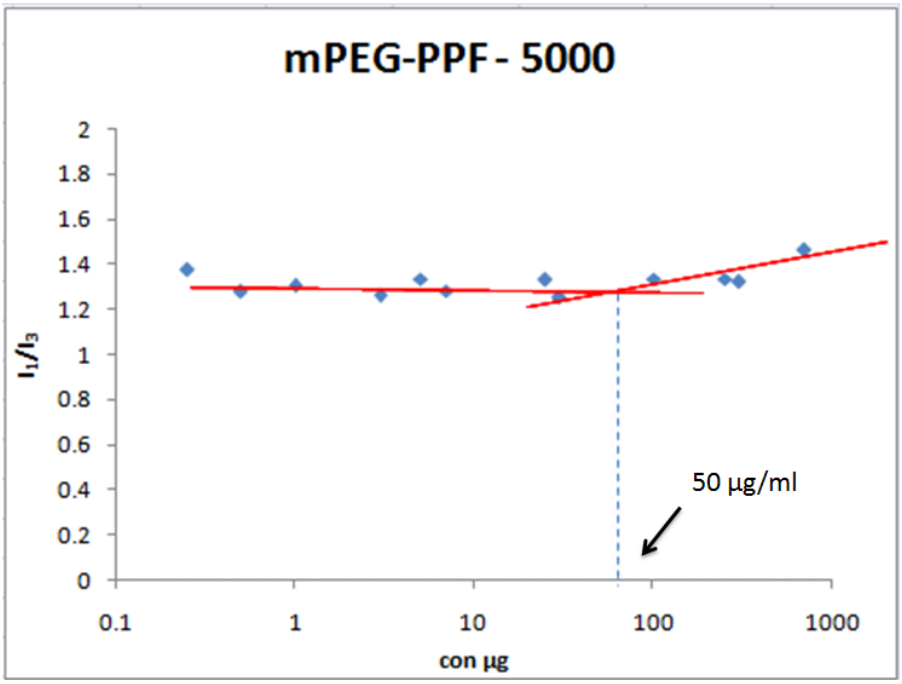


Figure 9b: CMC of mPEG(5000)-PPF

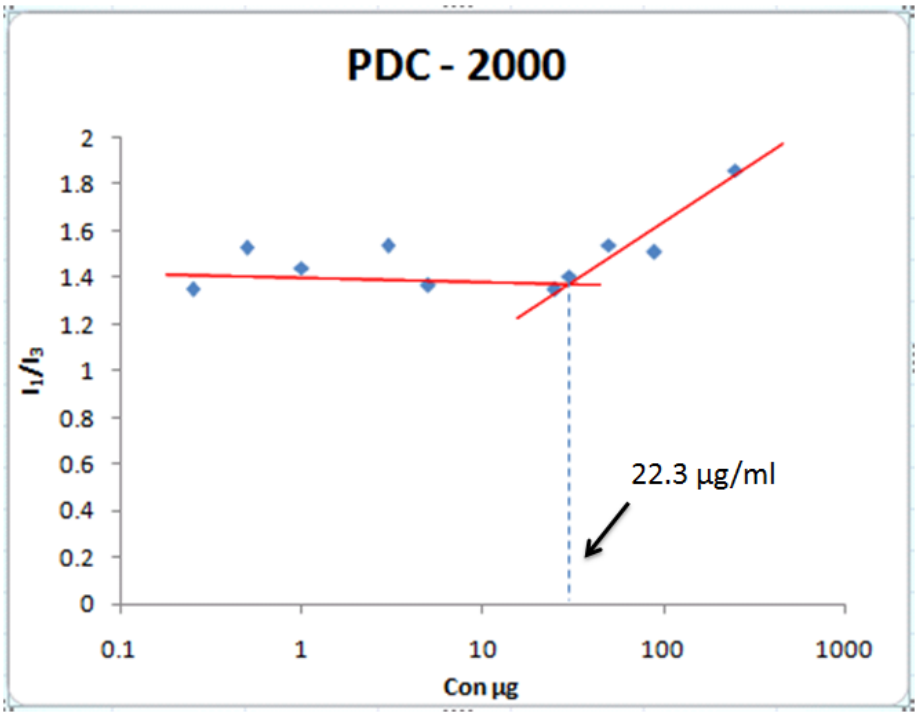


Figure 9c: CMC of PDC 2000

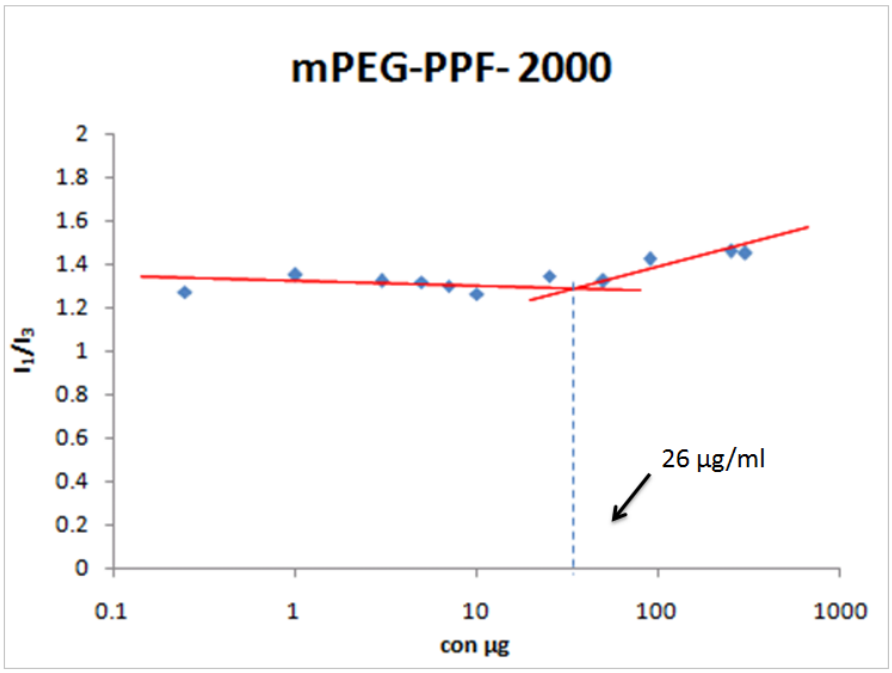


Figure 9d: CMC of mPEG(2000)-PPF

In vitro drug release

Drug release property of DMAEMA cross linked PDC micelle was analyzed *in vitro* condition using HPLC using 35% acetonitrile in sodium acetate solution as mobile phase in three different pHs, 9, 7.4 and 5. The retention time of Ibu was found to be around 5.4 – 5.7 min.

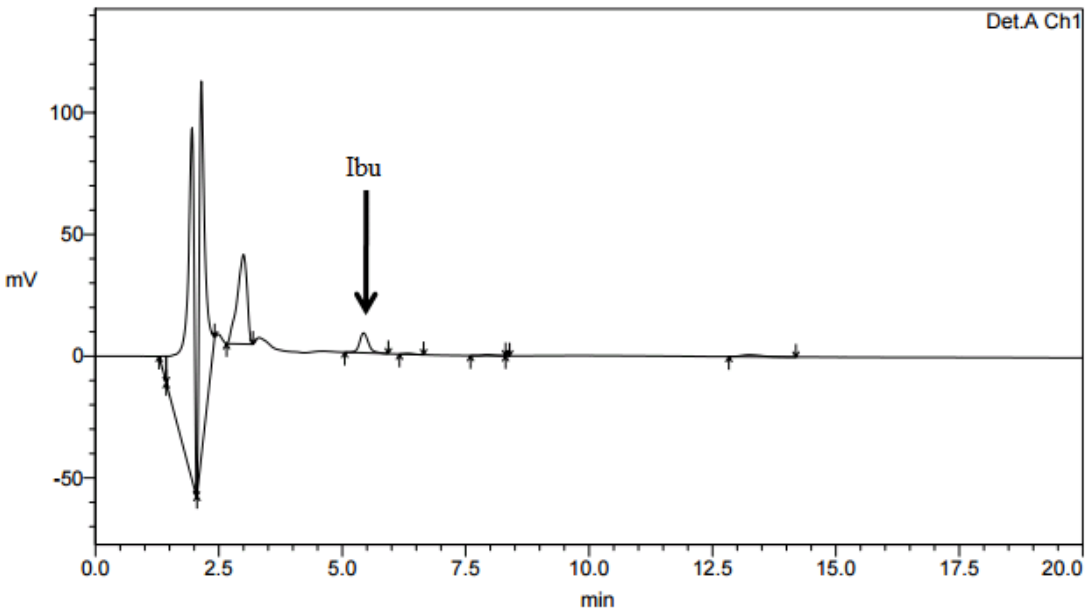


Figure 10: HPLC chromatogram of Ibu

In pH 9, drug release increased gradually and maximum drug released was ~45% during the 80 h time period. On the other hand, drug released from pH 7.4 plateaued and was steady at around 20% during the 80 h study period. In pH 5, drug release was significantly higher compared to pH 9 and 7 and 100% drug release was achieved within 80 h.

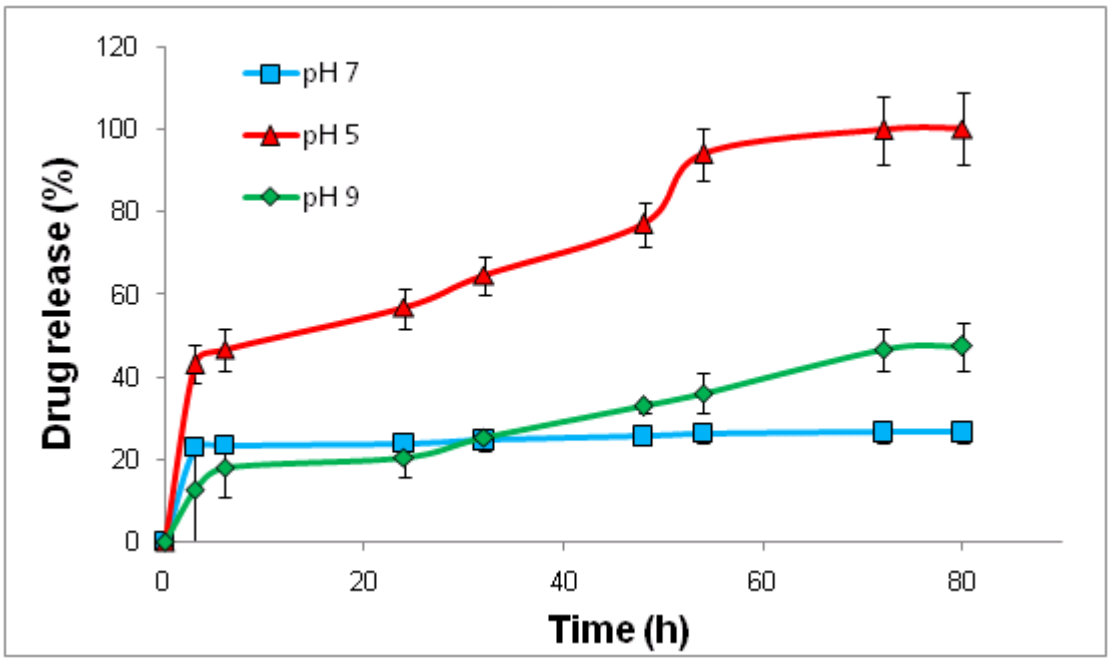


Figure 11: Controlled drug release from PDC- 5000 with 25% DMAEMA crosslinker

This might be attributed to the swelling of micelles in acidic pH as the amine groups in DMAEMA get protonated. This allows water inside the core thus anhydride bond got hydrolyzed and ibuprofen was released into the medium.

Cytotoxicity of amphiphilic micelles

The cytocompatibility of micelles was evaluated using MTT assay for evaluating its potential use for drug delivery applications. MTT assay gives significant indication of metabolically active cells on exposure to material.

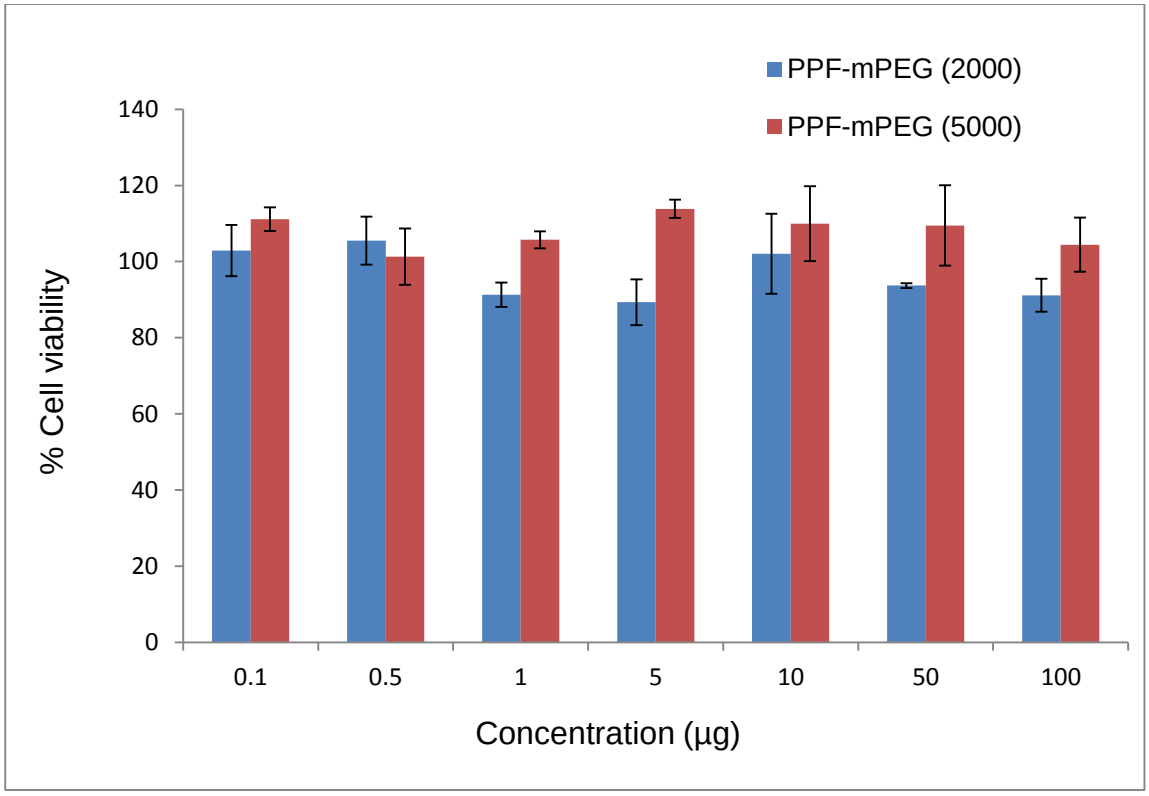


Figure 12: Cell viability of Hela cells with polymeric materials

Amphiphilic copolymer mPEG-PPF showed 80% viability at a maximum concentration of 100 µg/ml in HeLa cells. mPEG(5000)-PPF exhibited more cytocompatibility than mPEG(2000)-PPF copolymer. Graphical representation of the percentage viability of cells with different concentration of micelle solutions is given below.

Cell uptake

HeLa, a cervical cancer cell line, was used for cellular uptake studies. Particularly, PEGlayted micelles internalization was through dynamin and caveolin mediated pathway [61], this supports the internalization of mPEG-PPF comacromer micelles. Micelles loaded with fluorescein confirmed the internalization of comacromer micelles inside the cell, in

cytoplasmic region mainly in lysosomes [62] as shown below. This demonstrates that the prepared PDC micelles show good cytocompatibility *in vitro*.

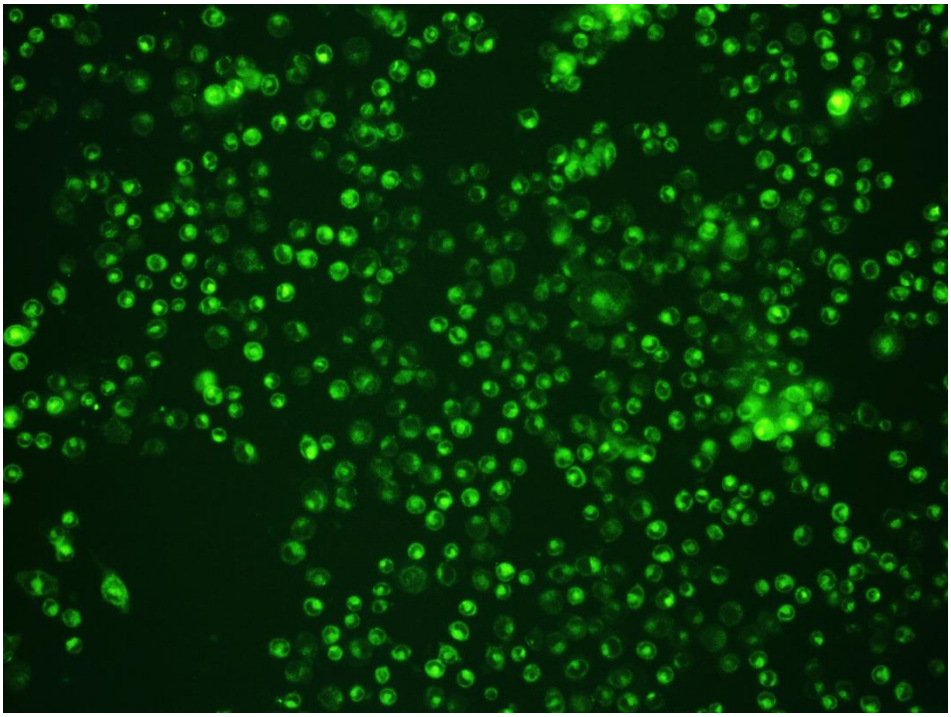


Figure 13: Cell uptake of fluorescein mPEG-PPF micelles in Hela cells

Chapter 5

Conclusion

The present study focuses on synthesizing pH-sensitive smart polymeric micelles for anti-inflammatory drug delivery applications. Here, hydrophobic carboxyl-terminated PPF was synthesized for preparation of polymeric drug conjugates. Polymeric drug conjugates (PDC) were obtained through via polycondensation reactions of monomers of mPEG, PPF, maleic anhydride and ibuprofen. PDCs were synthesized with two different molecular weights of mPEG, 2000 and 5000. The FTIR and H^1 NMR studies revealed the formation of drug tethered conjugates with fumarate, ester and anhydride bonds. The molecular weight and polydispersity through GPC confirmed the oligomeric nature of PDC. The synthesized PDC readily dispersed in water to form micelles whose formation was confirmed through SEM and DLS. CMC was evaluated with the hydrophobic probe pyrene. Results showed that CMC of PDC was lower compared to blank mPEG-PPF micelles which might be attributed to the stronger hydrophobic core of PDC. Cross linking of PDC with DMEAMA, a pH-responsive crosslinker, made the micelles pH -sensitive as DMAEMA protonates in

acidic pH. *In vitro* drug release studies in pH 9 and 5 with 25% crosslinker concentration confirmed higher drug release at pH 5 than at pH 9. Biocompatibility of the polymer drug conjugate was evaluated on Hela cells by MTT assay. Results showed that PDC were minimally cytotoxic with >80% cell viability after a 16 h incubation *in vitro*. Cell uptake studies with micelles loaded with fluorescein dye showed significant uptake of micelles in Hela cells. Cumulatively, these studies demonstrate that designed mPEG-PPF micelle system shows great promise as pH responsive vehicles for drug delivery.

Further studies will be carried out in a cell culture model with HIG82 synovial cell line *in vitro*. HIG82 cells will be stimulated with monosodium urate to activate the release of various inflammatory markers such as prostaglandin E2 (PGE2). As ibuprofen is a COX inhibitor, the upregulation of PGE2 expression induced by sodium urate crystals should be significantly lower compared to untreated cell controls. We will analyze the PGE2 levels in cells using commercially available prostaglandin assay kit.

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