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1. Introduction 1.1 What is cancer? The cancer is a class of disease which involves uncontrolled division of abnormal cells. There are 100 or even more different types of cancer, each of them are classified by the type of cells, tissues or organs those are affected first. The cancer cell can rapidly spread to other part of the body by the help of blood stream and lymph system. The process by which cancer successfully spread to other parts of the body and destroy the healthy tissues is called metastasis. Around 8.2 million cancers related deaths happened world width in recent past and 14 million new cases are investigated in 2012 and about 70% increase in the new case is expected in the years to come.1 The human body is basically a combination of different types of cells. These cells are grown and separated into new cells as it is required for a healthy body. The old cells or damaged cells are replaced time to time by newly born cells. However, sometime damage or changes of the genetic material i.e. DNA of the cell may produce mutation promoting the growth of normal cell and their separation. This utility leads to increase the number of redundant cells without the replacement of old cells. The supplementary cells are forming a lump or masses of tissue called tumors. All tumors are not cancerous. They are appeared in human body as benign and malignant. The cells in benign tumor can“t spread to other part of the body. Once, the tumor is removed from the body they may not be reproduced again and those are called as benign tumor, which are not cancerous. The cells in the malignant tumor can rapidly spread to other part of the body and invade the local tissues. The malignant tumors are cancerous and the phenomenon of spreading of cancer in the body is called metastasis. There are many symptoms which are helpful to identify the different types of cancer. The symptom of the cancer precisely depends upon its size and location. Some cancers can felt through the skin, as a lump on skin. The skin cancer may convert to a wart or mole which can be easily identified. The appearance of white patch or spot on mouth or tongue may be the indication of some form of oral cancer.2 The pancreas cancer is very small and there is no physical symptom. If they interfere with liver function, produce yellowing of skin color and eye, known as jaundice. The symptoms like constipation, diarrhea and change of stool size may be considered due to colon cancer. More irregular (frequent or infrequent) urination may also be the indication of bladder of prostate cancer. The symptoms like unusual fever, fatigue, anemia and unexpected weight loss are may be because of alternation of hormone function created by the cancerous cells. These symptoms are also pretty common for other diseases as well. The frequently feeling of vertigo and headaches of a patient may reflect spreading of brain cancer. Shortness of breath and coughing may be the indication of spreading of lung cancer. There are few types of cancer which are commonly viewed in the human body as: bladder cancer, melanoma, brain cancer, non-Hodgkin lymphoma, breast cancer, ovarian cancer, cervical cancer, pancreatic cancer, colorectal cancer, prostate cancer, esophageal cancer, skin cancer, kidney cancer, thyroid cancer, liver cancer, uterine cancer, lung cancer and leukemia. In the present thesis we will focus only on cervical cancer and its

corresponding cell line. The cancer which arises at the tissue of the cervices called cervical cancer. It is the third most common cancer for woman in the united state (first one is skin cancer and second one is breast cancer).3 These types of cancer are very slow growing and may not leave any symptom for its spread at the early stage. But later, it reveals the symptom, like pain during sexual intercourse, abnormal vaginal bleeding and pelvic pain. In most cases, around 90% cervical cancer is due to Human Papillomavirus (HPV) infection.4 The shape of the virus is like a golf ball having an average diameter around 55 nm. There are around 200 types of HPV viruses. Among them 15 are causing cancer called high risk HPV. The high risk HPV types are type 16, 18, 31, 33, 34, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68, and 70.5 Woman having many sexual partners or being a frequent smoker has a greater chance to be affected by cervical cancer. The early stage of cervical cancer can be identified by Papanicolaou test (Pap smear). This cancer can be avoided by using vaccine before affected by the HPV. Women were recommended to take vaccine (Gardasil and Cervarix) at the age of 9 to 26 and vaccine can easily performed the role of cancer protection for 4 to 5 years. There are six number of cervical cancer cell lines available namely, HeLa, Caski, MS751, C33A, SW756 and SiHa. Among them one is HPV negative (C33A) and others are HPV positive.6 In this thesis we 2 shall be mainly dealings with HPV positive HeLa cell line for in vitro studies. The name HeLa originated from the name of the cancer patient Henrietta Lacks. HeLa cell is the first cell line which survived in vitro in a test tube. The HeLa is the immortal cell which is widely used in the biological research for its quick growing nature. The HaCaT and L929 are the two normal cell lines which are usually used as a control. The HaCaT is the aneuploid immortal keratinocyte cell line of the adult human skin. The L929 is the murine fibrosarcoma cell line. The cell line was first derived in 1948. The stain L and clone 929 were the first established strains and clone respectively. The sarcoma 180 is the cancer cell line implanted in the Swiss albino mice model for studying in vivo cancer treatment.7 1.1.1 What is anticancer drug? A chemical substance which can be used either for treatment, cure and prevention or for diagnosis of the disease is called a drug. A group of drugs which are used for the treatment of malignancy or cancerous growth of cells are called anticancer drug. Depending on the solubility of the drug it is primarily classified in to two groups. One of them is hydrophilic and other hydrophobic in nature. However, other than that electrically charged and a neutral group of anticancer drugs also exist there. The delivery of drug through the nano-carrier to the required pathological site essentially depends upon the nature of the drug which is being used for delivery. The nature of the drugs affects the encapsulation and release efficiency and finally leading to its therapeutic performance. 1.2.1 Delivery of hydrophobic anticancer drug The tricky nature of the hydrophobic drug towards the biological system makes a challenge to deliver it to the required site. Because of the hydrophobic nature, a drug can“t be able to pass through the aqueous environment such as body and tissue fluids of the biological system. It is unable to penetrate the cell membrane to reach the intercellular sites. Furthermore, the agglomeration tendency of the hydrophobic drug promotes it to show the embolisms, creating local toxicity and there by acting as an obstacle after the intravenous injection. The delivery of hydrophobic drugs through encapsulation into the nano-carriers formed by the amphiphilic moieties are widely

applied in drug delivery. The most important issues to deliver such types of drug are encapsulation efficiency and release. The hydrophobic part of the amphiphilic moiety crucially takes care of that. The hydrophobic drugs which extensively used for nano- carrier based delivery are Doxorubicin, Paclitaxel, Cisplatic, Methotrexate, Adriamycin, Docetaxel and SN-38 etc. 1.2.2 Delivery of hydrophilic anticancer drug The hydrophilic drugs also have some undesirable attributes on the biological system like, poor uptake by the cell and very low bio-availability. The hydrophobic (lipid rich) nature of the cell membranes don“t allow to uptake these drug into the cell. The proteolytic and hydrolytic degradations along with very short half-life in the circulatory system are some of the causes of low bio-availability. Unlike hydrophobic drug, the delivery of hydrophilic through the nano-carrier is not so easy task. The nano-carrier formed by amphiphilic moiety has the inner hydrophobic corona to accommodate and carry the drug. In such delivery, drug loading efficiency becomes the main issue because hydrophilic drugs are poorly miscible with hydrophobic corona of the nano-carrier.8 The hydrophilic drugs used for nano-carrier based drug delivery are Bevacizumb, Cetuximab, Cyclophosphamide, Gremcitabine, Ibritumomab, L-asparaginase, Panitumumab, Ristuximab, Tositumomab and Transtumab etc. 1.2.3 Delivery of charged anticancer drugs Like hydrophilic drugs, highly charged drug also have same characteristics such as very low cellular uptake and degradation inside the physiological system. Furthermore, mononuclear phagocytosis system and kidney filtration quickly clear the charged drug molecule before reaching onto the required pathological site. Even today it has been a challenging task to deliver the charged drug with efficient therapeutic efficiency. The nano-carrier based delivery of such charged drugs are mainly based on electrostatic interaction between carrier and charged drug molecules. DNA, siRNA and miRNA are the charged drugs or biomolecules used for nano-carrier based delivery. 1.2.4 How does DOX work? In the thesis we have solely used DOX, the hydrophobic anticancer drug for the corresponding biological study. The DOX is an anthracycline antibiotic isolated by RB Weiss in 1992 from the fungus Streptomyces peucetius.9 It was applied in the cancer 4 therapy at 1996. The chemical structure of DOX is displayed in Fig. 1.1a. The DOX is prone to form free radial by enzymatic reduction. A semiquinone structure (Fig. 1.1a) of the DOX is obtained by one electron enzymatic reduction.10 The semiquinone DOX donates its electron to oxygen under aerobic conditions followed by formation of superoxide. Such redox reaction can start to form superoxide radical with a minute amount of DOX. This radical can damage the DNA and prevent further DNA synthesis and hence, prevents the growth of cancer. Additionally, because of the special type of structure, DOX can intercalate in the DNA base pair.11 In such a way, DOX blocks the cell replication, inhibits topoisomerase II and accrued the final cell death. Figure 1.1 (a) The chemical structure of DOX, (b) DNA binding of DOX and (c) Streptomyces peucetius 1.2.5 Advance Drug Delivery System (ADDS) The advance drug delivery entirely depends upon the brilliant performance of the carrier towards the biological system. Here, carriers are dedicated to act as sensor as well as actuator, mimicking the behaviors of antibodies, membrane receptor and recognize the role of enzymes in the living organism. Thus, the advance drug delivery system (DDS) not only solves the problems those happen with new problematic drug candidate but also it improves and fully exploits the

therapeutic potential of the already used drugs. The drugs unveil their activity as a result of molecular interaction of itself with the biological system. Thus, drug should reach to the required site and it should show its activity by following any kinds of administration route (oral, intravenous, transdermal, local) at a particular concentration. The advance drug delivery means to deliver the drug at right place at right concentration and for a right period of time. But, most of the time it is impossible to do so by selecting any of the administration routes. The problem can be resolved by taking into account the drug with carrier, collectively called as DDS. The administration of drug into the body through DDS leads to the change in the percentage of administered dose with time in the different organisms i.e. changes in the pharmacokinetics and bio-distribution of the body. There are some obstacles those arise in the drug delivery such as, very low solubility of drug, enzymatic degradation of drug, unspecific toxicity, fast clearance from blood vessel, inability of the drug to cross the cellular barrier, etc. These can be resolved by means of a suitable DDS. Thus, the challenge of increasing the therapeutic efficiency of the administered drug by minimizing the side effect can be achieved by perfect designing and engineering of the DDS. In the thesis we are mainly focused on cancer cell targeted drug delivery. The successful invention of new drug can be a potential candidate for fighting against cancer. But direct administration of these drug leads to the creation of several unwanted side effects. The normal and cancerous cells are nearly similar in many biological behaviors. It is very difficult for a drug to indentify whether they are normal or cancerous, that results in the damage of normal cell along with cancerous cells. Thus, the discovering of a drug which is toxic for cancer and non-toxic for normal cell is quite difficult. Discovered, such a carrier which can safely deliver the drug by encapsulating it and conveying to the specific tumor site and release the same at a clinically desire rate is a more practical solution in that respect. 1.2.6 Dosage of Drug The administration of drug to the required site leads to the prevention of disease. Thus, one can say that the administration of very high dose of drug may lead to a very quick recovery of the patient. However, this is not always true. It may also lead to adverse effect and the patient may also succumb to death. There is some particular concentration of each drug depending on their activity towards the biological system. The drug has therapeutic effect (ED), toxic side effect (TD) and lethal effect (displayed in Fig. 1.2). The dosage of drug which produce 50% toxic effect to the animal known as TD50 and 50% lethal effect known as LD50. The dosage of drug which produces 50% therapeutic effect is known as ED50. The relation among these three parameters is represented as: 6 …….Eq.1.1 Figure 1.2: Drug safety and therapeutic index A drug having a therapeutic index value „2“ means that 2 unit of dose creates 50% toxic effect whereas, 50% therapeutic efficiency shows by 1 unit dose of the same drug (relatively unsafe). Higher the value of TI, greater is the prospect of the drug to be used safely. 1.3 Nano-materials for drug delivery The materials having at least one of the dimensions in the nano scale (0.1 to 100 nm) are generally called nano-materials. The nano-material was first invented by great physicist Richard Feynman on the basis of his thought “There“s plenty of room at the bottom”. The nanotechnology has remarkable blessings for the rapid advancement of medical science. The largely growing implication of nanotechnology reflects in an area of medical science, known as nano-medicine.

The advance nanotechnology has good impact on prevention, diagnosis and treatment of disease.13 Therefore, a lot of attraction has been focused on nano-material mediated drug delivery. The presence of some lacunas (like, low solubility, low bio-availability, quick clearance of the drug by reticuloendothelial system, etc.) in the traditional drug delivery system promotes the nano-technology to stand in the front.14 The advancement of medical field, especially drug delivery system by the application of nano-material is because of their shape, size and uniformity. These unique characteristics have direct influence on the drug loading, drug release and stability of the drug both on in vitro and in vivo systems.15 For instance, small particles have very longer half-life of circulation on in vivo system but they are very difficult to store. Also for in vitro applications they quickly form aggregates. The nano-material has many advantages over their counter micro-material from the drug delivery point of view. The fact is already established that a 100 nm nano-material shows 2.5 times and 6 times greater Caco-2 human epithelial colorectal adenocarcinoma cellular uptake compared to the 1 μm and 10 μm size materials, respectively.16 Thus nano-size material can easily penetrate different cell membranes and able to enter the cellular system. The nano-size material also has the ability to penetrate the blood-brain-barrier by passing through tight junction of hyper osmotic mannitol solution.17 The sustained release of drug from some nano-material supports their implication. Some nano-materials can also produce sustain release of drug, when implanted for the treatment of brain tumor and similar difficult-to-treat diseases.18 Like size, shape of the nano-material also has similar importance in drug delivery. The spherically shaped nano-materials are the potential candidate for drug delivery. The anisotropic structured nano-material some time shows higher efficiency because of their structural anisotropy and large surface area to volume ratio. But the anisotropic structure with sharp edge and corners can injure the blood vessels.19 Thus, for an ideal nano- material for drug delivery should have the following characteristic such as, biocompatibility, biodegradability and ability to pass through biological membrane, able to maintain the drug integrity, recognition of target and finally control release of drug. The widely used nano-carriers or nano-vehicles for drug delivery are: (a) Liposomes (d) Polymersoms (g) Nano-hybrid (Inorganic-Organic) (b) Nanoparticles (e) Polymeric micelle (c) Dendrimer (f) hydrogel 1.3.1 (a) Liposomes Liposomes are basically spherical in shape having the size in „nano“ range. The liposomes are mainly formed by the emulsification of lipid in the aqueous medium. The spherical layer of the liposome is formed by the lipid bilayer as displayed in Fig. 1.3.20 As liposomes are formed in the aqueous environment, an aqueous layer is present inside the lipid bilayer.21 Liposomes are widely used for the treatment of infectious disease and for the purpose of anticancer drug delivery. The liposome is first exploited in drug delivery at 1960.22 There are some external parameters such as, temperature ionic 8 strength and presence of some special molecule those can affect the properties of the liposome. In some case, liposomes are rapidly cleared from endoplasmic reticulum endothelial system (RES) before it can reach to the required site. The stability of such liposome in RES can be increased by modifying its surface with hydrophilic moieties. The most frequently used hydrophilic moiety for the modification of liposome surfaces include poly(ethylene glycol), phosphatidylinositol and gangliosides.23 Such stable liposomes are known as „stealth“

liposome. The liposomes are widely used for the delivery of both hydrophilic and hydrophobic drug molecules. The hydrophilic drugs are encapsulated in the aqueous core and hydrophobic drugs are encapsulated inside the lipid bilayer of the liposome. The suitably modified and drug loaded liposome remarkably increase the pharmacokinetic behavior, reduce the toxic side effects and improve the tumor accumulation which promotes its wide implication in the anticancer drug delivery. 1.3.2 (b) Nanoparticles The particle having the size range in between 0.1 to 100 nm are called nanoparticle. The nanoparticles can be prepared from both the organic and inorganic sources. The widely used organic sources for the preparation of nanoparticles are natural polymer (chitosan, sodium alginate, agarose, etc.), lipid (fatty acid, sterol) proteins (gelatin, albumen) and synthetic polymers (poly(lactic acid), poly(acrylic acid), poly(ethylene carbonate), poly(acrylamide), poly(N-vinylcaprolactam), poly(ε-caprolactone), poly(vinyl acetate), poly(lactic-co-glycolic acid), poly(butylcyanoacrylate), etc.). The widely used inorganic sources for the preparation of nanoparticles include iron oxide, gold, silica, calcium phosphate, etc. The reported procedures for the preparation of nanoparticles are spray drying, spray congealing, microemulsion, hot and cold homogenization, solvent emulsification and evaporation, interfacial polymerization, solvent diffusion and nano precipitation.15, 24 The influential property of the nanoparticles is their small size which allows them to pass through the small capillary and get accumulated to the targeted site. The nanoparticles can protect the drug from degradation by encapsulation, decrease the toxic side effect of the drug, increase the bioavailability and changes the pharmacokinetics of drug. They allow a sustained release of encapsulated drug from the nanoparticles i.e. a longer period of release of the drug. 9 Although hydrophobic drug can easily be encapsulated inside the nanoparticles but encapsulation of hydrophilic drug is quite difficult.25 Still there are some critical methods which allow the entrapment of hydrophilic drug inside the nanoparticles.26 These methods are double emulsion to produce hollow nanoparticles and loading of drug onto the surface of the nanoparticles by judicious fabrication. Thus, a well designed nanoparticle can act as an efficient nano-vehicle for targeted drug delivery. 1.3.3 (c) Dendrimers Dendrimers are a highly branched polymer like a tree spreading their branches away from them. The dendrimer was first reported in the year of 1980.27 The dendrimers are synthesized by step wise way either from core initiator or from the terminal. The former one is the most commonly used method. Depending on techniques of the preparation a high level of order can be brought about on the size and degree of branching with narrow D-stroke (Đ) or dispersity of the dendrimer. The properties of the dendrimer (water solubility, degree of branching, size, molecular weight and flexibility) are largely controlled by the choice of core initiator and repeating unites. The dendrimers interact with nearby molecules by their terminal functional groups. The inner part can be made either hydrophilic or hydrophobic by changing their terminal i.e. peripheral groups. The dendrimer can acts as a host-guest complex with a suitable therapeutic agent (e.g. drug molecule). Depending on the position (inner core or periphery) of complex formation of the drug with dendrimers, release kinetic gets differed. The higher generation dendrimer produces a smaller hydrodynamic radius compare to their linear analog. The dendrimer have some unique characteristics like, presence of cavity inside the molecules and having globular shape. The

internal cavity allows the entrapment of the drug molecule and the drug molecule can also be attached onto the peripheral functional group of the dendrimer. The hydrophobic dendrimer can be tailored to amphiphilic structure by the introducing hydrophilic moieties like, poly(ethylene glycol) or poly(acrylic acid) through its peripheral functional groups. Any hydrophobic drug can be easily entrapped inside the amphiphilic dendrimer. Thus, the dendrimer are widely used for anticancer drug delivery. The drug delivery can be made as targeted by modifying its surface with cancer cell targeting moiety. In gene therapy, carriers which carries the gene are called vectors.28 The 10 dendrimer like vectors encapsulated with gene can quickly transport the gene by passing through the cell membrane to the nucleus.29 1.3.4 (d) Polymersome The polymersome are similar to liposome but one of the fundamental differences is that liposomes are formed by the lipid bilayer whereas; polymersomes are formed by the amphiphilic diblock copolymer.30 There are certain advantages of polymersome compared to the liposome, such as very high flexibility, stability and easy tailoring of bilayer characteristics. The multiblock (pluronic F27) copolymer can also be used to prepare polymersome.31 The polymersomes are formed by the bilayer structure with inner aqueous solvent trapped system. Like liposome, polymersome can also deliver hydrophilic as well as hydrophobic drug molecules. 1.3.5 (e) Polymeric micelle (PM) The polymeric micelle is formed by the self-assembly of the amphiphilic block copolymers. The polymeric micelle is prepared from the block copolymer having well defined and controllable size and shape. The reason for the self-assembly of the amphiphilic block copolymer is the hydrophilic and hydrophobic interaction (for water as a dispersion medium). In aqueous medium, hydrophobic part of the amphiphilic polymer clubs together leading to the formation of assembly. The assembly has different structure as like, sphere or rod (micelle), bilayer structure in planar form (lamellae) and bilayer structure in the closed spherical shape (called vesicles). A very low concentration of amphiphilic polymer is good enough for the formation of micelle. With increasing the size of the micelle, surface/volume ratio decreases. As a result, the water facing surface for each amphiphilic molecule diminishes in the surface of the micelle. The steric repulsion among the hydrophilic parts (water facing part) of the amphiphilic molecule prevents the formation of self-assembled structure. Thus, apparent size of the micelle is a result of negotiation of packing between hydrophilic and hydrophobic parts of the amphiphilic block copolymer. The critical micelle concentration (CMC) of an amphiphilic polymer is the concentration at which micelle gains thermodynamic stability i.e. when molar Gibbs free energy of association becomes zero. The predication of the shape of the micelle can be done from the packing parameter (P). 11 For an imaginary surfactant molecule, let us consider the length and volume of hydrophobic chain as „l“ and „v“, respectively. The cross-section of the hydrophobic part is (v/l). The optimal head area is symbolized by a0. The shape of the aggregates remarkably depends up on the ratio of optimal head area (a0) and hydrophobic cross- sectional area (v/l) i.e. packing fraction (P).32 $P = (l \times a0)/v$ …….Eq.1.2 Now, four situations can arise: (1) When, $P < 1$ then invert micelle will be formed (2) When, $P > 3$ then micelle will be formed (3) When, P = 1-2 then vesicles will be formed (4) When, P = 2-3 then wormlike structure will be formed The various probable structures which are formed at different „P“ values are shown in the Fig. 1.3. The micelle can encapsulate

the hydrophobic drug inside its hydrophobic core. The hydrophilic drug can also be functionalized onto the surface of the micelle. The drug molecules are also safe from the degradation and unwanted or premature release. The micelle can be used for targeting any specific biological system by attaching required targeting moiety on its surface. Thus, the suitable engineering micelle prepared from biodegradable or biocompatible polymer can be used as nano-vehicle for the targeted anticancer drug delivery. 1.3.6 (f) Hydrogel The hydrogels are three dimensional network of water miscible polymer which can able to trap the water, therapeutic agents or physiological fluid inside its network.14 Both synthetic and natural polymers are used for the preparation of hydrogels. The natural resources of hydrogel are hyaluronic acid, alginate, collagen, dextran and citosan.33 Poly(2-hydroxypropyl methacrylate), poly(vinyl alcohol), poly(ethylene oxide) and poly(acrylic acid) are the synthetic resource for hydrogel.34 The three dimensional network of gel can be created by physical or chemical cross linking. The physically cross linked hydrogels are potentially formed on the basis of van der Waals, hydrophobic, electrostatic and hydrogen bonding interactions. The physical gels are sensitive to temperature, ionic strength and concentration of the cross linking agents.35 The association of amphiphilic polymers and complex formation of the positively and negatively charged polymers enforce the quick gel formation. The chemically cross linked hydrogels are potentially formed by covalent cross-linking of available functional group in the polymer chains. Unlike physical gel, chemical gels are strong enough and not affected by the temperature, ionic strength like nominal factors. The cavity inside the hydrogel can be controlled by controlling the crosslink density. The cavity inside the hydrogel plays important role for loading of therapeutic agents. The presence of aqueous part inside the hydrogel enhances its biocompatibility by reducing the interfacial tension with biological fluids. The hydrogel can also be prepared in the form of nano, known as „nano-gel“. The nano-gels are widely used for anticancer drug delivery. The therapeutic agent locked in nano-gels can be released very slowly by controlled diffusion, erosion or swelling process. There exist some stimuli responsive hydrogels which release the therapeutic agents by the sudden cleavage of three dimensional networks. Figure 1.3 The expected size and packing parameter of the self-assemble structure (a) to (d) and liposome (e). 1.3. 7 (g) Nano-hybrid Like organic nano-materials, inorganic nano-materials have also gained their importance in the field of drug delivery. The inorganic nano-carriers are superior to organic analog by some critical characteristics like, magnetic, optical, electrical and other physical properties. The release of the drug from these nano-carriers can easily be controlled by 13 the application of external stimuli, according to their responsiveness. There are four different types of inorganic nano-materials namely metal, oxide, sulfide and carbonaceous.36 The nano-carrier can display different types of morphology, among them nano-tubes, nano-shells and mesoporous nano-particles have got most attraction for drug delivery. These special morphological structures help to encapsulate the drug molecules inside its void space. Nano-material devoid of vacant space inside them but can be fruitfully utilized in drug delivery provided those are modified with biodegradable or biocompatible polymers. The polymeric layers on the surface of the nano-material perform as a base for holding the drug molecule and also increase the biocompatibility. The nano-materials can also be used for

targeted drug delivery by external stimuli or can be suitably decorated with targeting moiety. There are a list of nano-materials such as, mesoporous silica nanoparticles, gold nanoparticles, carbon nano-tubes and magnetic nanoparticles (MNP) which are extensively used for drug delivery. 1.3.7.1 Magnetic nanoparticles (MNP) The engineering nanoparticles which can be manipulated by the application of external applied magnetic field called MNP. The MNPs are mostly prepared from „Ni", „Co", „Fe" and their oxides. Depending on magnetic behavior, MNPs are classified into five different types, namely diamagnetic, paramagnetic, ferromagnetic, anti-ferromagnetic and ferrimagnetic. Diamagnetic material The material having no resultant magnetic moment known as diamagnetic material Paramagnetic material The materials having resultant magnetic moment by the random orientation of magnetic vectors (Fig. 1.4a) are known as paramagnetic material. Ferromagnetic material The material which can be easily converted to permanent magnet or attracted towards magnet called ferromagnetic material. Unlike paramagnetic, ferromagnetic material shows very strong interaction. The interaction is originated from the parallel or anti- parallel arrangement of atomic moments (Fig. 1.4b). These material shows paramagnetic behavior after a certain temperature called Curie temperature (TC). 14 Anti-ferromagnetic material These materials are very similar to ferromagnetic material but the only difference is the anti parallel alignment of atomic moments (displayed in Fig. 1.4c). The alignment is due to the exchange interaction between the neighboring atoms. The anti-ferromagnetic material shows the paramagnetic behavior after a certain temperature known as Neel temperature (TN). Ferrimagnetic material This type of magnetic behavior is only observed in compounds or alloys not in pure atoms. The exchange interaction leads to the parallel alignment of atomic moment in one crystal side and anti-parallel alignment on other side (Fig. 1.4d). The breaking of magnetic material into magnetic domains results in ferromagnetic material with very low saturation magnetization. Figure 1.4 A schematic presentation of alignment of magnetic moments (a to d) and magnetization curve of (f) ferromagnetic; (g) superparamagnetic material. Single domain structure: origin of superparamagnetism The most widely studied effects of the size of nanoparticle on magnetism are due to single domain limit (i.e. single domain ferromagnetic nanoparticle) and superparamagnetic limit (i.e. superparamagnetic nanoparticles). In a large magnet a number of domains are separated by domain walls i.e. large magnets appear as multi- domain. In multi-domain system each domain has uniform magnetization. A magnetization at which all the magnetic moments are aligned to a particular direction (for both superparamagnetic and ferromagnetic nanoparticles) called saturation magnetization (Ms) (Fig. 1.4f). There exist remaining of a measurable amount of magnetization even after removal of the external magnetic field. The remaining amount of magnetization is known as remnant magnetization (Mr). The application of magnetic field in the opposite direction can bring the magnet from remnant state to its initial state. The amount of the magnetic field required in the opposite direction to bring the magnet to its initial state is known as coercivity (Hc). The bulk magnetic material and the single domain materials differ from each other by the mechanism of magnetization. In large magnetic material the sector of uniform magnetization are separated from each other by the formation of domain wall. The formation of domain wall is a process of

maintaining the balance between magnatostatic energy (ΔEMS) and domain wall energy (Edw). The volume of the particle has proportionality relation with ΔEMS. The Edw is also proportionally related with interfacial area between the domains.37 Thus, there is a critical diameter of a spherical particles below which ΔEMS = Edw i.e. the particles exist as a single domain state. Thus, the critical diameter (Dc) is represented as: ……Eq. 1.3 Where, A implies the exchange constant, K implies anisotropy constant, μ0 is the permeability of vacuum. The critical diameters for single domain state are located in the range of 10 to 100 nm for most of the magnetic materials. But in some case they may of the order of several hundred of nanometer for large anisotropic materials.38 For a single domain magnetic material the energy required to preserve the magnetization is proportional to (KV)/(KBT). Where, V is the particle volume, KB is the Boltzmann constant and T is the temperature. When, thermal energy overcomes the anisotropy energy then the magnetization becomes unstable and the material is said to be present in superparamagnetic state (Fig. 1.4e). The temperature at which thermal energy overcomes the anisotropy energy is known as blocking temperature (TB).38 In the superparamagnetic state, the material behaves as a giant magnet. The high Ms at low magnetic field with absence of Mr promotes the wide application of MNPs in the biomedical field. 1.4 Cancer cell targeting approach In the field of drug delivery, most important parameter of the drug delivery is their targeting aptitude. The targeting propensity of the nano-carrier increases the efficiency of the drug delivery by reaching to the predetermined organs or site. The cancer cell can be targeted in two different ways namely, passive targeting and active targeting. The external stimuli like magnetic field may also be applied for targeting the tumor cells. The magnetic stimuli can efficiently deliver the therapeutic agent (drug) to the right place at right time.39 Application of high frequency AC magnetic field on the MNP encapsulated nano-carrier produces rise in temperature called hyperthermia. The hyperthermia damages the cancer cells by thermal heating. As, MNP itself not able to carry therapeutic agent by encapsulating into it, so a suitable modification of MNP surface with biodegradable and/or biocompatible polymer is necessary. The polymeric layer presents on the surface of the MNP can hold and deliver the therapeutic agent to the magnetically targeted site. The release of drug from the MNP encapsulated nano-carrier may happen in two different ways e.g. hyperthermia and magnetically induced vibration. Hyperthermia some time leads to shrinkage of thermoresponsive polymeric layer and release of drug molecules.40 Magnetic vibration leads to the rupture of MNP (and drug) encapsulated nano-carrier followed by instant release the drug molecules.41 1.4.1 Passive targeting By taking the advantage of the nature of tumor cell and porosity of the blood vessel it can be targeted by passive accumulation technique. The tumor cell in the malignant sate grows rapidly. The rapid growing of the cell has an urgency to create new blood vessels. The newly formed blood vessels are abnormal in their nature (i.e. abnormality in porosity and architecture). The fenestrations created on the newly formed blood vessel are about 100 to 800 nm which is much larger in size compared to the pore size of the normal blood vessels (around 6 nm).42 The leakage and unsuccessful lymphatic drainage (Fig. 1.5) of the nano-carrier allows them to passively accumulate in the tumor cell compared to the normal cell.43 This phenomenon is known as enhance permeability and retention (EPR) 17 effect.

The nano-carriers having a size range from 30 to 200 nm are efficiently accumulated in the tumor tissue by EPR effect.44 The nano-carriers larger than 200 nm are rapidly cleared from blood stream by the spleen. However, the treatment of solid tumor by passive accumulation is somewhat difficult. The heterogeneous distribution of blood vessel followed by heterogeneous distribution of drug in the solid tumor makes it difficult to treat solid tumor. 1.4.2 Active targeting The efficiency of the drug delivery can be further enhanced by attachment of small targeting ligand such as, peptide, antibody, antibody fragment, vitamins and nucleic acid onto the surface of the nano-carriers. The receptors presents on the surface of cell allow specific binding with the targeting ligand attached onto the surface of the nano-carrier. The ligand-receptor binding phenomenon promotes the enhanced internalization of nano- carrier through receptor mediated endocytosis path way.45 The technique of targeting the cell with the help of molecular recognition is known as active targeting. Although, targeting moiety or ligand increases the therapeutic efficiency yet it does not mean that the attachment of excess number of ligands will show even better efficiency. The attachment of more than sufficient number of targeting ligand onto the surface of nano- carrier leads to the decrease of the efficiency of diffusion, uniformity of distribution and depth of penetration.46 Thus, it is necessary to optimize the number of targeting ligands which would be sufficient for showing effective performance in drug delivery. 1.4.2.1 Antibody for cancer cell targeting Antibody is basically a protein widely used in immune system for detecting the foreign material inside the body. The antigen human epidermal growth factor 2 (HER2) is widely overexposed onto the surface of the malignant tumor cell and weakly exposed in the surface of the normal cells.47 Thus, antibody against HER2 is widely used in the targeted drug delivery. The endothelial-leukocyte adhesion molecule, vascular cell adhesion molecule- 1 and intercellular adhesion molecule- 1 are other special types of antibody are extensively applied in targeted drug delivery.48 1.4.2.2 Peptide for cancer cell targeting Peptides are short chain of amino acids developed by forming the „–CONH-“ linkage between the acid groups of one amino acid with the ammine group of other. The attachment of peptides onto the surface of the nano-carrier enhances the efficiency of drug delivery. The TAT,49 RGD50 and NGR51 are extensively used for targeting the cancer cells. Figure 1.5 Passive targeting on normal and tumor cells through EPR effect. 1.4.2.3 Folic acid (FA) for cancer cell targeting The folic acid is „B“ group vitamin also known as folate. However, from the structural (chemical) point of view, the deprotonated structure of FA is the folate. Nano-carrier conjugated with FA on its surface acts as superior targeting system for efficient delivery of anticancer drug to the cancer cell. The presence of FA receptors in most of the cancer cells helps the nano-carrier to bind with it. The tumor cells in the malignant state are rapidly divided into new cells. This process essentially requires nutrients like FA because of the fact that it is essential for the biosynthesis of nucleotide. Usually, FA receptors are not available in normal cell as it present on the apical side of the epithelial cell. Once the cell becomes malignant, the cell membrane losses the polarity and FA receptor can appears at basal surface and then it can successfully able to receive FA.52 In the present work, we plan to target the cancer cells by the guidance of FA. 1.5 Controlled drug release In case of nano-carrier based drug delivery, the rate of drug release from the carrier is the key factor for

efficient cancer therapies. The release of the drug is largely controlled by design of the nano-carrier. The drug release can be classified into two different categories namely, sustain drug release and stimuli responsive drug release. The mode of drug release absolutely depends upon the condition of the patient and stage of the disease. 1.5.1 Sustained drug release In this method, the drug molecules are released from the carrier at a constant and predetermined rate. The constant rate is maintained over the whole period of drug release. This method of drug release is widely applied for such types of drug which either can be metabolized or eliminated from the body just after the administration. The carrier releases the drug at the same rate as it gets eliminated from the body and thus the constant rate of drug release is maintained. The carrier encapsulated drug as soon as comes in contact with aqueous fluid of the body, the drug molecules try to quickly reach the require pathological side. In order to make a sustain drug release, the drug molecule should be shielded by a polymeric shell that allows to slowly release of drug by diffusion or slow degradation of the shielded polymeric layer.53 The first technique is known as diffusion control drug release and second one is the erosion control drug release. 1.5.2 Diffusion control As mentioned earlier, the drug molecules are shielded from the aqueous biological system by a water insoluble polymeric shell. This process of drug release can be maintained by reservoir based or matrix based drug release.54 In the reservoir based system, the drug molecules are encapsulated inside the reservoir and outer surface are covered by reservoir layer. The drug molecules are first diffused from the core and then appear at the surface of the reservoir and finally start to diffuse slowly. In the matrix based system, the drug molecules are encapsulated inside the insoluble matrix. The drug molecules present on the surface are diffused first followed by reaching of the core drug molecule onto the surface (Fig. 1.6). Thus initially, noticeable rate of drug release known as burst release is observed.55 In the next part, the drug molecules those reach onto the surface are then slowly released to the biological system. 1.5.3 Erosion control This method involves the release of drug molecules by the degradation of polymeric shell of the carrier. The biodegradable or erodible polymers are applied for the preparation of erosion controlled carriers. The rate of drug release can be tuned by the proper selection 20 of polymer and techniques of encapsulation. A number of synthetic as well as natural polymer e.g. poly(lactic acid), poly(glycolic acid) and poly(lactic-co-glycolic acid) are frequently used for the preparation of such carriers. The polymers are degraded by the hydrolytic cleavage of ester bond followed by formation CO_2 and H_2O.56 The rate of degradation as well as the release of drug is controlled by the hydrophilicity of the carrier. Generally, the drug appears on the surface and then released first by surface degradation. In the next step, slow degradation process promotes the moderate rate of drug release. Finally, full degradation of carrier leads to the rapid release of the drug molecules (Fig. 1.6). Figure 1.6 The Schematic depiction of three fundamental release mechanisms 1.5.4 Stimuli responsive release The situation where constant or sustain release of drug is not sufficient for showing therapeutic efficiency, the stimuli responsive release system is a fruitful solution. Thus, for the treatment of the disease like tumorigenesis and spreading of cancer required a smart, intelligent and environmentally sensitive carrier for efficient therapy. In the bio-system, the responsiveness can be predicted, reproduced and it is proportional to the intensity of

the response. The different stimuli used for triggering the drug release can be classified into two major categories namely, external and internal stimuli. The internal stimuli responsive carriers are activating or modulating the release behavior by the change of any biological medium (e.g. temperature, pH, enzyme, redox, bio-chemicals etc.).57 The internal stimuli responsive release is known as self-regulating release. The external stimuli responsive carrier shows the pulsed release by the effect of external stimuli (e.g. magnetic, light, electric, ultrasound etc.).58 1.5.4.1 pH or ionic strength responsive release The variation of pH of pathological organs from the normal healthy tissue provides a key component for bringing the control on drug release. This variation in pH promotes the nano-carrier to deliver the drug selectively to the specific organ e.g. vagina, gastrointestinal track or inter cellular compartment of the body e.g. endosome, lysosome. The healthy and normal tissues have standard pH 7.4 but the cancerous tissue has the pH considerably lower i.e. 6 to 3. There are four different approaches for pH responsive drug release. First approach is the neutralization of acid by protonation followed by release of bound drug by the reduction of electrostatic interaction between polymer and drug molecule.59 Second approach is the pH responsive swelling or shrinkage of the hydrogel followed by release of drug from the drug loaded hydrogel.60 Third approach is the incorporation of cleavable (pH responsive cleavage) bond into the nano-carrier or attachment of drug through the cleavable bonds. The acetal, hydrazine and hydrazone type of bonds show such pH induces bond cleavage.61 The pH responsive cleavage of these bonds benefits the release of the drug molecules. Forth approach is the use of functionalized polymer which shows considerable change in density of ionic charges with the change of pH. The polymers used for this approach of drug release are poly(acrylic acid), poly(amide), Poly(methacrylic acid), poly(methyl acrylate) and poly(diethylaminoethyl methacrylate).62 All of these polymers has active acid (– „COOH“) groups. In the bio-system, acid groups should be present on the outside of the nano-carrier facing the aqueous system. The acid group present on the surface of the nano-carriers controls its hydrophilicity (followed by deprotonation of acid group) or hydrophobicity (followed by protonation of acid group) depending on the pH of the biological medium. The protonation or deprotonation leads to the change in electrostatic interaction (or ionic strength) and pH dependent drug release. The very last approach is attempted in the thesis to perform the pH responsive drug release. 1.5.4.2 Enzyme sensitive release The enzymes are globular proteins. Like other proteins they are formed by three-dimensional array of linear amino acid chains. The enzyme can accelerate or catalyzed the biological process by performing alone or in its complex form. The enzymes can covalently conjugate with polymer and subsequently self-assembled to micelle. The enzyme can rupture or form some specific bond.63 The attachment of drug with polymer through enzyme-sensitive linkage leads to the enzyme responsive release of drug.64 The alternation of the enzyme expression in different pathological environment can also promote the enzyme responsive drug release.61b The peptide bond linked drug can shows trigger drug release by the enzyme (proteases). The polysaccharide based carrier can similarly be activated by glycosidase. The lipase can break the structure of the liposome by hydrolyzing the phospholipid building block and release the bound drug molecule. The process of hydrolysis can also be used to hydrolyze the

gate-keeping moiety of mesoporous nano-particles and allows the releasing of bound drug molecules.65 There are some enzymes which can modify certain chemical groups or can break the covalent bond by altering the balance between hydrogen bonding, electrostatic, π-π interaction.66 The enzyme, hydrolase can also trigger enzyme responsive release according to one of the above mention ways. The reversible breaking of covalent bond by the enzyme, kinases and phosphatage proffered the enzyme responsive release of the drug molecule.67 1.5.4.3 Thermoresponsive release Temperature is the extensively used stimuli for triggering the drug release. The thermoresponsive release involves a sharp change in the physical property which triggers the release of the encapsulated drug molecules. The triggering temperature for drug release should be close to the body temperature i.e. in between 37 to 42 °C.68 Above that temperature range the many bio-molecules like protein gets denatured. The nano-carrier like liposome or nano-particles or micelles are used for such temperature triggered drug release. The temperature induces the structural or conformational change in the lipid bi- layer that helps to release the encapsulated drug molecules.69 A thermoresponsive bubble forming liposome is also fruitful for triggering the drug release. The bubbles of CO2 are formed by decomposition of NH4HCO3, present inside the liposome. The bubbles try to 23 escape leading to formation of holes in the lipid layer and drug molecules find their way out.70 For the polymer based micelle or nano-particle, the phase change occur at above or below the lower (LCST) and upper (UCST) critical solution temperature. It leads to the structural change of the polymeric layer from coil to globular form or vice-versa.71 The thermoresponsive polymer becomes hydrophobic above the LCST and takes the globular shape. Below the LCST, the polymers are soluble in the solvent. The change in the shape of the polymer triggers the release of encapsulated drug molecules. For cross-linked thermoresponsive network, the temperature induced swelling and shrinking of the networks trigger the release of arrested drug molecules.72 The thermoresponsive polymers widely used in drug delivery are enlisted as, poly(N-vinyl caprolactone), Pluronics, poly(N-isopropyl acrylamide), poly(N,N diethylamide), poly(methyl vinylether) etc.73 In this thesis we are focused on poly(N-vinyl caprolactone) and poly(N- isopropyl acrylamide) for performing thermoresponsive drug release. 1.5.4.4 Redox responsive release The reduction responsive drug release involves the rupturing of „–S-S-“ bond by the reduced glutathione (GSH). Thus, nano-carrier attached with drug through „-S-S-“ linkage can able to show reduction responsive drug release. The „-S-S-“ bond get reduced to „- SH“ by the GSH.74 The concentration of GSH present in inter cellular compartments (cytosol, nucleus, mitochondria) is about 2 to 10 mM which is about 2 to 3 times higher than GSH present in the extra cellular compartment (10-20 μM).75 However, the tumor tissue has the GHS concentration about 4 times higher than normal tissue.76 Hence, the reduction responsive nano-carrier can be easily targeted towards the tumor tissue. The nano-carrier involving „-S-S-“ crosslink bond can swell or disassemble in the presence of GSH and concurrently release the arrested drug molecules. 1.5.4.5 Light responsive drug release The light is the stimuli which can be used for „remote control“ release of drug molecules.77 The light responsive release can be achieved in three different ways e.g., by changing the conformation of light sensitive material or by breaking the covalent bond between drug and carrier molecule or by generating

the local heat. The light sensitive molecules like azobenzene, nitrozene, pyrene, cinnamoyl and spirobenzopyran undergo 24 trans- to cis- (or vice-versa) conformational transition in presence of UV or visible light, respectively.78 The reversible conformational change enables the nano-carrier to trigger release of bound drug molecule. Sometime, hydrophobic and hydrophilic phase change by the cis- to trans- transition may promote the release of drug molecule.79 The nano- carrier conjugated with drug through light sensitive covalent bond known as pro-drug. The irradiation of light with specific wave length leads to the breaking of the linkage and release of the bonded drug molecule from the pro-drug.80 Because of the poor depth of penetration and strong scattering of UV light by the soft tissue, the recent attention for activation has been shifted in the near infrared light (NIR) region.81 The NIR light can able to deeply penetrate the soft tissue without any local heat effect and damage. The plasmonic nanoparticles can able to transfer the absorbed NIR light into heat and shows the light responsive release study.82 The photo-thermal effect of Au nanoparticles leads to the generation of local heat which favors the dehybridization of DNA helices and release of drug attached with cytosine-guanine base pair.83 Recently, non- biodegradability of the inorganic nano-carrier promoted the wide application of organic transducer for converting the absorbed NIR light into heat energy.84 The indocyanine green and FDA approved fluorescence dyes are the recently used transducers for light sensitive drug release.85 1.5.4.6 Magnet responsive drug release Magnetic field is also an important external stimulus for triggering the drug release. As discussed in the earlier section that the MNP should be coated with suitable polymer to encapsulate and carry the drug molecules. The superparamagnetic MNP have three different prospects in drug delivery. First, it can be monitored in the body by the MRI. Second, it can be targeted to a specific site or organ in the body. Third, it can release the accumulated drug by the application of AC magnetic field.86 The Fe_3O_4 and γFe_2O_3 are the two non-toxic materials used for biomedical applications.87 The thermoresponsive polymer modified MNP offers the thermal induced shrinkage by hyperthermia and release the encapsulated drug molecules. The manipulation of AC frequency to the radiofrequency region leads to the generation of high heat which would destroy the cancer cell. The cancer cell can sustain up to 43°C but above that temperature they start 25 to be destroyed.88 However, the normal cell can sustained up to and above that temperature. Again, the increase of the frequency of AC magnetic field to higher level helps to rupture the carrier and shows the burst release of the drug molecule.73a In this thesis we are planned to use superparamagnetic Fe_3O_4 nanoparticles in order to achieve the magnetic responsive drug release. 1.5.4.7 Electric field responsive drug release The electric field is an interesting stimulus although it is not so extensively used in the drug delivery. The electric field stimulated drug release can be performed by using two different types of materials namely, intrinsically conducting material and polyelectrolyte material.89 The intrinsically conducting material can able to show magnetic, electric, electronics and photo responsive behavior. The source of electricity is the uninterrupted and π-conjugated back bone.90 The electrical signal change the redox state of the intrinsically electric material and allow the release of the bound drug molecule. The electric field responsive drug delivery can also be achieved by using polyelectrolyte with high charges containing functional groups which can able to show the pH

responsive drug delivery. The electric responsive delivery systems are appeared as a sheets, microparticles and in situ gelling injectable system. An electro-conducting patch is placed on the skin and allows for passing the electric signals. The potential difference between the two electrodes establishes the change of local pH. The pH change offers the release of drug by swelling or shrinking of the crosslink polyelectrolyte. The electric filed responsive drug release is successfully tested for the insulin from the microgel prepared from poly(diethylaminopropyl acrylamide).91 The electric stimulus was successfully applied for the release of drug and hormones from the microchips which is already incorporated in the body. This type of system can be successfully adopted for releasing paclitaxel from stents.92 1.5.4.8 Ultrasound responsive drug release Although ultrasound triggering release of drug is not so common, yet it can be used in some special applications. The ultrasound is superior from other ordinary stimuli because it can penetrate the deep of the tissue and exactly target a particular tissue. The ultrasound generates the local heat that helps the nano structure to penetrate into the depth of the 26 tissue and bubble cavitations.93 This stimulus is applied on polymeric micelle and recently on the layer-by-layer assembly. The bubble cavitation allows the trigger release of drug from the micelle based carrier. The low frequency ultrasound is extensively used for the treatment of in vivo tumor present on animal models.94 The externally applied ultrasound produces, pulsatile drug release.95 The rate of drug released can be manipulated by changing the frequency, pulsate length and power density. 1.6 The impact of the architecture of polymer in drug delivery In the field of self-assembly and drug delivery, polymer have brought a new direction of advancement.96 In the biological filed it is necessary to use material say, polymer having no toxic effect. Thus, a biodegradable and/or biocompatible polymer is recommended for such applications.97 The contribution of polymer in the field of biology is unavoidable. The synthetic polymers are superior because of their stability and versatility, less- expensive procedure of preparation.98 Recently, well defined polymer with wide varieties of architecture of polymers like branch, hyperbranch, hybrid, star and block can easily be prepared for biomedical applications.99 34, 100 Recently, branched architectures of the polymer are extensively exploited for self- assembly. As a result of self assembly of branched polymer, a variety of structures are obtained as micro or nano-micelle, vesicles, tubes, fibers, film, etc.101 The three dimensional globular architecture of the branched polymer offers large terminal functional groups those can interact with biological system. The low solution viscosity offers easy movement of them inside the biological system. The high aqueous solubility (for amphiphilic polymer) offers long circulation in the biological system. The creation of void space inside the structure during self-assembly allows the encapsulation of large amount of drug molecules. These characteristics of branched polymer promote the wide application of them (compare to the linear analog) in the form of self-assembly for drug delivery.102 1.7 Polymerization There are different types of polymerization techniques available for the synthesis of different types of polymer with well defined architecture depending on the functionality and structure of monomers. In the thesis we have reported synthesis of a library of 27 polymer forms by step growth polymerization utilizing mainly two different polymerization techniques namely atom transfer radical polymerization (ATRP) and ring opening polymerization (ROP). 1.7.1 Atom

Transfer Radical Polymerization (ATRP) It is a special type of control radical polymerization involving transition metal mediated catalysis reaction. The Cu, Ni, Fe and Ru are the transition metals usually used for ATRP reaction. Among them, the first one is extensively used to catalyze the ATRP reaction. The reaction involves a dynamic equilibrium between growing radical and large amount of dormant species. The term, atom transfer comes from the mechanism of the reaction. It involves the transfer of atom through a key elementary step. The atom transfer is the responsible step for the uniform growth of polymer chains in ATRP. The concept of ATRP was originated from the reaction involving the formation of carbon-carbon bond between organic halide and alkenes through „Ru" catalysis. The „Ru" catalyzed reaction is known as „Kharasch addition". The atom transfer radical addition (ATRA) is slight modification of „Kharasch addition" reaction which are usually initiated by light or conventional radical initiators.103 Scheme 1.1: The mechanism of ATRP reaction. The first step of ATRP reaction involves the formation of active species or radical (from alkyl halide, P-X) through a transition metal-ligand complex (Mn-Y/ligand, Y is the counter part of metal) mediated reversible redox reaction. The metal-complex gets oxidized to next higher oxidation state (X-Mn+1-Y/ligand) by donating concomitant electron to the halide. The reversible process occurs with a rate constant of kact and kdeact for the forward and backward reactions, respectively (Scheme 1.1). The rate of polymerization of ATRP reaction depends on the magnitude of the equilibrium constant (keq= kact/kdeact). If the keq is too small then the ATRP reaction may not happen or may happen at a very slow rate. If keq is too large then also there is a possibility of domination of termination reaction because of large radical concentration. Monomers react with 28 intermediate active radical and form the polymer chains with a rate constant kp. The termination reaction produces in ATRP at a rate constant kt. The termination reaction may happen through disproportionation or radical coupling reaction. The oxidized metal complex (X-Mn+1-Y/ligand) exists as persistent radical in the reaction mixture and decrease the stationary concentration of the growing radical followed by reduction the termination reaction.104 However, the termination can also be reduced to a few percent by meticulously controlling the ATRP reaction. Thus for successful ATRP, the reaction should pass through a very few percent of termination along with uniformly grown of polymer chains. A typical ATRP is a multi-component system with monomer, initiator (having transferable halogen) and catalyst (consist of transition metal with their complimentary ligand). For a successful ATRP the nature of the solvent and temperature of the reaction should also be taken into account. A variety of monomer was successfully polymerized through ATRP reaction, especially those which can stabilize the propagating radical. The initiator used in ATRP is generally alkyl halide with active substituent (aryl, allyl, carbonyl etc.) at the α-carbon. The initiator can determine the number of growing chains. If the initiation is fast then transfer and termination would be negligible. In this circumstance, the degree of polymerization i.e. the theoretical molecular weight will increase proportionally with the initial concentration of the initiator. ……Eq. 1.4 In order to achieve a narrow molecular weight distribution and well defined polymer structure, the halogen group (X) of initiator should quickly and selectively migrate between growing chains and transition metal-complex. Among the halogen groups, bromine, chlorine and sometimes iodine containing initiators

are applied in the ATRP reaction.105 The fluorine substituted initiator has not been used in ATRP reaction. Because the C-F bond is very strong and not easily cleave homiletically to form the radical.106 The nature of the catalyst is the key factor in ATRP reaction. In order to serve as a catalyst in ATRP, the transition metal of the catalyst should have the following characteristic such as, two oxidation states separated by one electron difference, very 29 high affinity towards halogen, capable to expand the co-ordination sphere upon oxidation and can able to form a strong complex with ligand. The ATRP be adopted both in bulk and solution polymerization. For solution polymerization, the choice of solvent is the most important factor. The solvent used in ATRP should not interact with catalyst and the chain transfer to the solvent should minimum. The increase of temperature leads to the increase of radical propagation rate constant and atom transfer equilibrium constant. Thus, finally increases the rate of ATRP reaction. However, at higher temperature the rate of termination and other side reactions become prominent. Generally, the dissolution of catalyst increases at higher temperature but higher temperature may also lead to the decomposition of the catalyst. Thus, the optimal temperature of the reaction is largely controlled by the monomer, catalyst and decided by targeted molecular weight. 107 ……Eq. 1.5 The rate of ATRP reaction mainly depends upon three important parameters normally concentration of monomer, concentration of growing radical and rate constant of propagation according to the equation 1.5. In the thesis we have used ATRP reaction to produce a four armed polymer with precisely control molecular weight and low dispersity (Đ).108 Furthermore, ATRP maintained the control architecture in terms of chain topology (star, cyclic, brash, and regular network), chain composition (block, graft and gradient copolymer) and diverse functionality. 1.7.2 Ring opening polymerization (ROP) The ROP of the lactone-like monomers can be carried out either in bulk or in solution polymerization or in emulsion or in dispersion polymerization. For bulk polymerization at around 100 to 150 $^{\circ}$C and for solution polymerization at around 0 to 25 $^{\circ}$C temperatures are applied in order to get effective control over polymerization process. The solvents generally used in the solution ROP include THF, dioxane, toluene and DMSO. The organometallic compounds like oxide, alkoxides, carboxylates are mainly used as initiator for ROP.109 Enzymes can initiate and catalyze the ROP.110 There are three established mechanisms for ROP namely anionic, cationic and coordination- insertion. In this thesis we have mainly used coordination-insertion polymerization technique for ROP. 1.7.2.1 Co-ordination-insertion The coordination-insertion polymerization is used for the of synthesis aliphatic polyesters. The initiator used in the polymerization must have vacant d-orbital to co- ordinate with carbonyl group of the lactone. The commonly used initiators include aluminum and tin alkoxides and carboxylates. The nucleophilicity of the carboxylates are slightly lower that alkoxides. In the reaction, carboxylates act as a catalyst rather than an initiator. Active hydrogen containing compound can act as a co-initiator. The co- ordination of initiator with carbonyl group of the monomer makes the monomer more susceptible to nucleophilic attack by the initiator. The polymerization follows acyl- oxygen cleavage followed by incorporation of monomer into the metal oxygen bond. The high reaction temperature and long time of reaction increase the dispersity of the synthesized polymer because of trans-esterfication reaction. The tin 2-

ethylhexanoate is the commonly used catalyst for polyester synthesis. The polymerization of lactone is carried out in presence of tin catalyst with active hydrogen containing compound as an initiator.111 The monomer forms the coordinate bond with carbonyl carbon and makes it active. The initiator attacks on active monomer and forms the polymer chains. Both initiator and monomer are attached with catalyst during the propagation reaction (scheme 1.3). After the complication of the reaction by hydrolysis, the hydroxyl group terminated polymer is formed. The polymerization of lactone or lactide follows the first order kinetics with respect to the monomer and initiator.112 Scheme 1.3: The mechanism of ring opening of ε-caprolactone 1.8 Literature review In the last decades, delivery of anti cancer drug through nano-carrier safely to the cancer cell has received much attention. Different types of nano-carriers e.g. micelle,113 vesicle,114 inorganic or organic nanoparticles,115 polymer modified inorganic nanoparticles41, 116 have been employed for such drug delivery. The nano-carriers to be use for drug delivery must have the spherical or nearly spherical shape with average size ranging from 10 to 200 nm.117 The carrier having size scale below this range are easily eliminated by renal system and above this size range have exhibited very less circulation time in bloodstream. The nano-carrier having the above mention size range can efficiently reduce the nonspecific interaction and increase enhance permeability and retention effect (EPR).118 Among the different nano-carriers, micelles prepared from amphiphilic block copolymer are extensively exploited for drug delivery.119 Micelle has two distinct parts, inner hydrophobic core and outer hydrophilic corona. Micelles can hold hydrophobic guests (e.g. drug, protein, gene, etc.) inside its core and safely transfer them to the required site. Outer, hydrophilic part of the micelle can stabilize them into the biological system.120 Although these carriers can carry the drug molecule by engulfing them inside but release of drug through them may not be so effective. As a result, they have failed in many occasions to exhibit their effective performance as nano-carriers for site specific drug delivery. The performance of such drug loaded polymeric micelle has been upgraded by employing stimuli (as like temperature, redox, ultrasound, magnetic and acidic pH) sensitive moieties for quick release of encapsulated drug molecules. This can also reduce the usual side effects leading to increase of the therapeutic efficiency.121 The cancer cells possess elevated acidity (i.e. relatively low pH at some solid tumor sites/tissues) compare to physiological pH of normal blood.122 Thus acid sensitive deswelling or collapsing of polymeric micelle can be administrated as acid stimulated release of drug molecules. By taking the advantage of acid sensitive hydrogen bonding interaction between adenine and uracil, Jianquan Fanamp and his coworkers have prepared a pH responsive polymer micelle by covalently linking these two complimentary groups on the backbone of amphiphilic block copolymers.123 The pH responsive formation and breaking of co-ordination bond also affords efficient carrying and delivering of anticancer drug to a low pH therapeutic site.124 Mesoporous silica nanoparticle grafted poly(methacrylic acid-co-vinyl triethoxylsilane) has been administered as a core-shell nano-carrier that exhibits an effective release of drug at low pH.125 The shell forming polymer, poly(methacrylic acid) is the responsible block that allows to express the pH sensitivity. Recently, a robust pH responsive polymeric micelle based on phenylboronic acid−catechol interaction has been synthesized by Jie Ren et al. for selective delivering of

protein.126 In order to manufacture a better drug delivery system, hydrophobic segment of the amphiphilic polymer, poly(ethylene glycol)-b-poly(ε caprolactone) block has been functionalized with hydroxyl group. The drug molecules (DOX) are hydrogen bonded through the hydroxyl group at the physicological pH of blood and thus enabling acid responsive release of drug molecule following weakening of these bonds.114, 127 A theoretical study on nanoparticles, micelles and vesicles also furnished a pH actuated self-assembly and disassembly of these nano-carriers.128 A PM prepared from block copolymer poly(γ-2-[2-(2-methoxyethoxy)ethoxy]ethoxy- CL)-b-poly(γ-octyloxy-CL), having LCST 38 °C affords secure delivery and temperature responsive release of Nile red and DOX.117 The synthesis of thermoresponsive micelle by Poly(NIPAM-co-3-(trimethoxysilyl)propylmethacrylate)-b-poly(2-(diethylamino) ethyl methacrylate) block co-polymer has been performed by Chang et al. and these micelles were subsequently used for thermoresponsive release of DOX.129 Bessaet et al. have synthesized elastin-like nanoparticles by thermoresponsive self-assembling of elastin like polypeptide derived from Val-Pro-Gly-Xaa-Glypentapeptide and subsequently used for combined release of bone morphogenetic proteins (BMP-2 and BMP-14).130The self- assembling of diblock copolymer poly(NIPAM)-b-2-(dimethylamino)ethyl acrylate, below their LCST leads to the formation of PMs.131 This micelle favors the fruitful delivery and release of oligo-DNA by self-catalyzed degradation. Similar type of thermoresponsive amphiphilic block copolymer has also been synthesized by De et al. for stimuli induced assembly and release of model hydrophobic drug, dipyridamole.132 The lack of sufficient stability of some thermoresponsive micelle in biological medium has reduced their therapeutic efficiency. The formation of unimolecular micelle from hyperbranched block copolymer affords formation of stable micelle that securely delivers the anticancer drug, paclitaxel to the cancer cell.133 A number of cross-linked PMs has also been synthesized with improved stability and therapeutic efficiency.134 A series of thermoresponsive water soluble block copolymer poly(ethyleneoxide)-b-trans-N-(2- ethoxy-1,3-dioxan-5-yl) acrylamide has been synthesized by Qiao et al.135 The synthesized block copolymer registers the LCST close to the human body temperature. Thus the formation of PM followed by loading of hydrophobic drug (DOX) at body temperature leads to safe delivery and beneficial therapeutic efficiency. Recently, a new class of thermoresponsive copolymer, poly(acrylic acid)-g-poly(N-vinyl caprolactam) shown to be potentially efficient nano-carrier for drug delivery.136 The attachment of cancer cell targeting moiety (folic acid, biotin, etc.) onto the surface of stimuli responsive PM helps in targeting to the cancerous cells. The presence of FA receptor provides efficient trafficking of folic acid decorated PM to the cancer cell.132 The external magnetic field can also be used to target the magnetically active micelle to the specific organs or tumor cell41. The MNP encapsulated poly(trimethylene carbonate) block poly(glutamic acid) polymer shows a magnetic targeted delivery and high frequency AC magnetic field influenced delivery of the drug molecules.137 The targeted delivery approach enables carrying most of the drug molecules to the cancer cell and reducing the side effects. The shape and characteristics of PM largely depend on architecture of the base polymer. The impressive property of branched polymer towards solution and self assembled morphologies promote its application for drug delivery. A tadpole (nonlinear) like linear- cyclic amphiphilic

block copolymer (cyclic-PNIPAM)-b-(PCL), offer a high rate of thermoresponsive drug release compare to its linear analog.138 Recent study on „Y" shaped i.e. three armed thermoresponsive amphiphilic block copolymer prepared from hydrophobic poly(undecylenic acid) and hydrophilic poly(N- isopropylacrylamide) has been reported by Young et al. It is subsequently used successfully for thermoresponsive drug delivery.139 Additionally, two „Y" shaped thermoresponsive amphiphilic copolymer based on poly(ε-benzyloxycarbonyl-L-lysine) and poly(L-lysine) have been synthesized by Li et al. and subsequently those have been 34 used for drug delivery.140 The synthesis of „Y" shaped poly(L-lactide)2-b-poly(g-benzyl- L-glutamic acid) copolymer and formation of its self assembled form have been reported by Sun et al.141 The polyamidoamine dendrimer has been synthesized by Singh et al. and afterwards it was used for anticancer drug delivery.142 A polyester based amphiphilic hyperbranched polymer (HBP) has been reported by Chen et al. It was synthesized by extending the chains of commercially available hyperbranched aliphatic polyester Boltorn H40 with PCL and then it was coupled with PEG.143 A self assembly fluorine based HBP polymer has been synthesized by Du et al. for 19F MRI agent.144 Unlike linear, nonlinear/branched or hyperbranched polymer have many advantages like wonderful template forming ability, effective solution property and provision of incorporating large number of functional groups leading to improved polymer-drug interaction.102a The entanglement-free self-assembly of the branched (i.e. nonlinear) polymer affords micelle with higher drug loading as well as the releasing efficiency of the nano-carrier can be uplifted.102b 1.9 Scope of the work A few numbers of literatures has been focused to encompass branched amphiphilic biocompatible PCL based nano-carrier for efficient cancer therapy. But there is no meticulously reported literature on branched PCL based polymer that discusses about a smart nano-carrier for both in vitro and in vivo cancer therapy comprehensively. Hence, there exist a large number of scopes for the preparation of branched PCL based smart nano-carrier towards in vitro and in vivo cancer therapy. By taking the advantage of the branched architecture of PE-PCL, different stimuli responsive moieties such as pH responsive acrylic acid based polymer or thermoresponsive PNIPAM, PNVCL, PEC based block or reduction (glutathione) responsive sulfur-sulfur linkage can be grown from it. The branched PE-P(CL)x-b-P(stimuli responsive monomer)y block copolymer with different segmental chain length may produce different size of stimuli responsive nano-carriers with variegated sizes in aqueous system. The encapsulation of fluorescent moiety may offer these nano-carriers to track their in vivo pathway remotely. In order to upgrade the efficiency of nano-carriers, MNP can also be encapsulation into it. The MNP encapsulated nano-carrier may act as a targeting entity. It can be located to a specific 35 place or organ by the application of external magnetic field. The application of high frequency AC magnetic field on the MNP encapsulated nano-carrier may lead to disassembly of nano-carrier followed by release of encapsulated drug. Targeting moieties, such as FA and peptide can also be attached onto the surface of these nano- carriers which may boost them to show effective performance in cancer therapy. The stimuli responsive crosslinker such as Fe3+ and di-hydroxydisulfide compound can be used to prepare a stable stimuli responsive nanogel from the amphiphilic block copolymer. The nanogel may able to load high amount of drug molecule and may show effective

performance in cancer therapy. All the above mentioned nano-carriers can be administered both in in vitro and in vivo systems and their therapeutic efficacy may be evaluated. 1.10 Objectives of the present work The thesis is mainly focused on the preparation of external and internal stimuli responsive smart PM (i.e. nano-carrier) from a branched biodegradable or biocompatible PCL based polymer. In order to accomplish this, smart PMs with the following crucial characteristics such as, (i) small size with EPR effect; (ii) high drug loading capacity; (ii) effective trafficking of drug loaded PM towards cancer cell and (iv) stimuli responsive release of drug molecule, have been fabricated to achieve following objectives: ? Synthesis of PE-PCL and grafting onto the surface of MNPs. ? Preparation of MAPM for cancer cell targeted and on demand drug delivery. ? Synthesis of FA attached PM (from PE-PCL-b-PEC-FA) and its in vitro cancer therapy. ? Synthesis of FA attached pH responsive PMs (from PE-PCL-b-PAA-FA) and their application in in vitro cancer therapy. ? Synthesis of FA attached thermoresponsive PMs (from PE-PCL-b-PNIPAM-FA and PE-PCL-b-PNVCL-FA) and study of their in vitro and in vivo cancer therapeutic efficacy. Chapter 2 2.1 Introduction This chapter of the thesis reports the details about the source of the solvents and reagents along with their grades and further purification procedure. This chapter also reports different techniques of characterization adopted in the thesis for the characterization of synthesized homo and block copolymer along with their corresponding nano-carrier. It describes procedures for measurement and evaluation of drug release from the nano- carrier both at in vitro and in vivo condition, cellular uptake and cytotoxicity are also presented here. The chapter also described detail about the preparation of sample for different types of characterization. 2.1.1 Reagents 2.1.1.1Monomers Table 2.1: Monomers with their special characteristics, source and purity Monomers Special characteristic Grades Resource ε-Caprolactone (CL) Seven member cyclic ester; polymerized by organometalic catalyst (scheme 1a); PCL semi crystalline, Tm = 55 to 65 °C and Tg = -60 °C; hydrophobic in nature, water contact angle around 70 to 75°; biocompatible as well as biodegradable. 97% pure Ethylene Carbonate (EC) Five members carbonate eater; polymerized by organo-metalic catalyst (scheme 1b); PEC decomposed above the 180 °C; Tm varies from15 to 25°C; PEC is hydrophilic with respect to PCL; biodegradable in nature. 98% pure tert-Butyl acrylate (tBuA) Aliphatic ester containing an active double bond; polymerized by ATRP (scheme 1c); hydrolyzed form of PtBuA is poly(acrylic acid) (PAA); PAA displays pH responsive behaviour (scheme 1d); biocompatible in nature. 98% pure Sigma Aldrich, USA N-isopropyl acrylamide (NIPAM) Aliphatic amide contains an active double bond; polymerized by ATRP (scheme 1e); PNIPAM shows a temperature responsive phase change; LCST, near to the human body temperature. 97% pure N-vinyl caprolactam (NVCL) Cyclic aliphatic amide containing active double bond; polymerized by ATRP (scheme 1f); LCST of PNVCL varies from 32 to 45 °C; biocompatibility in nature. 98% pure 35 Chapter 2 Scheme 2.1: Different types of monomers and their corresponding polymers 2.1.1.2 Other reagents Table 2.2: Other reagent with their purity grades Reagent Grades Resource 4' 4' Methylenebisdiphenyl diisocyanate (MDI) 98 % pure Dibutyltindilaurate (DBTDL) 95% pure Pentaerythritol (PE) 98% pure Tin(II)ethylhexanoate (Sn(Oct)2) 95% pure Sigma (3-aminopropyl)trimethoxysilane (TMAS) 97% pure Aldrich, Doxorubicin hydrochloride (DOX) 98% pure USA Folic acid (FA) 97% pure N,N'-

dicyclohexylcarbodiimide (DCC) 98% pure N,N,N′,N′′,N′′-Pentamethyldiethylenetriamine (PMDETA) 99% pure Trifluoroacetic acid (TFA) 99% pure 2-bromopropyonil bromide 80% pure Iron (III)acetylacetonate (Fe(acac)3) 99% pure Ferric chloride (FeCl3) 96% pure Merck Ferrous sulphate (FeSO4) 98% pure Chemicals Mumbai, Copper bromide (CuBr) 99% pure India Aqueous NH3 25% pure N-hydroxysuccinimide (NHS) 98% pure Spectrochem Mumbai, Sodium azide (NaN3) 98% pure India Triphenyl phosphine (P(Ph3)3) 98% pure 2.1.2 Purification of reagents The catalyst, CuBr was purified by washing with glacial acetic acid and diethyl ether. The washed CuBr was dried in vacuum oven under reduced pressure. The monomer, EC was purified by recrystallizing from cold hexane. The monomer, CL was purified by 36 vacuum distillation from calcium hydride and monomer mixture. The monomer, tBA was passed through basic alumina in order to remove the inhibitor and after that used as such for polymerization. The initiator, PE was dried in vacuum oven in order to remove the moisture. The solvent, THF was used after drying from sodium and benzophenone. 2.1.3 Solvents Solvents like tetrahydrofuran (THF), hexane, ethanol, acetone, chloroform, hexane, N- methyl pyrrolidone and methanol, DMF and toluene (all are analytical grade) were procured from Merck Chemicals Mumbai, India. 2.2 Different experimental procedures adopted 2.2.1 Determination of CMC The critical micelle concentration (CMC) of synthesized block copolymer was determined by spectroscopic method. A number of scan for different concentration of polymer (10-3 to 0.1 mg/mL) were collected from fluorescence spectroscopy by using pyrene as a fluorescent probe at 37 $^{\circ}$C. The ratio of the intensity of two peaks I3 and I1 of pyrene were plotted against concentration of the added polymer.144 The concentration corresponding to the sudden change in the trend of peak ratio (I3 / I1) is taken as the CMC. 2.2.2 Evaluation of pH responsive property of micelle In order to inspect pH responsiveness, polymer micelle was prepared in five different buffer medium (pH = 7.4, 6, 5, 4, and 3). After a fixed time period of shacking in, the hydrodynamic sizes of the PM were measured by DLS using different pH conditions. 2.2.3 Evaluation of thermoresponsive property of PM The thermoresponsive property of the PM (concentration: 2 mg/mL) was checked by temperature dependent DLS and UV-Vis measurements. In DLS, the changes in hydrodynamic size with increasing temperature scan (from 30 $^{\circ}$C to 45 $^{\circ}$C) were conducted. The variation of the size of PMs with temperature is recorded at 12 numbers of steps. In UV-Vis, the transmittances (at 500 nm) were noted by scanning the temperature from 25 to 55 $^{\circ}$C using temperature dependent UV-vis absorption spectroscopy. 2.2.4 Study of in vitro magnetic responsive release In order to determine the release kinetics, a 5 mL of DOX loaded MAPM solution (at a concentration of 1mg/mL) was placed inside the dialysis bag (cut off mol. weight 3.5 kDa). The dialysis bag was floated into a 50 mL beaker containing 25 mL PBS solution. The beaker was placed under the influence of 750 kHz frequency of alternating magnetic field at a filed strength of 2.5 mT. The experiment was carried out at 37 $^{\circ}$C with a constant interval of filed exposure time (say 5 minute). The amount of drug release was quantified by taking the UV-Vis absorbance (at 488 nm) of the outer solution against time. In order to evaluate the concentration corresponding to the UV absorbance we used pre-determined calibration curve. An in house AC magnetic field generating instrument has been fabricated by our group. The sketch of hardware arrangement along with in

instrumental setup is displayed in Fig. 2.1to 2.3 Figure 2.1: The sketch of hardware arrangement setup 38 Figure 2.2: The overall hardware arrangement setup Figure 2.3: The hardware arrangement setup of sample holder 2.2.5 Study of in vitro pH responsive release In vitro DOX release study was conducted by dialysis method. In brief, 5 mL of DOX loaded PM (at a concentration of 1 mg/mL) was placed inside the dialysis bag (cut off mol. wt. 3.5kD). The PM loaded dialysis bag was then floated on 35 mL phosphate buffer (at pH 7.4) and acetate buffer (pH 5. 0), respectively. The whole system was placed on water bath at 37 °C under stirring at 100 rpm. After each predetermined time interval, a fixed volume (2 mL) of outer solution was taken out from the system. In order to 39 maintain fixed volume inside the system, the same volume of fresh buffer was replaced. The amount of DOX present in the resultant solution was then measured by taking UV absorption at 485 nm and by using the calibration curve (that was made using known concentration of DOX molecule). The cumulative percentage (%) of DOX release was then calculated by the following equation 2.1. Each release study was performed thrice and average results were reported. Ve n1−1 Ci + V0Cn Er % = □□□□ × 100 … … … … … Eq. 2.1 Where, Er = amount of DOX release at t time; V0 = whole volume of release medium; Ve= volume of medium replaced; Cn = concentration of DOX in nth sample; mDOX = amount of DOX present. 2.2.6 Study of in vitro thermoresponsive release of DOX The thermoresponsive release of the DOX loaded PM was conduct at below and above the LCST. A 5 mL of DOX loaded PM having a concentration of 1 mg/mL was sealed into a dialysis bag (cut off mol. wt. 3.5kD, cellulose membrane). The sample was then dialyzed at two different ranges of temperatures (30-37 °C and 37-40 °C) against 45 mL phosphate buffer (pH 7.4) solution. The quantity of released drug was measure by taking out 2 mL of external buffer solution at various predetermined time intervals. To maintain the fixed volume, exactly same volume of PBS solution was added into the system. The amount of released DOX in the external solution was then determined by putting the UV absorbance (at 485 nm) of the resultant solution to the predetermined calibration curve. The cumulative percentage (%) of DOX release was then calculated by the following equation 2.1. The release studies were conduct in triplicate and average of the results are represented here. 2.2.7 In vitro cytotoxicity assay The human cervical cancer cell line (HeLa) and immortal human keratinocyte (HaCaT) cell lines were employed for the determination of cytotoxicity of the blank and DOX loaded PMs. Both the HaCaT and HeLa cells were seeded into 96 well plates at a concentration of 1×104 per well in 200 µL Dulbecco's modified eagle medium (DMEM) with 10% FBS. The seeded cells were allowed to adhere to culture plates for 24h under 5% CO2 atmosphere at 37 °C. The medium was then replaced by the same volume of 40 DMEM containing different concentrations (0 to 100 µg/mL) of blank and DOX loaded PMs. The cells were allowed to grow for next 24h at 37 °C. The wells with MTT solution were allowed to incubate for next 4h under humidified 5% CO2 atmosphere at 37 °C. The used medium was then replaced from each well by 200 µL DMSO solution. The absorbance (A) (at 595 nm) of the DMSO dissolved formazan crystal was captured from spectrophotometer. The cell viability of the blank and DOX loaded micelles were calculated as (Asample/Acontrol)×100. In chapter 5 cytotoxicity assay was carried out using L929 cell line in place of HaCaT cell line by following the same procedure. In order to

recognize the temperature responsive effect (in chapter 7) of the DOX loaded PM, one set of HeLa cell line was separately allowed to grow at 40 °C (for B series of sample, above LCST) for 24h. 2.2.8 In vitro cell uptake study HeLa (human cervical cancer cell, FA overexpressing) cells were cultured in Minimum Essential Medium (MEM, supplemented with sodium pyruvate and non-essential amino acids) containing 10% heat-inactivated fetal bovine serum (FBS) at 37°C in a humidified atmosphere of 5% CO2. The cellular uptake studies were performed by flow cytometry and fluorescent microscopy. For flow cytometry, cells were seeded at a density of 106 cells/well in six -well culture plates and grown overnight. The cells were treated with free DOX and DOX-loaded PMs (Sample A4 and B4) for a period of 1 and 4h, respectively. After the required time interval of treatment, the culture medium was discarded, and cells were washed thrice with PBS and harvested by trypsinization. The cell pellets were suspended in PBS and measured for the fluorescence intensity (excitation: 488 nm; emission: 575 nm) on a BD FACS ARIA III flow cytometer. For fluorescent microscopy, cells were seeded at a density of 0.5x105 cells/well and incubated overnight to allow the cells to adhere. Following this, the cells were treated with free DOX, DOX-loaded PM (sample A4 and B4) for a time interval of 1h, 2h and 4h. At the end of incubation, the cells were washed with PBS thrice, fixed with 4% paraformaldehyde for 30 min and the nuclei were stained with DAPI (4',6-diamidino-2-phenylindole). Cells were mounted and observed on a fluorescence microscope (Olympus, 1X81). In order to investigate the magnetic effect on DOX loaded MAPM on cell uptake, one set of a static magnet culture plate (field strength around 3.5 mT) was placed under the influence of magnetic field according to scheme 2.2. Scheme 2.2: Schematic presentation of cellular uptake study under the influence static magnetic field at a filed strength of 3.5 mT. 2.2.9 Cell cycle analysis For cell cycle analysis, cultured HeLa cells (at a concentration of 1 ×106 per well) were treated with IC50 concentration of DOX loaded PMs for 24 h at 37 °C under 5% CO2 atmosphere. The DOX loaded PMs were prepared from the respective polymers having different compositions. The cultured cells were harvested by trypsinisation and followed by fixing with chilled EtOH (70%). The fixed cells were then stored at -20 °C. The cells were then washed with PBS solution (pH 7.4) followed by incubation with 20 mL of DNase free RNase (10 mg/mL) along with 20 mL of DNA intercalating dye, propidium iodide (PI) (1 mg/mL) for 1 h at 37 °C under dark. The distribution of cells in different cell cycle phase was also approved by institutional animal ethical committee of Indian Institute of Technology (Kharagpur, India). Sarcoma 180 cells were taken from other mice in growing phase and suspended in phosphate buffer saline (PBS) and injected intradermally into the right flank of every mouse. After 10 days, when tumor size reached approximately 100 mm3, animals were divided in three random groups containing five animals in each group. Before injection, tumor volume and body weight of animals were measured. Animals were then injected through tail vain according to following manner: (i) Sterile phosphate buffer saline (control group); (ii) Free DOX (2 mg/kg); (iii) DOX loaded PM (2 mg/kg). The systemic toxicity and inhibition of tumor growth of the mice were analyzed periodically. Animals were injected for 28 days leaving one day gap between two injections. After 28 days of treatments all mice were sacrificed and tumor volumes were measured by vernier calipers. The volume of tumor (width2×length/2, mm3) of treated as well as

control animals was determined. 2.2.10 In vivo accumulation of DOX in tumor Location of DOX in sarcoma 180-induced tumors in mice was inspected by florescent imaging. For this study, mice bearing sarcoma 180 tumors were divided in three groups as following (i) Sterile phosphate buffer saline treated (control group); (ii) Free DOX treated (2 mg/kg) and (iii) DOX loaded PM treated (2 mg/kg). After the growth of tumor about 100 mm3, the tumor site of the mice was dipped into the pre-heated water bath at 40°C and other part of the body was isolated from temperature bath.145 The sterile phosphate buffer or free DOX or DOX loaded polymer was then intravenously administrated into the mice and allowed the tumor site keeping into the water bath for 30 minutes. The mice were then sacrificed and tumor tissue samples were fixed in formalin. Then, formalin fixed tumor tissue was embedded in paraffin. A thin section of it was made by microtomy. After preparation of tissue section, paraffin was removed by xylene and rehydrated by graded alcohols (100%, 90% and 80% v/v) and washed in water. The DAPI was used as a counter stain. Tissue samples were air dried in dark and mounted on DPX. Then, images were taken by using a florescent microscope at 20× magnification (Carl Zeiss). 2.3 Characterization techniques adopted 2.3.1 FTIR spectrum FTIR spectra were recorded using a Perkin-Elmer FTIR-spectrophotometer (model spectrum RX-I), within a range of 400-4000 cm-1 using a resolution of 4 cm-1. An average of 16 scans has been reported for each sample. A dilute THF solution of the polymer was placed on to the KBr cell and the spectrum was recorded. In case of powder sample each samples were grinded with (10 times by weight) KBr powder and made a pellet for FTIR scan. 2.3.2 UV-Vis spectrum UV-Vis absorption spectroscopy was recorded from 700 to 200 nm on Perkin Elmer's Lambda 35 instrument at a slit width of 1 nm. The samples are dissolved in THF or water 43 at a concentration of 5×10-4 mole/L and placed onto the UV-Vis spectrometer. In case of DOX, a calibration curve was made from a concentration vs. absorbance intensity (at 482 nm) curve. For release study, the outer medium of the solution were scanned using UV- Vis spectrometer time to time and finally the concentration was calculated from the pre- maid calibration curve. 2.3.3 NMR spectrum The samples for NMR were dissolved with the deuterated solvent like DMSO-d6 and CDCl3 (having small amount of TMS as internal standard) into the NMR tube and placed inside the tube holder for generating the spectrum. NMR spectrum was recorded on a 200 MHz Bruker Avance Instrument at an average of 16 number of scan. To avoid the overlap of signals between initiator (PE) carbon and DMSO- d6 carbon, 13C NMR spectrum was also recorded in CDCl3 using the same instrument. 2.3.4 TEM The distribution of magnetic nanoparticles, estimation of the size of polymer micelles and polymeric nanoparticles were captured by using a High Resolution (HR) TEM (JEOL 2000) operated at an accelerated voltage of 200 kV. For TEM, the samples were dispersed into appropriated solvent and a drop of dispersed sample was placed on copper grid (300 mesh size). After drying, the copper grid was placed into the sample holder of the TEM instrument. 2.3.5 XRD The wide angle X-ray diffraction (WAXD) was performed using Rigaku Dmax 2500 diffractometer with Co target (k = 0.179 nm) at room temperature. The system consisted of a rotating anode generator, operated at 40 kV and at 30 mA beam current and a wide angle goniometer. The samples were scanned from 2θ = 10– 70o at the step scan mode (step size 0. 0380, preset time 2s) and the diffraction pattern was recorded

using a scintillation counter detector. 2.3.6 GPC The molecular weight of the synthesized homo and block copolymers were determined from Agilent GPC 1200 series by using HPLC grade THF as an eluent. The calibration was made by using polystyrene of narrow dispersity as a standard with a solvent flow rate of 1mL/min. The GPC was furnished with three PLgel-10μm mixed bed columns which 44 were preceded by a guard column. The molecular weights were evaluated from the Agilent GPC software. 2.3.7 MALDI-TOF MALDI-TOF mass analyses were carried out using a Perceptive Biosystems Voyager Elite MALDI-TOF mass spectrometer, equipped with a nitrogen LASER (wavelength 337 nm). For MALDI-TOF mass analysis, the matrix (2, 5-dihydroxybenzoic acid) with 2-hydroxy-5-methoxybenzoic acid, called super-DHB was dissolved in THF at concentration of 40 mg/mL. The sodium trifluoroacetate salt was dissolved in THF at a concentration of 3 mg/mL. The polymer was dissolved with a concentration of approximately 10 mg/mL. The matrix, salt, and polymer solutions were mixed at the ratio of 5:1:5, respectively and then spotted on target plate. Both the linear and reflection modes were used to collect the data. The data collection involved delayed extraction, with a delay time of 350 ns. 2.3.8 DSC Differential scanning calorimetry (DSC) analysis was carried out on a TA (DSC Q100 V8.1 Build 251) instrument at a heating rate of 20 °C/min under nitrogen atmosphere. In this case, the samples were heated from +25 oC to +100 °C with a heating rate of 20 °C/min then cooled down to −100 °C with a rate 20 oC /min and then heated again to +100 °C with the same rate. The second heating cycle was used to estimate the Tm of the synthesized polymers. 2.3.9 TGA Thermogravimetric analysis (TGA) was carried out on a TA (TGA Q50 V6.1 Build 181) instrument. In this case, a small amount of sample (approximately 8 mg) was heated from room temperature to 600 °C at a heating rate of 20 °C/min under nitrogen atmosphere and change of weight of the samples was monitored against temperature. 2.3.10 SQUID-VSM The magnetic measurements were performed on a SQUID magnetometer (MPMS; Quantum Design) under a magnetic field of 30 kOe at 27 °C. The sample (weight 3 to 4 mg) was wrapped with teflon tape and placed onto the sample holder. 2.3.11 XPS A small film of sample was placed on the sample holder and irradiated with X-ray photoelectron (Mg Kα). The surface composition of polymer immobilized MNP were recognized by X-ray photoelectron spectroscopy (XPS) [PHI 5000 Versa Probe II (ULVAC-PHI Inc., Japan)]. 2.3.12 DLS DLS measurement was performed using a Malvern Nano ZS instrument. The experiment was conducted using a 4 mW He−Ne laser (λ = 632.8 nm) at 25 °C. In Zetasizer Nano ZS, the detector angle is fixed at 90°. The sample with appropriate concentration was taken into the cuvette and placed inside the cuvette holder. 2.3.13 Fluorescence spectroscopy Fluorescence spectra were recorded by using Jobin Yvon-Spex Fluorolog-3 spectrofluorimeter equipped with a thermostatic cell holder having 1 cm path length quartz cuvette. The excitation wavelength of 325 nm was used in all cases for selective excitation of pyrene and emission spectra were recorded from 360 to 600 nm. 2.3.14 AFM The phase image of PM was evaluated by AFM (Agilent Technologies, Multiview- 1000TM; 70 μm AFM/ NSOM (Nd: YAG Laser) Scanner, in tapping mode using silicon cantilever. The resonance frequency of the cantilever is 20 kHz with a force constant of 0.38 N/m. The AFM sample was prepared by making a film of sample on a glass slide by spin coating at 2500 rpm. 2.3.15 Contact angle measurement

The water contact angle of the synthesized polymers was measured by CA Goniometer (Rame-Hart instrument Co. Model no.- 190F2). The film of synthesized polymer was casted on glass slide and subjected to contact angle measurement. The error bar in the contact angle was found to be 1.5. 3.1 Introduction In recent years, the nanotechnology has been extensively used for upgradation of the properties of materials. The nanotechnology helps gearing up of engineering properties as well as helps to develop biomedical properties of materials. Among the different types of particulate nanomaterials, magnetic nanoparticle (MNP) is of great importance for their both medical146 and engineering applications. 147 Polymer grafted magnetic nanoparticles have many applications, such as, magnetic targeted drug delivery, 148 contrast enhancement in magnetic resonance imaging, 149 purification and separation of biomolecules, 150 catalytic applications, 151 and shape-memory applications. 152 Dispersion of MNP into polymer in liquid state produces ferrofluids. Ferrofluids are those liquids whose physical and rheological properties can be controlled by application of external magnetic fields. 153 Among the different polymers, poly(ε- caprolactone) is potentially important as biodegradable polymeric component of polymer grafted MNPs. Because it is aliphatic polyester, it can be easily degraded by enzymatic and hydrolytic processes and its nontoxic degradation products make it biodegradable. 154 MNP has an inherent tendency to form agglomerates due to its high surface energy and magnetic nature. Agglomeration of MNP leads to deterioration of most of its attributes for biomedical applications e.g. MRI contrast enhancement and magnetic targeting drug delivery. Because of this vital disadvantage, magnetic nanoparticles can be removed by macrophages or the reticuloendothelial system before it can reach at the targeted site. 155 For both of these applications magnetic nanoparticles need to be homogeneously dispersed. Additionally, targeted drug delivery techniques are used to reduce the unsafe side effects. For drug delivery purpose it is always favored to deliver the drug by incorporating into biodegradable polymeric shell to reduce the side effects. Thus both the agglomeration and side effect problem can be resolved successfully by modifying magnetic nanoparticles with biodegradable polymeric shell. Among the different available techniques, grafting of polymers onto MNP is one of the simplest and efficient techniques. This technique produces core-shell structures with magnetic core that is stabilized by a surrounded polymeric shell. J. A. Park et al. have prepared a PCL coated MNP by simple solvent casting method and it was used subsequently for studying magnetic properties and MRI.156 A. M. Schmidt et al. have 49 reported preparation and characterization of core-shell nanoparticles by surface initiated ROP of poly(ε- caprolactone) and also by atom transfer radical polymerization (ATRP) of 2-methoxymethacrylate from the surface of MNP. 157 Scheme 3.1 (a) Schematic of in-situ coating onto MNP surface by TMAS modification and (b) Grafting of four armed PE-PCL onto TMAS coated MNP. Microwave assisted grafting of poly(ε- caprolactone) on MNP have been reported by A. Nan et al. 158 Grafting of poly(ε- caprolactone) on (3-isocyanonatopropyl)triethoxysilane modified MNP has also been reported by C. Flesch et al. 159 Linear PCL has been used for modifying the surface of MNPs in almost all previously reported works. It is presumed that in comparison to linear poly(ε- caprolactone), four armed poly(ε- caprolactone) has many advantages like low viscosity, high encapsulating efficiency and high

grafting density onto MNP surface. This in turn would lead to significant improvement of dispersion of the MNPs and in long run it would help in increasing the drug carrying capacity of PCL grafted MNPs. To the best of our knowledge, the grafting of branched poly(ε- caprolactone) onto MNP surface is not yet reported in literature. The objective of the chapter is the synthesis of branched biodegradable polymer PE-PCL grafted MNP for biological application. In order to achieve the objective, the synthesis of MNP by hydrothermal method followed by coating of TMAS was performed. Synthesis, kinetic study, and establishment of chemical structure of PE-PCL were also done successfully. MALDI-TOF analysis was used to detect the end groups of PE-PCL. Grafting of PE-PCL onto TMAS coated MNP was also performed successfully. MDI was used as an intermediate linker for linking PE-PCL with TMAS coated MNP. The dispersion of MNP into polymer matrix was studied by HRTEM. The thermal (DSC and TGA) and magnetic properties of polymer grafted nanoparticles so formed was also evaluated successfully. The biocompatibility of polymer grafted nanoparticles was investigated by MTT assay. 3.2 Synthesis 3.2.1 Synthesis of MNP MNP was synthesized by hydrothermal process. The salt Fe(acac)3 (1 g, 4 mmol) was dissolved in 20 mL of N-methyl pyrrolidone. The N2 gas was purged for 30 min to remove the dissolved O2 inside the solution. The reaction mixture was then refluxed for 1 h. The methanol (20 mL) was added into the reaction mixture as soon as the temperature came down to ambient temperature (25 °C). Then the reaction mixture was centrifuged (3000 rpm for 5 min) to collect the precipitated particles. The precipitated particles were washed three to four times by acetone and dried in vacuum oven at 40 °C at a reduced pressure of 400 mm Hg. 3.2.2 Synthesis of TMAS Coated MNP. The particles so formed were dispersed in distilled water (100 mg/150 mL) by ultrasonication for 1h. The aqueous NH3 solution (2 mL) was added into the dispersion of MNP to make it basic. The dispersion was stirred (around 1500 rpm) by mechanical stirrer followed by dropwise addition of TMAS solution (0.1 mL in 10 mL of water) at room temperature (shown in scheme 3 .1a). The stirring was continued up to 6 h. Following these, TMAS coated MNP was washed thoroughly with acetone and water alternatively for removing unbound silane traces. The TMAS coated MNP so formed was dried in vacuum oven under reduced pressure. 3.2.3 Synthesis of PE-PCL Ring-opening polymerization (ROP) ofε-caprolactone (CL) initiated by pentaerythritol (PE) was conducted by bulk polymerization method. The required amounts (shown in Table 3.1) of CL and PE were taken into a 25 mL two-neck round-bottom flask. The reaction mixture was put into a preheated oil bath at 120 °C under dry N2 atmosphere with continuous stirring at around 450 rpm. The catalyst, Sn(Oct)2, was added after homogenization of the reaction mixture. The polymerization was carried out under a constant w/w ratio of monomer and Sn(Oct)2 (1000:1). Small aliquot of reaction mixture was taken out after various time intervals for checking conversion by gravimetric method (time count started after adding catalyst). After 97−98% conversion, the reaction mixture was cooled down to room temperature. Then the reaction mixture was dissolved in CHCl3 and precipitated in hexane. Reaction product was dried in vacuum overnight at 60 °C. Table 3 .1 Ring opening Polymerization of pentaerythritol (PE) initiated ε-caprolactone (CL) for different compositions of PE and CL at 120 oC. Expt. no. PE /CL Time 1 (mol/mol) (minute) 1:40 40 (%) 98 5000 4500 1.1 38 a Yieldc Mnb (GPC) Mn (Theo.) Đb Xca (%)

Sample code 2 1:100 60 98 13100 11000 1.2 39 b 3 1:150 80 97 17600 16000 1.2 43 c aDetermined from DSC measurement. bDetermined from GPC. cDetermined for gravimetric measurement. Table 3.2 Various constituents and reaction condition PE-PCL (1 MDI (6 DBTDL Rotation Temp. (oC) MNP TMAS- equiv.) equiv.) 5% (w.r.t (rpm) & content (%) MNP (g) (wt. in g) (wt.in g) total wt.) Time (h) 1 0.011 1 0.116 0.056 600-700 60 3 0.033 & 5 0.056 6 7 0.078 aDetermined from DSC by usingΔHo = 139.5 J/g. Xc a (%) 32 28 26 12 Sample code MNP 1 MNP 3 MNP 5 MNP 7 3.2.4 Grafting of PE-PCL (Sample B) onto TMAS Coated MNP All the reactions were performed under dry N2 atmosphere. MDI (0.116 g) was taken in a 250 mL two-necked RbF at dry N2 atmosphere. Dry THF (30 mL) was injected into the RbF through a rubber septum. PE-PCL (1 g), the required amount of TMAS coated MNP, and 0.056 g of DBTDL catalyst were taken into a 25 mL RbF with 10 mL of dry THF (scheme 3 .1b) and sonicated for 1 h. This sonicated solution was added dropwise (continued up to 1 h) into 250 mL RbF which already contained dissolved MDI. The temperature of the reaction mixture was maintained at 60 °C. The constituents and conditions of reactions are showed in Table 3.2. After 6 h of polymerization, reaction mixture was precipitated in cold methanol. The precipitate was collected with filtration, washed by methanol 3 to 4 times, and dried in vacuum oven at 80 °C for 24 h. 3.3 Results and discussion 3.3.1 Synthesis and Modification of MNP Successful synthesis of MNP and coating with TMAS are confirmed by FTIR, HRTEM, and XRD analysis. FTIR spectra display characteristic bands at 574 cm −1 and 3436 cm−1 which correspond to Fe−O and surface O−H stretching frequency of iron oxide nanoparticles (NP), respectively. 160 The chemisorption of TMAS onto NP surface is indicated by the Si−O−Si stretching band at 1088 cm−1 and a band at 3386 cm−1 which corresponds to surface N−H stretching frequency of TMAS coated NP (Fig. 3.1b). The HRTEM photomicrograph of TMAS MNP (Fig. 3.1) demonstrates that the NPs have a nearly spherical shape with average diameter of 5 nm. Interestingly, NPs do not appear very well separated from each other in the HRTEM photomicrograph (Fig. 3 .1), which may be due to small thickness of TMAS coating. Figure 3.1 (a) HRTEM photomicrograph of TMAS coated MNP and (b) FTIR spectra of pristine MNP and TMAS -MNP. The crystal structure of NP core is evaluated by X-ray diffraction study (XRD). XRD patterns reveal six peaks (Fig. 3.2) at 2θ values of 30.2°, 35.5°, 43.5°, 54.0°, 57.5° and 62.9°, respectively which are the characteristics of Fe3O4 NP.161 According to the literature these six peaks correspond to (200), (311), (400), (422), (511) and (440) planes, respectively. 162 Figure 3.2 XRD pattern of pristine Fe3O4 MNP 3.3.2Structure Analysis of PE-PCL PE initiated CL polymerization has been carried out according to the procedure as described in earlier section. The four armed structure of PE initiated CL polymer is confirmed by 1H and 13C NMR spectra. The 1H NMR spectrum shows three triplets at 4.0 ppm, 3.6 ppm and 2.3 ppm (Fig. 3.3) which correspond to two−CH2− protons for H-(5), two protons for H-(4) of the terminal−CH2− and –CH2- protons for H-(3), respectively. Two multiplets appeared at 1.5 ppm and 1.3 ppm which correspond to H-(2) and H-(1), respectively. A singlet appeared at 4.36 ppm corresponds to eight −CH2− protons of the initiator (PE). The 13C NMR spectrum (Fig. 3. 4) shows a peak at 43.7 ppm, corresponding to tertiary carbon (C-1) of the initiator. It also shows a number of intense peaks at 173.72 ppm, 64.30 ppm, 34.27 ppm, 28.49 ppm, 25.68 ppm and 24.72 ppm

corresponding to carbonyl carbon (C-3), C-8, C-4, C-7, C-6 and C-5 of the repeating units, respectively. A number of less intense peaks also appeared (inset window): which are assigned to their corresponding terminal carbons. 3.3.3 End Group Analysis MALDI-TOF mass spectra are enormously helpful for detection of end group and repeating unit in polymer chains. Fig. 3.5 exhibits the MALDI-TOF mass spectrum of cleaved PCL prepared by using PE as an initiator. This displays a number of species, each separated by 114 mass (m/z) units, which is the molar mass of the repeating unit CL. The mass series (M) emerge in MALDI-TOF mass spectrum can be calculated by equation (Eq. 3.1).163 Figure 3.3 1H NMR spectrum of PE-PCL (Sample a) in d6 -DMSO Figure 3.4 13C NMR spectrum of PE-PCL (sample a) in CDCl3 M = M[PE] + nM [CL] + M[H] + M[Na+].....Eq. 3.1 Where, M[PE], M[CL], M[H] and M[Na+] are the masses of initiator (M[PE] = 132), monomer (M[CL] = 114), end groups (4M[H] = 4) and cation (M[Na+] = 23), and 'n' denotes the degree of polymerization. The simulated peaks derived from Isopro-3 software were used to compare the isotopic distribution of peaks emerged in MALDI- TOF spectrum, and finally the peaks were consigned cautiously. The peak at mass (m/z) of 1986.63 is ascribed to scheme 3.2 where n = (w + x + y + z) = 16. Thus, from MALDI-TOF analysis it is clearly proven that macromolecular chains contain four armed PE as an initiator and monomer CL as a repeating unit. 3.3.4 Kinetics of PE Initiated CL Polymerization Kinetic study of PE initiated CL polymerization has been done by gravimetric analysis. The conversion of CL with time was monitored by gravimetric analysis for various concentration of initiator. A linear dependence of ln(1−x)−1 vs time at different concentrations of initiator was observed, which clearly demonstrated the first order kinetics of the given polymerization (Fig. 3.6). The figure displays a gradually increasing rate of reaction with increasing initiator concentration according to sample codes C, B and A, respectively (shown in Table 3.1). The number average molecular weight (Mn), degree of polymerization, and PDI of each set is also reported in Table 3.1. Figure 3.5 MALDI-TOF mass spectrum of PE initiated CL. Scheme 3 .2 Proposed chemical structure of PE-PCL, as confirmed by MALDI-TOF. 3.3.5 Structure Analysis of −NCO Terminated PE-PCL The grafting of PE-PCL onto MNPs has been performed according to scheme 3 .1b. Initially, −NCO terminated PE-PCL has been synthesized and the chemical structure of that is confirmed by 1H NMR analysis in DMSO- d6 (Fig. 3.7a). In the 1H NMR spectrum each signal is assigned to the corresponding protons of four branches of −NCO 56 terminated PE-PCL. 1H NMR shows two triplets at 4.08, 2.24 ppm and two multiplets at 1.5, 1.3 ppm corresponding to two −CH2− protons for H-(4), H- (6) and two −CH2− protons for H-(7), H-(8), respectively, in PCL chains. A singlet signal appears at 3.8 ppm which corresponds to H-(5) of the central −CH2− of MDI. The signals appearing at 7.08 and 7.3 ppm signify aromatic H-(3) and H-(2) protons of MDI. The triplet signal appearing at 3.6 ppm corresponds to H-(4) of terminal −CH2− of PE-PCL (Fig. 3.3), and disappears in Fig. 3.6. Accordingly, a new singlet for -NH- protons appears at 9.45 ppm (symbolized by 1) which signifies effective bonding of MDI with PE-PCL. FTIR spectrum also displays two characteristic band at 2350 and 3345 cm−1 (Fig. 3.7b) for - NCO and –NH groups, respectively. Thus both 1H NMR and FTIR spectra suggest the chemical structure that is displayed in Fig. 3.7a. Figure 3.6 Kinetic plots of PE initiated CL with CL:PE ratio of (a) 1:1/40, (b)1:1/100 & (c) 1:1/150. During all

three polymerization the monomer by catalyst ratio remained the same (1000:1 by weight). 3.3 .6 FTIR Analysis of Grafted Polymer In situ grafting of PE-PCL onto MNP has been done according to scheme 3 .1b. The nomenclature of various PE-PCL grafted MNPs are displayed in Table 3.2. The FTIR spectra of TMAS-MNP, PE-PCL and MNP 7 are shown in Fig. 3.8A. The spectra clearly shows a number of bands at 574 cm−1, 1723 cm−1, 2848 cm−1 and 2933 cm−1 which are characteristics of Fe−O stretching of MNP, C=O stretching, and symmetric and asymmetric −CH2− stretching of PE-PCL, respectively. The band at 3448 cm−1 corresponds to terminal free −OH stretching of PE-PCL polymer. Figure 3.7(a) 1H NMR spectrum of -NCO terminated PE-PCL in DMSO- d6 and (b) FTIR spectra of-NCO terminated PE-PCL. It is most interesting that the band which appeared at 3386 cm−1 for free -NH2 stretching of TMAS-MNP shifts to a lower frequency region at 3370 cm−1. This observation clearly proves that there must have been some sort of interaction between polymers and MNP. Again, the FTIR spectra of PE-PCL, MNP 1, MNP 3, MNP 5 and MNP 7 together (Fig. 3.8B) reveal that there is a definite increase in intensity of -NHCO- band at 1690 cm−1 with increasing MNP content. From these two consecutive observations, we conclude that there must have been chemical interaction between MNP and polymers. 3.3.7 XRD Analysis of Grafted Polymer XRD patterns of pristine MNP, MNP 1, MNP 3, MNP 5 and MNP 7 are shown in Fig. 3.9. For a particular amount of polymer, the intensity of peaks corresponding to MNP is gradually reduced with decreasing MNP content. This indicates that successful immobilization of polymer has taken place on the MNP surface. 161 Using the half height width of peaks corresponding to (200) and (440) planes and the Scherrer equation, 164 the crystallite sizes for all compositions were determined. The crystallite sizes for all compositions remain approximately the same (not shown). Therefore, crystallite size is not affected by grafting of polymer onto MNP surface. 3.3.8 XPS analysis A general survey of XPS from the binding energy of 90 to 750 eV for TMAS coated MNP and four armed PCL immobilized MNP are shown in Fig. 3.10. Silica coated MNP shows a number of signals at 97, 283, 399, 529 and 709 eV which are assigned to the Si2p, C1s, N1s, O1s and Fe2p respectively.165 Figure 3.8: The curve a, b, c, d, e and f indicates FTIR spectrum of PE-PCL, MNP 7, MNP 5, MNP 3, MNP 1 and TMAS-MNP, respectively. (A) FTIR spectra of TMAS-MNP, PE-PCL and MNP 7, zooming from 3200 to 3600 cm-1 in the inset window and (B) FTIR spectra of PE-PCL and MNP 1 MNP 3, MNP 5, MNP 7 respectively, zooming from 1680 to 1780 cm-1 in the inset window. Figure 3.9: XRD of MNP 1, MNP 3, MNP 5, MNP 7 and PE-PCL. The inset window contains zoom portion form 2θ value of 30o to 70o. But the survey spectrum of polymer modified MNP only displays two prominent signals at 283and 529 eV these are assigned to the C1s and O1s of polymer only. The absence of N1s and Fe2p, signals of MNP core in the survey spectrum of polymer immobilized MNP clearly indicates the wrapping of polymer chains onto the surface of TMAS modified MNP. For further insight into semi-qualitative estimation of surface functionalities, high resolution XPS (HRXPS) spectrum of each detected element as 59 presented separately. HRXPS spectrum of Si2p, C1s, O1s and Fe2p from TMAS modified MNP are shown in Fig. 3.11. The HR spectrum of Si2p shows two prominent deconvoluted components at 99.6 and 100.2 eV, respectively which are assigned to the Si atoms from Si-C and Si-O of TMAS. The two prominent deconvoluted components at 282.6 and 283.7eV of C1s are

observed. These two peaks are assigned to the C atoms from C-Si and C-C of TMAS. HRXPS spectrum of O1s also shows two deconvoluted components at 528.4 and 529.8 eV which are assigned to O atoms from O-Fe and O-Si of TMAS coated MNP. Figure 3.10: Survey XPS spectra scanning from the binding of 90 to 750 eV. Figure 3.11: High resolution XPS spectra (a) Si2p, (b) C1s, (c) O1s and (d) Fe2p of TMAS modified MNP. The two major signals at around 709 and 722.5 eV are assigned to Fe2p3/2 and Fe2p1/2 of MNP core. The HRXPS spectrum of polymer immobilized MNP are shown in Fig. 3.12. HRXPS spectrum of C1s displays three prominent deconvoluted components at 282.8, 284.3 and 286.9 eV which are designated for C atoms from C-C, -CH2O- and -COO- of PCL main backbone.166 HRXPS spectrum of O1s exhibits three deconvoluted components at 528.6, 530.2 and 531.6 eV which are assigned to the O atoms from O-Si of TMAS, -COO- of PCL backbone and -OCONH- which are formed when terminal -OH react with -NCO group of MDI. The absence of Fe2p and Si2p signals in the survey and HRXPS spectra of PCL immobilized MNP signifies the fruitful immobilization of four armed PCL chains onto the surface of TMAS modified MNP. Figure 3.12: High resolution XPS spectra (a) C1s and (b) O1s of PE-PCL immobilized MNP. Figure 3.13 HRTEM photomicrographs of (a) MNP 7, (b) MNP 5, (C) MNP 3, (d) NMP 1 and (e) schematic presentation of MNP grafted polymer. 3.3.9 HRTEM Analysis The HRTEM photomicrographs were captured by dispersing the sample in CHCl3 and by placing a drop of it on a carbon coated copper grid. A uniform dispersion of MNP is observed for all samples as shown in Fig. 3.13. However with increasing MNP mass content, e.g., MNP 1, MNP 3, MNP 5 and MNP 7, respectively, the effective particle size increases approximately from 5 to 10 nm. This is because as the number of particles becomes more (as for MNP 5 and MNP 7) compared to the number of polymer chains, a few individual particles make a nano-aggregate and allow the polymer chains to graft onto their exposed surface (as shown in Fig. 3.13e schematically). The numerical number (1, 2, 3, etc.) on the MNP in Fig. 3.10e indicates schematically the number of MNP involved in nano-aggregation. In another way, individual grafted particles having less grafting density may form nano-aggregates due to dominating magnetic dipole−dipole interaction. 3.3.10 TGA Analysis From TGA thermograms (Fig. 3.14a) it is observed that the maximum degradation temperature (Tmax) of polymer decreases from 400 °C to 265−285 °C because of grafting on the surface of MNP. It is a general fact that the thermal stability of a polymer increases when it is grafted onto nanoparticles. But in our case, thermal stability of virgin polymers was found to be higher compared to that of MNP grafted polymers, which is overturned from the general case. The decrease in thermal stability due to loss of crystallinity (shown in Table 3.2) of polymer chains in favor of grafting onto the MNP surface already has been reported by C. Flesch et al. 159 Grafting leads to increasing the number of amorphous chains which accelerates thermal degradation of polymers by allowing easier diffusion of degradation products. The grafting density159 was determined by using Eq. 3.2. The grafting density (D) is given as: □ = %□× □□□ … … … … … … Eq. 3.2 □□□ − %□ × □ × (□ − □ × □) Where, 'S' is the specific surface area of MNP (128 m2 g−1) determined by BET isotherm and '%m' is the weight percentage of polymer loss in the temperature range of 160 to 500 °C. The number average molecular weight of PE-PCL and molecular weight of MDI are symbolized by 'M'

and 'L', respectively. Grafting density for different compositions is shown in Table 3. 3. From Table 3. 3 it is clearly observed that grafting density in our sample is higher compare to that of linear polymer grafted MNPs as determined earlier by C. Flesch et al. 159 Although linear polymer chains would face less steric clashes compared to the branched analog, they show a high value of grafting density because of their lower solution viscosity leading to higher mobility during the grafting reaction. Hence higher grafting efficiency is achieved with branched polymer. It is important to note that in the present case the grafting density has been at least 2−3 times higher than that of the literature reported values with linear PCL. It is observed that grafting density decreases linearly with increasing MNP mass content (Fig. 3.14b). As the MNP mass content is increased for a particular amount of polymer, the number of grafted polymer chains per MNP decreases, which leads to reduction of grafting density. Figure 3.14 (a) TGA thermogram curves of PE-PCL and MNP 1, MNP 3, MNP 5 and MNP 7 within the temperature range of 0° to 600 °C and (b) variation of grafting density with increasing MNP content. Table 3. 3 Grafting density on polymer grafted MNP by TGA. Sample code MNP contain Weight Grafting density (μ (%) loss (%) a mol m-2) b MNP 1 1 96 15.5 MNP 3 3 95 12.3 MNP 5 5 94 10.4 MNP 7 7 92 7.4 aDetermined by TGA curve, in the temperature range of 160 to 500 oC; bDetermined by using equation 2. Figure 3.15 (c) DSC thermogram of virgin and grafted polymers, (a) schematic depiction of intra-molecular H-bonding between PE-PCL chains, and (b) between grafted polymer chains. 3.3.11 DSC Analysis The DSC thermogram (Fig. 3.15c) displays two melting peaks in the second heating cycle. The emergence of two melting peaks may correspond to two types of organization of molecules. Recently, J. L. Wietor et al. reported the appearance of two melting peaks for ureidopyrimidinone end-capped PCL. One of them has been ascribed to melting of crystalline domains of PCL and the other is due to melting of stacked ureidopyrimidinone ends. 167 Accordingly, in the present discussion, the first melting peak originated from crystallization of linear polymeric chains and the second one as a result of intramolecular H-bonding formed by end groups (−OH) (shown in Fig. 3.15a). Figure 3.16 M-H plots of MNP 1, MNP 3, MNP 5, MNP 7 hybrids and TMAS-MNP (Fe3O4) in the inset windows. The intensity of the second melting peak is found to increase due to in situ modification of MNP. This observation is due to stacking of phenyl groups (from MDI) through more effective intramolecular H-bonding (as shown schematically in Fig. 3.15b) that occurs between urethane groups (−NHC=O). The DSC thermograph also shows a decrease in melting temperature during grafting of the polymer chains onto the MNP surface. The first melting peak shifted from 50 to 45 °C and the second from 54 to 51 °C. This is probably due to a decrease in overall crytallinity of PE-PCL chains caused by grafting onto MNP surface. 3.3.12 Magnetic Properties The magnetic measurements of pristine MNP and various PE-PCL grafted MNPs are displayed in Fig. 3.16. The superparamagnetic nature of pristine and grafted MNPs is clearly confirmed by the absence of coercivity and remanence on the magnetization loop. The magnetization curve has shown a very high value (65 emu g−1) of saturation magnetization (Ms) for TMAS-MNP. The Ms values of MNP 1, MNP 3, MNP 5 and MNP 7 are 0.70, 1.25, 2.64 and 6.21emu g−1, respectively. The increasing value of saturation magnetization from MNP 1 to MNP 7 is due to decreasing grafting density as explained in TGA analysis section. 3.3.13 Cytotoxicity of

Polymer Grafted MNP One of the major objectives of this chapter is to prepare biocompatible nanoparticles. Hence, as a first step, it is necessary to perform cytotoxicity test (in vitro) at different concentration of polymer grafted MNP and pristine MNP to determine whether it is cytotoxic or not. The cell viability of polymer grafted nanoparticles was performed using an MTT [3-(4,5- dimethylthiazol -2-ly)-2,5-diphenyltetrazolium bromide] assay with HeLa cells for 24 h after they had been incubated at 37 °C. As shown in Fig. 3.17, polymer grafted nanoparticles MNP 1, MNP 3 and MNP 5 display cell viability of about 80% at concentration of 25 µg/mL, whereas MNP 7 was found to be less compatible compared to MNP 1, MNP 3 and MNP 5 (about 50% cell viability at concentration of 25 µg/m). MNP 1 shows much lower cytotoxicity compare to others at equivalent concentration (µg/mL). The cytotoxicity gradually reduces with decreasing MNP content from MNP 7 to MNP 1. The IC50 values are 191.6, 89.0, 63.7 and 45.8 µg/mL for MNP 1, MNP 3, MNP 5 and MNP 7, respectively. The MNP 7 shows to some extent higher cytotoxicity compared to 65 other, due to low grafting density onto MNP surface, and may also be due to the presence of some ungrafted MNP. However, the cytotoxicity of MNP 7 is less as compared to pristine MNP especially at higher concentrations. So, these ensure secure application of polymer grafted MNPs for magnetic targeted drug delivery and MRI, especially at lower concentrations of MNPs. Figure 3.17 Column chart of in vitro cytotoxicity of polymer grafted MNP at different concentrations on HeLa cell measured by using MTT assay. 3.4 Conclusions Novel superparamagnetic and biocompatible polymer grafted nanoparticles have been prepared successfully by grafting four armed PE-PCL onto MNP surface. The grafting density of PE-PCL has been found to be significantly high, leading to much lower aggregation tendency of the MNPs. The synthesis of PE-PCL has been carried out by ring-opening polymerization. The polymerization follows a first order kinetics where the rate of reaction is found to increase with increase of initiator concentration (PE). The structure of four armed PE-PCL is successfully established by spectroscopic analysis, detection of end group, i.e., PE by MALDI-TOF analysis. The FTIR, XRD, HRTEM and XPS analysis are confirmed successful immobilization of PE-PCL onto the surface of MNP. In a given initiator and CL concentration, grafting density onto MNP is found to decrease with increase in MNP content. The dispersion of MNP into polymer matrix was significantly reflected in decrease of both overall crystallinity (from DSC) and thermal stability (from TGA) of the grafted polymers compared to the virgin one. The appearance of two melting peaks and increasing intensity of the second peak during grafting was successfully explained. The TMAS coated MNP and its PE-PCL grafted analogues display strong signatures of their superparamagnetic nature with a very high degree of saturation magnetization in SQUID magnetometric measurements. Increase in the value of saturation magnetization (Ms) with decreasing grafting density is clearly revealed by the magnetic measurements. Cell viability testing ensures that polymer grafted nanoparticles with low MNP content show higher cell viability compared to others having high MNP content. MNP 1 and MNP 3 exhibited uniform dispersion combined with superparamagnetic characteristics and low cytotoxicity at equivalent concentration compared to two other samples, namely, MNP 5 and MNP 7. Thus these novel PE-PCL grafted MNP particles are potential material for magnetic targeted drug delivery and MRI applications. 4.1 Introduction The superparamagnetic nanoparticles (MNPs)

have diverse range of applications in various facets starting from engineering to biomedical aspects. MNPs can be used for cell separation and purification,168 as a carrier of anti-bacterial polymer,169 for magnetic targeted drug delivery (MTDD),170 in magnetic resonance imaging (MRI)171 and RNA fishing172 along with other applications such as catalysis and shape memory. Now-a-day's out of these, two most important biomedical applications namely MTDD and MRI are getting utmost attractions in biomedical research. MNPs are extensively used for such applications, because of their easy controlled size synthesis and by virtue of its superparamagnetic nature. For MRI and MTDD, one can control the position of the MNP in biological system (or MNP tagged drug) or their magnetic moment can be manipulated by applying external magnetic field. These types of deliveries can efficiently guide to reduce the side effects.173 Unlike other nanoparticles, MNPs have higher tendency to form agglomerated structures owing to their inherent cohesion due to magnetic attraction.174 The agglomeration leads to increase in apparent size of MNP. The large size MNP leads to the reduction of their circulation time in blood stream.175 Agglomeration leads to the bio-elimination of magnetic nanoparticles by macrophages or recticuloendothelial system before it can reach at the vicinity of the targeted site. To resolve this dilemma, MNPs are normally immobilized using biocompatible polymers.148c This technique can reduce the tendency of agglomeration of MNPs and thereby the ability of carrying drug molecule by the hybrid vehicles can be significantly enhanced. Polymer immobilized MNP have already been synthesized and their dispersion behaviour has also been investigated in one of our recent communication.176 It has been well demonstrated that immobilization of polycaprolactone onto the surface of MNP by click reaction provided a material with improved state of dispersion.177 Polycaprolactone immobilized MNPs synthesized by ring opening polymerization (ROP) from ricilonic acid modified MNP present successful attachments with polymer.178 The immobilization of caprolactone (by ROP) and 2- methoxymethacrylate (by ATRP) onto the surface of MNP lead to a core-shell morphology.157a,179 The immobilization of hydrophilic poly(ethylene glycol) monomethacrylate onto MNP surface results in non-cytotoxic and stable aqueous phase dispersed material for potential biological applications.180 Poly(ethylene glycol) 69 Chapter 4 methyl ether methacrylate immobilized MNP has been demonstrated to be a potential protein resistant nanoprobe for magnetic resonance contrast enhancement.181 Chitosan immobilized MNP offers excellent adsorptivity for purification of pollutants like perfluorinated compounds from water and ultimately helps in purification of water.182 MNP grafted with glycidyl methacrylate followed by dendritic poly(amido amine) (PAMAM) displays fairly good dispersion characteristics.183 The hyperbranch polyglycerol grafted onto the surface of MNP by grafting from approach, provides a uniform dispersion of NP.184 The polymer immobilized MNPs presents a material having antifouling surfaces and encapsulating layers.185 The literature is scanty on studies regarding colloidal stability and dispersion behaviour of such MNPs. However a section of publication deals with dispersion characteristics of dendritic polymer grafted MNPs.183 Immobilization of polymer onto surface of MNP is not the sole aspect of the problem (agglomeration). The saturation magnetization (Ms) of MNPs having different size is to be considered with due importance. Different size of MNP offers a compatibility with different types

of bio- entities for instance protein (5-50 nm), gene (2 nm wide and 10-100 nm long) and virus (20-450 nm).186 It's a common knowledge that MNP with high Ms, leads to higher extent of aggregation, compare to others having low Ms. For Fe3O4 based MNPs, the Ms values are largely controlled by the size factor. It has been reported that reduction of particle size offers higher value of Ms.36 But this trend reverses below the critical particles size (nearly10 nm).187 In this chapter, we plan to prepare two different size range of MNPs, one having average size of 15 nm i.e. above critical size of superparamagnetism and other having size range of 5 nm below the critical size. These prepared nanoparticles were immobilized by four armed PE-PCL through urethane (-NHCO-) linkage separately. Magnetic properties of both types of pristine MNP and polymer immobilised MNP were measured by using SQUID magnetometer. The size dependent dispersion behaviour (from HRTEM) of both types of MNPs (15 nm and 5 nm) was realized by considering two fundamental forces e.g. magnetic dipole-dipole and van der Waals force, respectively. The thermal properties of polymer immobilized MNP were studied by DSC and TGA analysis. The uniformly dispersed polymer immobilized MNP (5 nm) was used to prepare MAPM. The hydro dynamic size and bulk morphology of MAPM were studied by DLS and HRTEM analysis, respectively. A 70 model anticancer drug (DOX) was encapsulated into the MAPM. The magnetic targeted cell uptake of DOX encapsulated MAPM was studied by fluorescence microscopy. The cell viability of MAPM, DOX encapsulated MAPM and free DOX was studied against HeLa cell by MTT assay. The HFAMF triggered on demand release of the DOX from the MAPM was studied by UV-Vis absorption spectroscopy. 4.2. Synthesis 4.2.1 Synthesis and modification of MNP (5 nm) (i) The synthesis and modification of MNPs were performed according to the procedure as described in the in section 3.2.1 and 3.2.2 of chapter 3. 4.2.2 Synthesis and modification of MNP (15 nm) (ii) MNPs were prepared by simple co-precipitation method. In a 250 mL round-bottom flask (RbF) fitted with mechanical stirrer, 0.33 g of FeCl3 and 0.28 g of FeSO4 (2:1 molar ratio) were dissolved into 50 mL deionised water. Then, after 1 h of N2 purging, the aqueous NH3 solution was added drop by drop into the solution followed by drop wise addition of ethanol and TMAS mixture (10:1 volume ratio) as shown in scheme 4.1. The temperature of reaction mixture was maintained at 80 ºC and stirred at high speed (approx. 1500 rpm). After 2h of reaction, heat source was removed and stirring was continued up to next 6h. Finally, precipitated particles were washed with ethanol and acetone alternatively to remove excess TMAS. The washed particles were dried in a vacuum oven, over night at 40 ºC and then stored in vacuum desiccators for long term use. Scheme 4.1: Schematic presentation of two different size ranges of MNPs, their synthesis and modification by TMAS. 4.2.3 Synthesis of four armed polycaprolactone (PE-PCL) The synthesis of four armed PE-PCL was performed according to the procedure as described in the 3.2.3 section of the chapter 3. 4.2.4 Immobilization of PE-PCL chains (mol. wt. = 13100 g/mol) onto modified MNP Grafting of PE-PCL onto MNPs of two different sizes was done by coupling with MDI (urethane linkage formation) as shown in scheme 4.2. In a two-neck round bottom flask (RbF, 250 mL) 0.116 g of MDI was taken and dissolved with 30 mL of dry THF under N2 atmosphere. In another RBF (25 mL) 1g of PE-PCL polymer, required amount of MNP (5 nm or 15 nm) and 0.056g of DBTDL were dissolved in 10 mL of dry THF under N2 atmosphere. The

above solution of MNP with polymer was sonicated for 1h. The homogeneous solution was taken into 10 mL glass syringe and added drop-wise into the RbF which already contained dissolved MDI. The temperature of the reaction mixture was maintained at 60ºC during addition of homogeneous solution. The composition and constituents of the reaction are shown in table 4.1. The reaction was continued upto 6 h and then cooled to room temperature. Afterwards, the reaction mixture was then precipitated into cold methanol and collected after filtering and washing with methanol (3 to 4 times). The precipitated product was then dried in vacuum at 70-80 ºC under reduced pressure. Scheme 4.2: Schematic representation of four armed PE-PCL immobilized MNP. 4.2.5 Preparation of magnetically active DOX loaded polymer micelle The polymer immobilized MNPs were dispersed into DMSO at a concentration of 1mg/10µL. Required amount of DOX was added into the solution and stirred overnight under dark condition.188 100 µL of the above solution was then slowly added into 10 mL PBS solution under sonication at room temperature. The addition and sonication processes were continued for 15 minutes. Owing to the hydrophobic nature, the DOX molecules might enter into the hydrophobic PCL chains of the PE-PCL immobilized MNP (scheme 4.3). Finally, DOX loaded magnetically active polymer nanoparticles was dialyzed (using dialysis bag of 3.5 kD cut-off mol. wt.) 72 against water for 12 h to remove unbound DOX. The amount of DOX loaded into the MAPM was determined by UV-Vis. absorption spectra at 482 nm. A calibration curve was obtained by plotting λmax against different concentrations of DOX. The calibration curve was used later to calculate the DOX loading contents (DLC) and DOX loading efficiency (DLE) of MAPMs. The DLC and DLE were calculated by using the Eq. 4.1 and 4.2. □□□ % = □□□□ ?□ □□ □□□ □□□□ □?□ □□□□□□□□□□□□□□ × 100 … … … … … . . Eq. 4.1 □□□□ ?□ □□ □□□□□□□□□□□□□ □□□□□ □□□ % = □□□□ ?□ □□□□□□□□?□□ □□□□□□□□□□? □□□□□□□□□□□□□□□□□□□□□□ × 100 … … … … … … □□. 4.2 Table 4.1: Composition of Polymer immobilized 5 nm and 15 nm MNP 5nm Sample code 15nm MNP content (%) PE- PCL(w t. in g) MDI (wt.in g) DBTDL 5% (w.r.t total wt.) 5nm MNP 15nm Xc a (%) of 1 aDetermined from DSC by usingΔHº = 139.5 J/g. MNP (5) 1 MNP (5) 3 MNP (5) 5 MNP (5) 7 MNP (15) 1 MNP (15) 3 MNP (15) 5 MNP (15) 7 1 3 5 7 0.116 0.056 32 28 26 12 37 34 30 27 Scheme 4.3: Schematic representation of DOX loaded MNP and DOX release under the influence of high frequency alternating magnetic field (HFAMF). 4 .3 Results and discussion 4. 3.1 Synthesis and modification of MNPs In situ synthesis and modification of MNP (15 nm) was done by co-precipitation method. Hydrothermal method was used for synthesis of MNP (5 nm) of small size and modification was done following sol-gel method. Successful synthesis of MNP (both 5 nm and 15 nm) was recognized by FTIR, HRTEM and XRD analysis. The FTIR spectra (Fig. 3.1b, chapter 3) shows a number of bands at 574 cm-1 and 3436 cm-1 which correspond to Fe-O and surface –OH stretching frequencies of unmodified MNP. TMAS modified MNPs show a few additional bands at 1088 cm-1 and 3386 cm-1 corresponding to Si -O- Si and -NH- stretching frequencies which are the characteristics of TMAS. HRTEM photomicrographs of both 15 nm and 5 nm TMAS modified MNP are displayed in Fig. 4.1a-c. Figure 4.1: HRTEM photomicrograph of (a) single 15 nm MNP, (b) TMAS coated 15 nm MNP and (c) TMAS coated 5 nm MNP. Fig. 4.1a is the image of a single

MNP with size approximately 10 to12 nm but the average size of these particles has been found to be around 15 nm. Fig. 1b shows that MNP particles are effectively coated with TMAS. Core-shell assembly of MNP is notified with inorganic core stabilized by organic TMAS shell. Interestingly, NPs do not appear to be very well separated from each other on the HRTEM photomicrographs at low magnification. By zooming on the image we get to see a clear separation of MNP (Fig. 4.1b). Similarly, HRTEM photomicrograph of TMAS modified small size MNP is shown in Fig. 1c which has an average size of 5 nm. X- ray diffraction pattern show (Fig. 3.2) six characteristic peaks of Fe3O4 at 30.2°, 35.5°, 43.5°, 54.0°, 57.5° and 62.9° which correspond to (200), (311), (400), (422), (511) and (440) planes, respectively. 189 4.3.2 Synthesis of four armed PCL chains The structure of the synthesized four armed polymer was successfully established by 1H and 13C NMR spectra as described in section 3.3.2 and 3.3.3 of the chapter 3. 4.3.3 Immobilization of PCL chains onto MNP The immobilization of the polymer chains onto the surface of the MNP was successfully confirmed by FTIR, XRD and XPS analysis as described in chapter 3. Figure 4.2: (a) DSC thermogram of pure PE-PCL and PE-PCL immobilized MNP. (b) Intra and (c) intermolecular crystallization represented schematically. (d) Schematic presentation of PE-PCL immobilized MNP. 4.3.4 Thermal analysis The DSC thermogram of virgin polymer and polymer immobilized MNP are displayed in Fig. 4.2. From DSC thermogram it is observed that overall crystallinity of polymer gradually decreases with increasing MNP content (from MNP1 to MNP 7) as displayed in table 4.1. This is schematically depicted in Fig. 4.2c and d. The four armed polymer can stack into a plane, and the plane can stack with one another before immobilization of polymer onto MNP surface (Fig. 4.2c). But after getting immobilized onto MNP surface, the polymer can't able to stack as before (Fig. 4.2d). The loss of stacking ability leads to reduction of the overall crystallinity of polymer. DSC thermogram displays two melting peaks one at higher temperature and other at lower temperature. The first one is attributed to the assembly of end groups that can be realized as intra-molecular organization (Fig. 4.2b). Second one is attributed to the main chain crystallization can be called as intermolecular crystallization (Fig. 4.2c).167a The melting peaks are shifted to lower temperature region because of immobilization of polymer chains onto MNP surface, leading to decrease in crystallinity of polymer. From the TGA thermogram (Fig. 4.3) it is noticed that thermal stability of virgin PE-PCL is higher (Tmax = 400 ºC) compared to PE-PCL which are immobilized on MNP. Usually thermal stability of polymer increases by adding filler particles into the polymer matrix. As we immobilize the polymer onto the surface of the MNP, the thermal stability of polymer immobilized MNP decreases from that of virgin polymer. The decrease in thermal stability can be rationally explained by considering the resultant decrease in crystallinity (table 4.1) of polymer during immobilization onto surface of MNP. This leads to an increase in the number of amorphous chains which are vulnerable to degradation at even lower temperature by allowing easier diffusion of degradation products. 190 The amount of polymer immobilised onto the surface of MNP (i.e. grafting density) is calculated from the weight loss (Fig. 4.3) in between the temperature range of 160 to 500 °C. The calculated grafting densities (from equation 3.2) for both 15 and 5 nm MNP are displayed in table 4.2. The grafting density for both the particles gradually decreases with increasing MNP content from MNP 1 to MNP 7 caused by decreasing

number of polymer chains available per particle. It is observed that the grafting density on the surface of 15 nm MNP is higher compare to 5 nm for each percentage of MNP content. As grafting density on the 15 nm particles is higher compare to 5 nm particles, the colloidal stability of later is supposed to be lower. But in practice, 15 nm MNPs form agglomerated structures. This agglomeration is due to strong dipole- dipole interaction energy (105.85 kT) that subdues the steric effects. Table 4.2: Thermo gravimetric weight loss and determination of grafting density Sample MNP Weight loss (%)a Grafting density (μ mol m-2) (%) 15 nm 5 nm 15 nm 5 nm MNP 1 MNP 3 MNP 5 MNP 7 a Weight loss in between the temperature range of 160 to 500 ºC 1 3 5 7 96.4 95.4 93.6 89.2 96.4 95.7 94.3 92.1 28.6 22.2 15.5 8.7 17.5 14.6 10.8 7.5 Figure 4.3: TGA thermogram of virgin PE-PCL and PE-PCL immobilized MNPs. Figure 4.4: HRTEM photomicrograph of 5 nm (upper part) and 15nm MNP (lower part) each part having sample designation as MNP 7, MNP 5, MNP 3& MNP1 (from left to right). 4.3.5 HRTEM Photomicrograph and intra-particles force of interaction The superparamagnetic nanoparticles have inherent magnetic moment which assists to form agglomerates. The extent of aggregation among the MNPs can be explained by the balance of two types of intermolecular forces of interaction namely, van der Waals and magnetic dipole-dipole interaction (gravitational force is ignored, for simplicity). The aggregation tendency can be reduced by decorating the MNP with four armed poly(ε-caprolactone) chain. Although large size (15 nm) MNPs are grafted more by branched poly(ε-caprolactone) chains, yet they show some fairly higher extent of aggregation (Fig. 4.4). Generally, the aggregation tendency comes into prominence with increasing MNP content for a given weight percent of polymer. At higher MNP content, a few number of small size particles forms nano-colony which leads to increase in effective particle size. Thus, by comparing the different dispersion behaviours it can be inferred that large size magnetic nanoparticles (15 nm) grafted with polymer shows prevalent aggregation (specially at higher MNP content) compared to small size MNP (5 nm). The phenomena can be made clear by calculating van der Waals (equation 4.3) and dipole-dipole interaction (equation 4.4) energy for both sizes MNPs separately. As dipole-dipole interaction is more sensitive towards radius of MNP compared to van der Waals interaction, dipole-dipole interaction becomes imperative component for explaining this type of dispersion behaviour.191 The individual forces of interaction as calculated (from equations 4.3 and 4.4) are presented in table 4.3. van der Waals interaction energy: □ □2 □2 □2 □□□□ = − 12 □2 − □2 □2 + 2 ln 1 − □2 … … … … … □□. 4.3 + Where, 'A' = Hamaker constant 'D' = Particle size 'r' = Centre to centre separation distance Dipole-dipole interaction energy: μ0□2 1 − 3□□□ 2Θ □□□ = 4□ □3 … … … … … □□. 4.4 Where, μ0 = magnetic constant. m = magnetic moment. θ = angle between the magnetic moment and distance vector between two MNP. For uniform dispersion the value of θ is considered to be zero. It is observed that in case of small size MNP (5nm) there is not much difference between van der Waals and dipole-dipole (0.174 kT and 0.170 kT, respectively) interactions energy. The van der Waals interaction energy is to some extent higher compared to dipole-dipole interaction which constitutes the basis for uniform dispersion of such particles. However, a very large difference exists between the values of dipole-dipole interaction energy and van der Waals interaction energy (105.85 kT and 16.66 kT, respectively) in case of large size

MNP (15 nm). This would certainly favour the formation of aggregates for large size MNPs. The superparamagnetic nature of polymer immobilized MNP and pristine MNP (in the inset windows) are displayed in Fig. 4.5. A very high value of saturation magnetization (Ms) (169.34 emu/g) is manifested by 15 nm pristine MNP compared to 5 nm MNPs (table 4.4). Because of this high value of Ms, the entire polymer immobilized 15 nm MNP exhibits associated dispersion behaviour. A low value of Ms is displayed by all the polymer immobilized MNP compared to the pristine one. 4.3.6 Magnetic measurement The Ms values of all the polymer immobilized MNP and pristine MNP are summarized in table 4.4. Each category (5 nm and 15 nm) polymer immobilized 78 MNPs pursued a decreasing trend of Ms value on moving form MNP 7 to MNP 1. The increasing the amount of polymer onto MNP surface (i.e. grafting density) is the responsible factor for deceasing Ms value from MNP 7 to MNP 1. Table 4.3 Calculated values of intra-particle interaction energies. Assumption Interactions 5 nm 15 nm 5 nm 15 nm energy (kT) particles particles r = (5+3)=8 r = (15+1)=16 van der Waals 0.1745 16.66 && Magnetic dipole- 0.170 105.85 D = 5 D = 15 dipole D = particles size and r = Centre to centre separation distance between two particles Figure 4.5: Magnetic measurement of (a) 15 nm and (b) 5 nm polymer immobilized MNP and pristine MNP (in the inset windows). Table 4.4: Saturation magnetization (Ms) of pristine MNP and polymer immobilized MNPs. Sample Saturation magnetization (emu g-1) 15 nm 5nm Pristine MNP 169.34 65.02 MNP 7 11.75 6.25 MNP 5 6.82 2.65 MNP 3 4.19 1.32 MNP 1 3.20 0.77 4.3.7 Characterization of magnetically active polymer micelles (MAPM) Polymer immobilized MNP 5 (5 nm) was mainly used for preparation of MAPM due to its favourable dispersion characteristics, according to the procedure as illustrated earlier in the experimental section. Bulk morphology of polymer micelle was inspected by HRTEM analysis (fig. 4.6). Figure 4.6 shows a uniform appearance of MAPMs (as uniform array). The black spots inside the MAPM are the signature of MNP presents inside the core of polymer MAPMs. The average particles size of the MAPM is around 100 nm which is very close to the hydrodynamic radius (116 nm) of MAPMs as determined from DLS measurement (Fig. 4.6b). The MAPM shows the zeta-potential around -28 mV. The negative zeta potential signifies exposure of unused amine group (present onto the surface of the polymer immobilized MNP) from core to outer surface during the preparation of MAPM. The MAPM gets stabilized by posing these amine groups towards water. The magnetic responses of MAPMs are shown in Fig. 4.6d. Figure 4.6c indicates formation of a stable water dispersed MAPMs. But MAPAMs are spontaneously attracted towards the magnetic fields from stable aqueous dispersions of MAPMs. The nanoscale MAPMs are found to be totally separated from aqueous medium with in 3h in presence of a bar magnet (Fig. 4.6d). 4.3.8 Cytotoxicity assay (MTT) The cell viability of free DOX, MAPM and DOX loaded MAPM were checked by MTT assay against HeLa cells. MTT assay were performed by incubating different concentration of earlier mention components with HeLa cells for 24h. Figure 4.7 displays the cell viability results of all these components together. Figure 4.6: (a) HRTEM photomicrograph of MAPM, (b) DLS and schematic presentation of MAPM, (c) water dispersed MAPMs at 0h and (d) after 3h in presence of external magnet. Figure 4.7(a) MTT assay of the above mention components shows in column chart model (b) MTT assay in line symbol format. MAPM (prepared from MNP 1 and MNP 3) are showing high degree of cell viability (about 90 %)

even at higher concentration (100 µg/mL). Both free DOX and DOX loaded MAPM (prepared from MNP 1 and MNP 3) reveal high cytotoxicity i.e. low cell viability compare to blank MAPM. Between free DOX and DOX loaded MAPM, free DOX shows high cytotoxicity compare to DOX loaded MAPM. Quick incorporation of free DOX into the cell as well as nucleus is the responsible factor for its high cytotoxicity. For DOX loaded MAPM, DOX molecules are present inside the micelle and take some additional time to diffused from it. This time dependent movement of DOX molecules (present inside the polymeric micelle) is the answerable feature for low cytotoxicity i.e. high cell viability of DOX loaded micelles compare to free DOX. The dose require for 50 % cell growth inhibition i.e. IC50 values of DOX loaded micelle and free DOX are 4.47 and 0.54 µg/mL, respectively. These results manifest that DOX loaded polymeric micelles are ready to incorporate into the cell and showing their expected biological action. 4.3.9 Magnetic targeted cell uptake study To investigate the enhanced cell uptake of DOX loaded MAPM, one set of DOX- polymeric micelle treated cultured plate was placed directly under the influence of magnetic field (~average field of 3mT) and the other set was kept as control (without magnetic field). Because of self-fluorescence (red colour) characteristic, intercellular distribution of DOX molecule can be prominently detected by fluorescence microscopy. Study was conducted by incubating both free DOX and DOX loaded polymeric micelle with HeLa cell line in presence and absences of static magnetic field for 0.5h, 2h and 4h, respectively as discussed in the experimental section. In 81 absence of magnetic field, fluorescence microscopic image (Fig. 4.8) displays a very small intercellular uptake of DOX loaded MAPM even after 4 h of incubation. While in presence of magnetic field, DOX loaded MAPM shows (Fig. 4.9) an effective intercellular uptake just after 0.5 h of incubation. The intensity of red fluorescence implying the signature of DOX loaded MAPM is gradually increased with time up to 4h. Figure 4.8: Fluorescence microscopic images of HeLa cell treated with DOX loaded MAPM in absence of static magnetic field where nucleus was stained by DAPI (blue). The mentioned scale bar is 40 µm. The red fluorescence of DOX loaded MAPM is significantly observed mainly in the cytoplasm but not in the nucleus. Appearance of DOX fluorescence mainly in the cytoplasm indicating uptake of DOX loaded polymeric micelle might be happening through endocytic path way.122 In contrast, incubation of free DOX with the cell shows (Fig. 4.10) significant red fluorescence in the nucleus just after 0.5 h of incubation. This phenomenon indicates rapid internalization of free DOX molecule into the nucleus through passive diffusion mechanism.113 The micelle with loaded DOX which is present in the cell media attracted strongly towards the magnetic field. The experience of strong magnetic attraction provides a quick contact of MAPM with the cytoplasmic membrane of the cell which then gets internalized into the cell through endocytic mechanism. In the absence of magnetic field, the MAPM is present as suspended state in culture medium and required more time to get in contact with the cell membrane. Thus shows very low internalization of DOX loaded MAPM even after 4 h of incubation. 4.3.10 Release study The anticancer drug, DOX was encapsulated into the MAPMs according to the procedure as described in the experimental section. The amount of drug loaded into the MAPM was calculated by the equation 2.1. The DLC of the MAPM prepared from the 5 nm MNP 1, MNP 3, MNP 5 and MNP 7 samples are 32%, 28 %, 18% and 12%, respectively. The decrease in the

trend of DLC from MNP 1 to MNP 7 is attributed to the decrease in the number of drug preserving branched PE-PCL chains per particles i.e. decrease in the grafting density (table 4.2). Figure 4.9: Cell uptake study of DOX loaded MAPM on HeLa cell in presence of static magnetic field where nucleus was stained by DAPI (blue) and scale bar is 40 μm. Figure 4.10: Fluorescence Microscopic image of free DOX treated HeLa cell at scale bar of 40 μm. The DOX loaded MAMPs were placed under the influence of high frequency alternating magnetic field (HFAMF) according to the procedure as described in the experimental section. Under the influence of HFAMF, the superparamagnetic nanoparticles generated heat energy. Neel's relaxation is the cause of internal heat effect.136 The amount of heat generated is proportional to the strength and expose time of applied HFAMF.39 The amount of DOX release with time is displayed in Fig. 4.11. Figure 4.11a shows a cumulative release of DOX from MAPM at 37 and 45 $^{\circ}$C. The release profile shows only 8.3 and 9.7 % (after 24h) release of the DOX molecule at 37 and 45 $^{\circ}$C, respectively. Therefore the differences in release behaviours at two different temperatures are not remarkable. Thus, Neel relaxation does not have significant influence on drug release. A step up increase in the rate of DOX released under the influence of HFAMF is displayed in the Fig. 4.11b. After 1h, MAPM shows about 51.3% and 29.1% release of DOX molecule from MNP 5 and MNP 3, respectively. The high Ms value of MNP 5 (compare to MNP 3, table 4.4) offers the MAPM to experience more effect of HFAMF and release high amount of DOX. The MAPM release the DOX molecule may be by forming a number of pores on the wall of the micelle or by rupturing of the self-assembled structure by application of HFAMF.40 Figure 4.11: (a), the release kinetics of DOX loaded MAPM prepared from MNP 5(5 nm) at 37 and 45 $^{\circ}$C and (b) the release kinetics of MNP 3 and MNP 5(5nm) under the influence of HFAM F at 37 $^{\circ}$C. 4.4 Conclusions The four armed polymer chain could be efficiently immobilized onto the surface of MNP. Large size (15 nm) polymer immobilized MNPs displayed associated structure because of their extremely high magnetic dipole-dipole interaction energy (105.8kT) compare to van der Waals interaction energy (16.6 kT). In comparison words, smaller 84 size (5 nm) polymer immobilized MNP displayed its colloidal stability because of comparable or slightly higher van der Waals interaction energy (0.174 kT) subduing magnetic dipole-dipole interaction energy (0.170 kT). Although the amount of polymer immobilized onto the surface of 15 nm MNP is higher compare to 5 nm MNP, still it displayed associated structure owing to its extremely high magnetic dipole-dipole interaction energy that stems from its superparamagnetic nature. The decrease in overall crystallinity during immobilization is attributed to the loss of staking ability of polymer chains. The appearance of two melting peaks (from DSC), one at higher and other at lower temperature is associated with intra and intermolecular organization of four armed PE-PCL chains. MAPM has been prepared successfully and its magnetic activity has been realized in presence of external bar magnet. HRTEM photomicrograph displayed a regular appearance of spherical MAPM having average size of 100 nm which is at par with the hydrodynamic size (116 nm) evaluated from DLS measurement. Biocompatibility test provided information that all MAPM are showing biocompatibility above 90% even at higher concentration (100 μg/ mL). Selective and high cellular uptake of DOX loaded MAPM is observed for magnetically targeted HeLa cell line compared to the untargeted one. Plenty

of DOX loaded micelles enters into the cytoplasm of the magnetically targeted cell just after 0.5 h of incubation. In contrary, there is no significant uptake of loaded micelle for untargeted HeLa cell line even after 4h of incubation. Unlike free DOX molecules, loaded micelles are found mainly concentrated on cytoplasm indicating endocytic pathway which they have followed for entering into the cells. The MAPM shows a quick on demand release of about 51.5 % DOX (after 1h) under the influence of HFAMF. Hence, using such novel MAPMs we can able to tailor the drug release rate according to the status of the patient (i.e. on demand) by switching 'off' or 'on' HFAMF device. Chapter 5 5.1 Introduction Nanocarriers are used extensively to deliver drug molecules to specific sites in the body. This approach is often used to decrease the side-effects of highly toxic drugs with a low solubility in water.192 Biocompatible/biodegradable polymer micelles (PMs) are one of the most promising nano-carriers for targeted drug delivery.193 The PMs used for the targeted delivery of drugs must have the following characteristics: (a) they must be stable in phosphate-buffered saline (PBS) (pH 7.4); (b) they must be able to encapsulate, carry and deliver the drug; (c) they should be biocompatible and/or biodegradable inside the biological system; (d) their size should be in the range 10–200 nm for effective performance;117b, 138 and (e) they must be site-specific.194 Biocompatible/biodegradable amphiphilic polymers are widely used for the preparation of such PMs. Amphiphilic polymers are mainly formed by tailoring blocks of hydrophilic and hydrophobic polymers,195 i.e. by incorporating hydrophilic functional groups onto the backbone of a hydrophobic polymer, or by incorporating hydrophobic groups onto the backbone of a hydrophilic polymer. The hydrophobic parts of these amphiphilic polymers serve as efficient shields with which to hold the toxic drug molecules, which usually have a low solubility in water. In contrast, the hydrophilic part of these amphiphilic polymers is responsible for the colloidal stability of the polymer nanoparticles in water. Poly(lactic acid-co-glycolic acid),196 polycaprolactone (PCL),166, 195a, 197 polyphosphazene,198 poly(propylene fumarate),199 chitosan,197a, 200 and their modified variants are widely used to design the hydrophobic part of amphiphilic polymers. Polyethylene glycol (PEG)201 is one of the polymers most widely used as the hydrophilic part of amphiphilic polymers. The PMs formed by linear amphiphilic poly(ethylene glycol)-b-poly(3 -caprolactone-co- g -hydroxyl- 3 -caprolactone) have been used as nano-carriers to deliver hydrophobic anticancer drugs, including DOX.113 Free DOX has a higher cytotoxicity than DOX-loaded PMs, indicating the effective encapsulation of the drug into the PMs. Liu et al.112b reported the synthesis of docetaxel-loaded PMs using poly(ethylene glycol)- b-poly-(caprolactone) and subsequently investigated its antitumor action. They also reported that the particles formed in this way had a high degree of penetration into the tumor cell. 87 Chapter 5 Scheme 5.1: Schematic presentation for the synthesis of folic acid attached PE-PCL-b- PEC-FA block copolymer. The linear amphiphilic triblock copolymer formed by hydrophilic PEG and hydrophobic oxime-tethered PCL has also been used as an efficient nano-carrier for delivering anticancer drugs.118a A special type of amphiphilic block copolymer was synthesized via the supramolecular interaction of adenine and uracil groups attached at the end of the hydrophobic PCL and hydrophilic PEG parts of the polymer, respectively.122 This non- covalently connected amphiphilic polymer acts as a stimuli-responsive (pH-responsive) carrier for anticancer drugs.

Compared with linear polymers, branched and hyperbranched analogues appear to be more useful as nano-carriers for drug delivery, as confirmed by a recent study by Yong et al.138 Recent study on a Y-shaped polymer i.e. three-armed thermo-responsive amphiphilic block copolymer prepared from hydrophobic poly(undecylenic acid) and hydrophilic poly(N-isopropylacrylamide). This polymer was subsequently successfully used for thermoresponsive drug delivery. 88 Unlike linear polymers, branched or hyperbranched polymers have many advantages, such as a high efficiency of encapsulation, the ability to form templates101a and high functionality, enabling a wide range of interactions with biological systems. Although PEG is widely used as a hydrophilic polymer for drug delivery, it still has certain limitations, such as (a) a potential hypersensitivity reaction (it may produce anaphylactic shock) by PEG itself or by side products formed during polymerization,202 (b) abnormalities in its pharmacokinetic behavior203 and (c) its non-biodegradability.204 We aimed to overcome these limitations of existing nano-carrier systems by replacing PEG with polyethylene carbonate (PEC). PEC is well known for its biodegradability205 and is also biocompatible in nature. PEG is more hydrophilic than PEC; however, PEC is more hydrophilic (water solubility 88.1 g L-1 at 20 °C; Sigma-Aldrich MSDS) than PE-PCL. This confirms the amphiphilicity of the desired block copolymer. The objective of this chapter is the preparation of FA conjugated four-armed PE-PCL-b- PEC-FA block by the simple ring-opening polymerization of ε-caprolactone (CL) and ethylene carbonate (EC) followed by esterification with FA. The objective also includes finding the potential application of polymeric micelles (prepared from synthesized block copolymer) for cancer cell targeted drug delivery. In order to achieve the objective, amphiphilic block copolymers of PEC (PE-PCL-b-PEC) were synthesized by the ring opening polymerization of different equivalent amounts of ethylene carbonate (EC) using four-armed PE-PCL as a macro initiator. The structures of the synthesized homo, block and FA tethered polymers were established by 1HNMR spectroscopy. The water contact angle of the synthesized block copolymer was measured by a contact angle goniometer. The FA-attached amphiphilic polymer (PE-PCL-b-PEC-FA) was then precipitated in water to form the PMs. The hydrodynamic size and bulk morphology of the PMs were studied by dynamic light scattering spectroscopy (DLS) and HRTEM. The critical micelle concentration (CMC) of the PMs was determined by fluorescence spectroscopy using pyrene as a fluorescent probe. The drug-loading efficiency (DLE), drug-loading content (DLC) and release profile of an anticancer drug (DOX) were studied by UV-Vis spectroscopy. We also studied the effectiveness of DOX loaded PMs towards specific uptake by cancer cells (HeLa cells) and the in vitro release and cytotoxicity of DOX. 89 5.2 Synthesis 5.2.1 Synthesis of branched PE-PCL Branched PE-PCL was synthesized by ring-opening polymerization, as reported previously.176 PE was taken into a 25 mL two necked round- bottomed flask (RbF) under an N2 atmosphere. The required amount of monomer (CL) was added to the RBF, which was then placed into a preheated oil-bath at 120 °C. After homogenization of the reaction mixture, the required amount of catalyst (Sn(Oct)2 at a constant 1000 : 1 w/w ratio of monomer and catalyst) was added to the reaction mixture and the reaction was allowed to continue at the same temperature until 97–98% conversion (confirmed by a gravimetric method); after this level of conversion, the reaction mixture appeared highly viscous. The reaction mixture was then

cooled down to room temperature. The resulting solid mass was dissolved in a minimum volume of chloroform and precipitated into hexane. The precipitated product was dried under vacuum at 60 °C under reduced pressure. 5.2.2 Synthesis of branched PE-PCL-b-PEC PE-PCL-b-PEC was synthesized by the ring-opening polymerization of EC206 using branched PE-PCL as a macro initiator. The calculated amount of PE-PCL and EC were taken into a 25 mL two necked RbF fitted with a condenser under a dry N2 atmosphere. The required amount of dry toluene (at a constant 1:10 w/v ratio of EC and toluene) was then added. After homogenization of the reaction mixture, the RbF was placed into a preheated oil-bath at 110 °C. The calculated amount of catalyst (Sn(Oct)2 at a constant 1000 : 1 w/w ratio of EC and catalyst) was injected into the RBF just after starting reflux of toluene. The reaction was continued for up to 20 h at the same temperature. Finally, the reaction mixture was precipitated into diethyl ether and dried in a vacuum oven under reduced pressure. 5.2.3 Synthesis of FA-tethered PE-PCL- b-PEC copolymer The FA-tethered PE-PCL-b-PEC copolymer was synthesized using a slight modification of a previously reported procedure (scheme 5.1).207 FA (4 equiv.) with DCC (4.5 equiv.) and N-hydroxysuccinimide (4.5 equiv.) was dissolved in DMF in a two necked RbF under an N2 atmosphere. The reaction mixture was cooled down from room temperature using an ice-bath. The block copolymer (1 equiv.) dissolved in DMF was then slowly added into the RBF containing FA. After 1 h, the ice-bath was removed from the reaction system and stirring was continued for up to 24 h at room temperature. The FA-tethered polymer was then filtered through a filter-paper to remove residual dicyclohexylcarbodiimide (DCU). The filtrate was dialyzed against DMF through a dialysis membrane (cut-off mol. wt 3.5 kD; cellulose acetate membrane) to remove any unreacted FA. The FA tethered polymer was precipitated into an excess amount of hexane and dried under vacuum. The final product was characterized by 1H-NMR spectroscopy. 5.2.4 Preparation of FA-conjugated, DOX-entrapped PMs The FA-conjugated block copolymer (10 mg) was dissolved in 100 mL of DOX solution (2.5 mg DOX/100 mL DMSO). The mixture of copolymer and DOX was stirred overnight in the dark. The resulting mixture was then poured slowly into 10 mL of double distilled water with vigorous agitation. The hydrophobic nature of DOX would help it to get entrapped preferentially in the hydrophobic core (PCL core) of the block copolymer (Scheme 5.2). The suspension of PMs with entrapped DOX was then transferred into a dialysis bag (cut-off mol. wt 3.5 kDa, cellulose acetate) and dialyzed against water to remove DMSO and unbound DOX. The dialysis was continued for up to 24 h at room temperature in the dark. Table 5.1: Synthesis of branched PE-PCL-b-PEC by taking PE-PCL (Mw=11.3kDa, Đ=1.2) as macroinitiator at 120 °C under N2 atmosphere. Exp. EC/PE-PCL No. (mol/mol) Mn a (kDa) Đa PCL CMCb content (mg/mL)× (%) 10-3 DLCc (%) DLEd (%) Sample code 1 100 16.5 1.3 ~68 4.21 14.7 59.0 A 2 150 19.6 1.2 ~58 6.32 12.4 49.7 B 3 200 24.3 1.2 ~46 8.75 11.0 44.1 C 4 250 26.1 1.3 ~43 9.32 9.9 39.9 D a Evaluated from GPC analysis. b Determined by fluorescence spectroscopy using pyrene probe. c,d determined from equation 4.1 and 4.2, respectively. The amount of DOX loaded into the polymer was calculated by taking the absorbance of a known amount of DOX loaded polymer at 482 nm and from a calibration graph for the absorbance of free DOX. The calibration graph was constructed by adding different concentrations of DOX into DMSO. The DOX loading content (the

DLC) and the DOX loading efficiency (the DLE) of the PM were calculated as follows: 5.3 Results and discussion 5.3.1 Synthesis and characterization of branched PE-PCL The branched PE-PCL was synthesized by the simple ring opening polymerization technique described in the earlier section. The chemical structure of the branched polymer was confirmed by 1H-NMR spectrometry (Fig. 5.1). The resonance signal at 4.3 ppm is assigned to the methylene proton (–CH2O–, a) of the initiator (PE). The spectrum displays two more signals at 4 ppm and 2 .3 ppm those are assigned to the methylene protons (–CH2O–, e) and (–COCH2–, b) attached to the main chain. A signal at 3.6 ppm is assigned to the terminal methylene proton (–CH2OH, f) of the polymer chains. Two signals at 1.3 ppm and 1.6 ppm are assigned to their corresponding protons (d and c, respectively) in Fig. 5.1. Scheme 5.2: Schematic representation for the preparation of folic acid conjugated block copolymeric micelle. 5.3.2 Synthesis and characterization of branched PE-PCL-b-PEC The block copolymer was synthesized by the ring-opening polymerization of EC described in the earlier section. The chemical structure and composition of the block copolymer were confirmed by 1H-NMR spectrometry, GPC and DSC. The 1H-NMR spectrum shows a number of resonance signals at different chemical shifts (Fig. 5.2). The 92 signals at 4 ppm, 2.3 ppm, 1.6 ppm and 1.3 ppm are assigned to the –CH2O– (b), – OCOCH2– (d), –CH2– (e) and –CH2– (f) protons of the PE-PCL sector of the block copolymer, respectively. The signals at 4.5 ppm and 3.6 ppm are assigned to the –CH2– (a) and –CH2O– (c) protons of the EC part of the block copolymer. We have synthesized block copolymers with different proportions of PE-PCL and EC. Figure 5.1: 1H NMR spectrum of branch PE-PCL in CDCl3. We used PE-PCL as a macro initiator with a fixed molecular weight (11.3 kDa) and the amount of EC was varied (Table 5.1).The formation of the block copolymer with different molecular weights was confirmed by GPC (Fig.. 5.3). The retention time of the block copolymers (samples A and B, Table 5.1) are shifted to the left (i.e. a lower retention time) compared with the homopolymer (PE-PCL). Figure 5.2: 1H NMR spectrum of branch PE-PCL-b-PEC in CDCl3. This shift in retention time recognizes the increase in molecular weight (compared with PE-PCL) and the formation of the block copolymers. The growth of the hydrophilic block (PEC) from the hydrophobic block (PE-PCL), i.e. the formation of the block copolymer, 93 was also confirmed by the reduction in the water contact angle from 77° (for PE-PCL) to 55° (for sample D) (Fig. 5.5). Figure 5.3: (a) GPC trace of homopolymer (PE-PCL), sample A and sample B. Figure 5.4: DSC thermogram of (a) homopolymer (PE-PCL) and (b) block copolymer (PE-PCL-b-PEC). Figure 5.5: Water contact angle of PE-PCL (77º), Sample A (71º). Sample B (67º), Sample C (59º) and Sample D (55 º). The formation of the block copolymer was confirmed by DSC (Fig. 5.4). The DSC thermogram of the homopolymer PE-PCL shows two melting peaks at 50 °C and 54 °C, respectively. These temperatures correspond to the molecular organization of main chain and end-group of the PE-PCL chains, respectively.176 The disappearance of the melting peak at 54 °C for the block copolymer is attributed to the growth of amorphous PEC chains from the end-group (–OH) of the homopolymer (PE-PCL). The emergence of a glass transition temperature at around 22 °C (the signature of PEC) along with a melting temperature of 50 °C (the signature of PE-PCL) in the DSC thermogram is attributed to the formation of the block copolymer (PE-PCL-b- PEC). 5.3.3 Synthesis and characterization of FA-

conjugated PE-PCL-b- PEC copolymer The FA-conjugated block copolymer was synthesized via the reaction of HO-PEC-b- PCL-PE with FA by DCC coupling reaction. Between the 'α' and 'γ' carboxylic acids of the FA-conjugated block copolymers, the latter copolymer has a superior biocompatibility with an increased circulation time in the bloodstream and allows the conjugate to act as an active targeting agent towards cancer cells.208 The conjugation of FA with the block copolymer was confirmed by 1H NMR spectrometry (Fig. 5.6). The spectrum shows a number of resonating signals at 4.5 ppm, 4 ppm, 2.3 ppm, 1.6 ppm and 1.3 ppm, which are assigned to the –CH2– (a), –CH2O– (b), –OCOCH2– (c), –CH2– (d) and –CH2– (e) protons attached to the main chain carbons of the block copolymer. In addition, another set of resonating signals emerged between 6.0 and 9.0 ppm and those were assigned to the protons of FA.207b, 209 We have quantitatively determined the percentage of FA conjugation in the block copolymer using UV-Vis spectrometry (Fig. 5.7). Unlike FA, the pure block copolymer did not show any absorbance in the range 370–280 nm. Figure 5.6: 1H NMR spectrum of folic acid conjugated branch PE-PCL-b-PEC in d6- DMSO. However, the FA-conjugated block copolymer shows two absorption maxima at 280 and 365 nm, confirming the successful conjugation of FA with the block copolymer. An attachment of about 4% (by weight) of FA is confirmed from the absorption intensity of the FA-conjugated block copolymer at λ=362 nm and from the calibration graph obtained with free FA in DMF. Figure 5.7: UV-Vis absorption spectra of folic acid (FA), PE-PCL-b-PEC and PE-PCL- b-PEC-FA. Figure 5.8: HRTEM (a) and AFM phase image (b) of folic acid conjugated polymeric micelle. 5.3.4 Preparation and characterization of FA-conjugated PMs FA-conjugated PMs were prepared as described in the earlier section. Fig. 5.8 shows the HRTEM and AFM images of the PMs. The average size of the PMs is 80 nm. The hydrodynamic size (Fig. 5.9) of blank (98±2 nm) and DOX loaded (111±2 nm) PMs are higher than those observed from the TEM photomicrographs; this may be because the 96 TEM micrographs were captured under dry conditions that may lead the PMs to shrink to a smaller size compared with those obtained by DLS. PE-PCL-b-PEC-FA forms micelles with a water-holding dynamic structure and therefore the dilution stability of the PMs is determined by the CMC. Figure 5.9: DLS histogram of (a) blank and (b) DOX loaded polymeric micelle with standard deviation. The CMCs for these block copolymers were calculated by fluorescence spectrometry using pyrene as a fluorescence probe (Fig. 5.10). All the block copolymers show a CMC of the order of 10-3 mg/mL (Table 5.1). The CMC value shows a slightly increasing trend from sample A to sample D (Table 5.1). This is a result of the increase in the chain length of the hydrophilic component (PEC segment) from sample A to sample D.210 In general, the lower the value of the CMC, the better the dilution stability. Table 5.1 gives the DLC and DLE values determined by UV-Vis spectrometry. The DLC of the block copolymer shows a decreasing trend from sample A (14.7%) to sample D (9.9%). Figure 5.10: Critical micelle concentration (CMC) of sample A by using pyrene as a fluorescence probe. As we have been dealing with a hydrophobic drug (DOX), this decrease in the DLC is attributed to the decrease in the percentage of the hydrophobic segment (PCL about 68% to 43%, i.e. an increase in the hydrophilic PEC segment) of the block copolymer from sample A to sample D. 5.3.5 Cytotoxicity assay To check the biocompatibility of the synthesized PMs, MTT assay was performed against both cancerous (HeLa) and normal (L929) cell lines (Fig. 5.11).

Samples B, D and the DOX loaded sample D did not reach 50% inhibition of growth for the L929 cell line (IC50) even at a concentration of 100 µg/mL. However, free DOX reached 50% inhibition of growth of the L929 cell at a concentration of 3.81 µg/mL. Therefore, samples B, D and the DOX loaded sample D shows very high cell viability (>70%) against L929 cells, even at high concentrations (100 µg/mL), which suggests that they may be suitable for drug delivery systems. The MTT assay of these samples was also investigated for the HeLa cell line. The IC50 values of free DOX and the DOX loaded sample on the HeLa cell line are 3.70 and 14.84 µg/mL, respectively. The similar IC50 values obtained for free DOX in both the L929 and HeLa cell lines indicates the comparable effect on both cell lines. However, the DOX loaded sample D shows a greater cytotoxicity against the HeLa cell line (IC50 = 14.84 µg/mL) compared to that with the L929 cell line. The high cytotoxicity of the FA-conjugated, DOX-loaded PMs towards the HeLa cell line demonstrates their potentially effective performance in cancer treatment. Figure 5.11: MTT assay of free DOX and DOX loaded polymeric micelle on HeLa (a) and L929 (b) cell lines. Figure 5.12: Fluorescence microscopic image of L929 cell treated with DOX loaded polymeric micelle with a scale bar of 40µm. Figure 5.13: Confocal microscopic images of DOX loaded polymeric micelle on HeLa cell at scale bar of 40µm. The nucleus is stain with DAPI. 5.3.6 Cellular uptake The cellular uptake of the DOX loaded PM was studied by confocal laser scanning microscopy for both the L929 and HeLa cell lines. To examine the targeting ability of the PMs, FA-conjugated DOX loaded PMs were incubated with both the HeLa and L929 cell 99 lines. The emergences of the red fluorescence of DOX were monitored as a function of time. In contrast to the L929 cell line (Fig. 5.12), a high level of red fluorescence was seen in the HeLa cell lines (Fig. 5.13) after just 1 h of incubation. The bright fluorescence of the DOX loaded PMs on the HeLa cell line is attributed to the presence of an FA receptor that directs the FA-conjugated PM specifically towards the HeLa cells.196b, 211 The very high cytotoxicity of the DOX loaded PMs against HeLa cells compared with L929 cells (Fig. 5.11) supports these results for cellular uptake. The uptake of DOX- loaded PMs by HeLa cells (Fig. 5.13) leads red fluorescence mainly in the cytoplasm and partly in the nucleus. The targeting ability of FA-conjugated PM against FA- nonconjugated PMs on HeLa cells is shown in Fig. 5.14 and 5.15. The very high intensity of red fluorescence in HeLa cells (Fig. 5.14) also clearly shows the targeting ability of FA. The appearance of red fluorescence after only 1 h of incubation may be attributed to the partial release of DOX in the acidic environment of the HeLa cell, which then diffuses through the endocytic compartment to the nucleus. However, over a longer period of time the intensity of the red fluorescence increases both in the cytoplasm as well as in the nucleus of the cell. This suggests that the uptake of the DOX loaded PMs may follow an endocytic mechanism.113, 118a Figure 5.14: Fluorescence microscopic image of HeLa cell treated with FA conjugated, DOX loaded PM with a scale bar of 40µm. 5.3.7 In vitro DOX release The pH sensitive release of a drug is crucial in the treatment of cancer. The in vitro DOX release of FA conjugated samples B and D was studied at 37 $^{\circ}$C in PBS (pH 7.4) and 100 acetate buffer (pH 5.0) (Fig. 5.16). The amount of DOX released is calculated from the UV-Vis spectrum (λmax = 482 nm) using the calibration graph and release equation as presented in earlier chapter (Eq. 2.1). At pH 7.4, only 12% and 13% of DOX are released within 24 h from the DOX loaded PMs

prepared from samples B and D, respectively. This slow release rate at the physiological pH of the bloodstream may decrease the side effects of the drug. Figure 5.15: Fluorescence microscopic image of HeLa cell treated with FA unconjugated DOX loaded PM with a scale bar of 40μm. Figure 5.16: In vitro release profile of DOX from DOX loaded polymeric micelles, prepared from sample B and sample D at pH of 5.0 and 7.4, respectively. At pH 5.0, however, about 49% and 58% of the DOX was released within 24 h from samples B and D, respectively. The comparatively faster release rate of DOX at the physiological pH (5.0) of cancer cells illustrates the potentially enhanced efficiency of it for cancer treatment. The release rates for DOX shows an increasing trend at low pH (acidic media) compared with high pH (neutral media); this is attributed to two important factors. The first factor is the greater solubility of DOX in acidic media (pH 5.0) that allows the DOX molecule to diffuse from the PM more quickly and efficiently than at the physiological pH (7.4) of the bloodstream.212 The second factor is the hydrogen bonding interaction of the primary amine (–NH2) and the carboxyl (–COOH) groups of the DOX molecule with the carbonyl groups of the PCL segment.113, 213 There is effective hydrogen bonding among these groups at the physiological pH (7.4) of the bloodstream. This hydrogen bonding prohibited the DOX molecule from diffusing through the PM, which is reflected in its release profile (Fig. 5.16). However, at the physiological pH (5.0) of cancer cells, the –NH2 and –COOH groups of the DOX molecule are protonated, which leads to weakening of the hydrogen bonding interactions between DOX and the polymer. This weakening effect allows the DOX molecules to diffuse easily from the PM to the outer medium.118b Samples B and D displayed different release rates at pH 5 than at physiological pH. At pH 5, a slow release rate of sample B compared with sample D can be explained by considering hydrophobic interaction. The PCL content is higher in sample B (about 58%, Table 5.1) compared with sample D (about 43%, Table 5.1). The hydrophobicity of DOX helps to create hydrophobic–hydrophobic interactions that may be responsible for the lower rate of DOX release. 5.4 Conclusions Branched PE-PCL-b-PEC has been successfully synthesized by ring-opening polymerization. FA is attached at its edge by a DCC coupling reaction. The shift of the gel permeation chromatogram towards a lower elution time and the appearance of a glass transition temperature (seen on DSC) at about 22 $^{\circ}$C (the signature of PEC) confirmed the formation of the block copolymer. The NMR signatures also established its chemical structure. The FA coupled block copolymer forms a biocompatible and biodegradable PM in aqueous media. The spherical micelles with an average size of 80 nm were confirmed by HRTEM, DLS and AFM. The low CMC value (4.21×10-3 mg/mL, sample A) of the block copolymer PMs (evaluated by fluorescence spectrometry) suggests their potential application in drug delivery systems. PMs produced from branched polymers can encapsulate DOX molecules and show a maximum DLC of 14.7% (sample A). The decrease in the DLC from sample A to sample D is clearly explained by considering the proportion of the hydrophobic (PCL) part in the different block copolymers. The very high degree of cell viability obtained when using the PMs, as evaluated from an MTT assay, is encouraging for its in vivo use in drug delivery systems. The very high cytotoxic effect of FA-conjugated DOX loaded PM on the HeLa cell line compared with the L929 cell line shows the selective interaction of the PMs with the HeLa cells. The cell uptake study also showed a bright

fluorescence with HeLa cell lines compared with L929 cell lines after an equivalent incubation time. These two results demonstrate the selective targeting ability of FA conjugated PM on the HeLa cell line. A high release rate of DOX from the PMs at the physiological pH (pH 5.0) of cancer cells was successfully explained by taking into consideration the better solubility of DOX in acidic media. The high release rate of DOX from the PMs at the physiological pH of cancer cells is responsible for the rapid treatment of cancer by supplying the required amount of DOX. In summary, the FA conjugated PMs prepared in this part of the works are promising nano-carriers for the targeted delivery of DOX or similar drug molecules to cancer cells. 6.1 Introduction In recent years, suitably decorated nano-carriers prepared from amphiphilic block copolymer present admirable performance for cancer cell-specific drug delivery. The nano-carriers are prepared by self-assembling214 the amphiphilic block copolymer. Inner part is formed by hydrophobic segments and outer part comprises hydrophilic segments of the block copolymer in aqueous environment.215 The inner part (hydrophobic) of the nano-carrier can embrace the drug molecules (mostly hydrophobic) through the favorable hydrophobic interaction.216 The outer part provides stability of the polymeric nano-carrier inside the biological system leading to increase of the circulation time. Hence, difficulty of the hydrophobic drug to deliver through biological system has been resolved by delivering them through polymeric micelle like nano-carriers.217 The spherical or nearly spherical micelles having size in between the range of 10-200 nm were widely applied for such drug delivery.116 These smaller micelles reduced the nonspecific interactions, thereby increased the enhance permeability and retention (EPR) effects at the cancer cells.117b, 218 The performance of such drug loaded polymeric micelle has been upgraded by employing stimuli (as like temperature, redox, ultra sound, magnetic and acidic pH) sensitive moieties for quick release of encapsulated drug molecules. This can also reduce the usual side effects leading to increase of the therapeutic efficiency.118c, 120b-e, 219 Acidic pH is considered to be perfect stimulus for targeted delivery of anticancer drug towards cancer cell.118b, 220 The cancer cells possess elevated acidity (i.e. relative low pH at solid tumor sites/tissues) compare to physiological acidity of normal blood.121 Thus acid sensitive deswelling or collapsing of polymeric micelle can be administrated as acid stimulated release of drug molecules. The pH responsive formation and breaking of co-ordination bond also affords efficient delivery of the anti cancer drug to a low pH therapeutic site.3, 123b A theoretical study on nanoparticles, micelles and vesicles also furnished a pH actuated self-assembly and disassembly of these nano- carriers.221 In a nut shell, acid susceptible polymeric micelle can be prepared mainly by three different ways: (a) attachments of hydrophilic and hydrophobic segments via acid sensitive linkages like, oxime, orthoester, acetal, hydrazone, imine, and cis-acotinyletc; (b) the attachment of drug molecule directly to amphiphilic blocks through these acid sensitive covalent linkage; (c) selective design of amphiphilic block copolymers where hydrophilic segment can able to show both activities together, e.g., acid sensitivity as well as hydrophilicity. The first two strategies are 105 very sophisticate but those require many synthetic steps. In contrary, last one is very simple and a high loading of drug molecule can be achieved effortlessly. The polymeric micelle prepared from pH responsive block copolymer has furnished an effective encapsulation and release of drug

molecules towards cancer cell (low pH). However, for efficient cancer therapy it is desired that the entire drug should be released after reaching to require pathological site. Moreover, all drug loaded polymeric micelle can't reach the required pathological site and thus creates a number of side effects. Effective reduction of side effect can be achieved by covalent attachment of cancer cell targeting moiety (e.g. folic acid, biotin, etc.) with the hydrophilic segment of these amphiphilic block copolymers.222 The existence of folic acid (FA) receptor in cancer cell provides successful trafficking of FA conjugated polymeric micelle to accumulate in cancer cell and to reduce the side effects. There are limited numbers of articles available on branch architecture of polymer to be used for drug delivery.139-140, 192 However, branch polymer has many fascinating advantages as a nano-carrier in terms of the drug loading, carrying and delivering of the drug molecules.101b The wonderful template forming ability favors smaller effective size of hyper branched polymer in a solution.101a Large number of functional group present in branched polymer offers high degree of interactions with the encapsulated drug molecule. Finally, branched polymer should create more porous structure compare to linear one depending on their molecular weight and state of association during preparation of polymeric micelle. This unique characteristic permits the micelles to accommodate high loading of drug molecule compare to its linear analogue. The ideal polymeric micelle for cancer cell targeted drug delivery should have the following characteristics e.g., (a) small size with EPR effect; (b) high drug loading capacity; (c) pH responsive release of drug molecule and (d) effective trafficking of drug loaded polymeric micelle towards cancer cell. The objective of this chapter is to synthesize FA conjugated PE-PCL and PAA based branched amphiphilic block copolymer. The chapter also includes preparation of a robust pH responsive micelle (from the synthesized polymers) for cancer cell targeted drug delivery. Thus, to fabricate such robust polymeric micelles, we synthesized a FA conjugated branched amphiphilic block copolymer. First we synthesized a four armed branched PCL chain by using PE as initiator. By taking this branched polymer as a macro initiator we grew another block, poly acrylic acid (PAA) on it by atom transfer radical polymerization (ATRP). As acrylic acid cannot be polymerized through ATRP reaction, we have first polymerized tert-butyl acrylate through 106 ATRP then hydrolyzed the tert-butyl group through selective hydrolysis. The bromine terminated amphiphilic block copolymer was converted to amine terminated one and then conjugated with FA through esterification reaction. The structure of the FA conjugated block copolymer was then established by 1H NMR spectroscopy. The amount of folic acid conjugated with polymer was determined by UV-Vis spectroscopy. The drug (Doxorubicin; DOX) loaded and blank polymeric micelles were prepared from FA conjugated pH sensitive amphiphilic block copolymer. The critical micelle concentration (CMC) of these branched polymers was determined by fluorescence spectroscopy using pyrene as a fluorescence probe. The morphological study of the polymeric micelles was carried out by HRTEM analysis. The hydrodynamic size and zeta potential of the polymeric micelles were determined by DLS study. The cell viability of these DOX loaded and blank micelles were studied by MTT assay. To investigate the cancer cell targeting cellular uptake, FA conjugated polymeric micelle was incubated with both the normal (L929) and cancer cell line (HeLa) separately and effective cell uptake were monitored after different time

interval using confocal laser scanning microscopy. FACS analysis was performed to analyze the cell cycle. The pH stimulus responsive release of DOX from loaded polymeric micelle was also performed at physiological pH of both normal blood cell (pH 7.4) and cancer cell (pH 5.0). 6.2 Synthesis 6.2.1 Synthesis of four armed PE-PCL-OH (1) Four armed PE-PCL was synthesized by ring opening polymerization of CL.176 The detailed of the synthesis procedure was already discussed in section 3.2.3 of the chapter 3. 6.2.2 Synthesis of 'Br' terminated four armed PE-PCL-Br (2) In order to attach ATRP initiator at the ends of four armed PE-PCL (Mn = 11,300, from GPC), 2 g (1 equiv, 1.77 × 10-4 mol) of PE-PCL was dissolved in 8 mL dry THF with excess amount of Et3N (0.16 mL, 2.17 × 10-3 mol) under N2 atmosphere. The reaction system was cooled from room temperature by placing it into ice bath. Then, 0.11 mL (6 equiv, 1.06 ×10-3 mol) of 2- bromopropionyl bromide was dissolved separately in 2 mL dry THF under N2 atmosphere. The bromide dissolved THF solution was then added drop wise into ice cooled reaction mixture. The cooled condition of the reaction mixture was maintained for 1 h. The reaction mixture was allowed to stir at room temperature for next 24 h. A white precipitate of triethylammonium hydrobromide was formed and subsequently separated out by filtration. THF was removed from 107 the filtrate using rotary evaporator and redissolved in ethyl acetate. Saturated solution of NaHCO3 was used to purify the product and it was extracted with ethyl acetate. The ethyl acetate solution of final product was then dried with MgSO4 and concentrated under vacuum. The vacuum dried product was then characterized by 1H NMR spectrum. 6.2.3 Synthesis of PE-PCL-b-PtBA-Br (3) PE-PCL-b-PtBA-Br was synthesized by ATRP method using four armed PE-PCL-Br as a macro initiator (Scheme 6.1). Required amount (5 equiv.) of CuBr was taken into dry reaction tube shielded with a septum. After continuous purging of N2 for 15min, require amount of PMDETA (5 equiv., dissolved in acetone) was added into the reaction tube through the septum. The macro initiator, PE-PCL-Br (1equiv.) dissolved acetone was added into reaction mixture followed by a blue colored complex formation between CuBr and PMDETA. Then, different equivalent (as shown in Table 6.1) of monomer (tBA) was added into the reaction mixture. The reaction tube was then transferred into preheated oil bath at 55 °C. After completion of the reaction, mixture was diluted with THF and passed through a column filled with neutral alumina to remove CuBr- PMDETA complex. Finally, polymer was precipitated in cooled MeOH and dried under vacuum. The structure of the dry polymer was characterized by 1H NMR spectrum. 6.2.4 Synthesis of PE-PCL-b-PtBA-N3 by end group modification (4) The 'Br' terminated block copolymer, PE-PCL-b-PtBA-Br (1 equiv.) was dissolved with NaN3 (4 equiv) in dry DMF under N2 atmosphere. The reaction was continued for 24 h at room temperature. After completion of reaction, excess amount of brine solution was added into the mixture and the product was extracted with ethyl acetate using a separating funnel. The ethyl acetate solution of the product was dried with MgSO4 and concentrated under vacuum. The azide terminated block copolymer was characterized by FTIR spectroscopy. 6.2.5 Synthesis of PE-PCL-b-PtBA-NH2 from azide terminated block copolymer (5) The azide terminated block copolymer PE-PCL-b-PtBA-N3 (1 equiv.) was dissolved in dry THF with PPh3 (4 equiv.) under N2 atmosphere and stirred at room temperature for 24 h. The azide terminated block copolymer formed a complex with PPh3.

Addition of catalytic amount of water to this complex leads to formation of phosphine oxide and NH2 functionalized block copolymer. The product was extracted from the reaction mixture using ethyl acetate. The ethyl acetate solution of the product was concentrated under reduced pressure and precipitated in cooled hexane. The precipitation procedure was continued for 3-4 times to remove phosphine oxide. Finally, NH2 terminated polymer so obtained was characterized by 1H NMR spectrum. Scheme 6.1: Schematic presentation of end group modified PE-PCL and subsequent polymerization of tBA by ATRP technique. 6.2.6 Attachment of folic acid (FA) onto -NH2 functionalized block copolymer to form PE- PCL-b-PtBA-FA (6) Attachment of folic acid was achieved by slight modification of DCC coupling reaction as reported in literature.209 FA attached block copolymer was synthesized by two steps, e.g.; formation of FA ester following the formation of active FA ester with NHS. In brief, 0.5 g (1.132 × 10-3 mol) of FA was dissolved in 10 mL of DMF with 0.3 mL of Et3N under N2 atmosphere. The temperature of the reaction mixture was cooled down using an ice bath. Then, 5 mL DMF solution containing DCC (0.35 g, 1.69 ×10-3 mol) and NHS (0. 196 g, 1. 70 ×10- 3 mol) were added into the reaction mixture. The reaction was continued for next 24 h under dark at room temperature. The activated FA ester was dried by evaporating DMF and Et3N. Vacuum dried ester was then dissolved in 5 mL DMF with 0.1 mL Et3N under N2 atmosphere. The ammine terminated block copolymer; PE-PCL-b-PtBA-NH2 was dissolved in DMF and added into activated ester solution with required amount of DCC under ice cooled condition. The low temperature condition was maintained for first 1 h and reaction was further continued for next 24 h under dark at room temperature. Finally, reaction mixture was dialyzed using 3.5 kD cellulose membrane against DMF to remove unbound FA acid. Vacuum dried product was then characterized by 1H NMR and UV-Vis spectroscopic techniques. Scheme 6.2: This scheme represents the method of preparation of FA attached block copolymer followed by selective hydrolysis of tBA group to form of pH responsive PE-PCL-b-PAA-FA copolymers. Table 6.1: The composition and property of block the copolymer Sample (kDa) Đ PCL Content (%) Contact Angle (º)b (%) (%) Sample code Mna DLCc DLEd PE-P(CL)99-P(AA)0 11.3 1.20 100.0 PE-P(CL)99-P(AA)38 16.2 1.23 69.7 PE-P(CL)99-P(AA)63 19.4 1.22 58.8 PE-P(CL)99-P(AA)87 22.4 1.22 50.4 PE-P(CL)99-P(AA)120 26.7 1.20 42.3 77 …. …. A 68 25.1 83.6 B 58 13.2 44.0 C 53 9.2 30.6 D 46 6.7 22.3 E a Determined from GPC. b Determined from goniometer. c ,d Determined from equation 4.1 and 4.2. 6.2.7 Hydrolysis of tBA groups of PE-PCL- b-PtBA-FA and formation of PE-PCL- b-PAA- FA (7) The hydrolysis of tert-butyl group from FA attached block copolymer was done selectively using TFA. PE-PCL-b-PtBA-FA was dissolved in dry CHCl3 under N2 atmosphere. At least 10 fold excess (by mol) of TFA compared to tert-butyl groups present in block copolymer was added into the reaction mixture. The reaction mixture was stirred for 24 h at room temperature. After evaporating CHCl3 from the reaction mixture, the dry polymer was redissolved into minimum volume of THF and precipitated using hexane. The vacuum dried product was characterized by 1H NMR spectrum. 6.2.8 Preparation of DOX encapsulated polymeric micelles FA attached block copolymeric micelle was prepared by precipitation method.223 FA conjugated block copolymer (10 mg) was dissolved in 100 mL DMSO. Resulting polymer solution was then slowly added into 10 mL PBS (pH 7.4) solution under sonication. The PBS solution was

sonicated for 1 h to maintain uniform distribution of micelles. The resultant solution was then dialyzed (cut off mol. wt. 3.5 kDa) against 80 mL freshly prepared PBS solution at pH 7.4. The prepared micelles were then characterized by TEM and DLS. In order to prepare DOX encapsulated polymeric micelles, we followed the similar procedure as depicted earlier by dissolving DOX and FA conjugated polymer together.224 The amount (or weight) of DOX loaded into polymeric micelle was then characterized by considering UV-Vis absorption of DOX loaded polymer (by dissolving in DMSO) and by using a calibration curve. In order to determine DOX loading content (DLC) and DOX loading efficiency (DLE), we used the following equations 4.1 and 4.2. 6.3 Results and discussion 6.3.1 Synthesis of four armed PE-PCL-OH The branched PCL was synthesized according to the procedure mentioned in the chapter 3. The chemical structure of the branched polymer is established by 1H NMR spectrum as shown in Fig. 5.1 in chapter 5. 6.3.2 Synthesis of 'Br' terminated four armed PE-PCL-Br The aim of this work is to prepare a pH responsive polymer. Thus we plan to introduce tBA block in the synthesized block copolymer through ATRP reaction. In ATRP reaction, the branched PCL is used as a macro initiator. Thus, functionalization of end hydroxyl with 2- bromopropionyl bromide (BPB) was done according to the procedure described in the earlier section. The resulting bromine terminated branched initiator displays a number of resonances signal at 4 ppm (t, 2H), 2.3 ppm (t, 2H); 1. 6 ppm (m, 4H) and 1. 3 ppm (m, 2H) which are assigned to backbone of the initiator (Fig. 6.1). Interestingly, resonance signal that is expected to 111 be appeared at 3.6 ppm (t, 2H) (in Fig. 5.1) is completely disappeared in Fig. 6.1. This is attributed to the functionalization of hydroxyl group of PE-PCL with BPB. Appearance of two additional resonating signals at 4.4 ppm (q, 1H) and at 1.7 ppm (d, 3H) corresponding to methyne proton (-OOC-CH(CH3)Br, 5) and methyl proton (-OOC-CH(CH3)Br, 6) of the BPB, respectively further confirms the successful functionalization of BPB with terminal hydroxyl group. 6.3.3 Synthesis of PE-PCL-b-PtBA-Br The PE-PCL-b-PtBA was synthesized by ATRP.225 The structure of the branched block copolymer is recognized by 1H NMR spectrum (Fig. 6.2). The NMR spectrum shows a number of signals at 4 ppm (t, 2H) 2.3 ppm (t, 2H), 1. 6 ppm (m, 4H) and 1. 3 ppm, (m, 2H). The emergence of these above mentioned signals are assigned to the macro initiator (i.e. PE-PCL). The appearance of resonating signals at 4.4 ppm (t, 1H) is attributed to the terminal methyne proton (-CH2-CHBr-COOtBu, 5) of the block copolymer. The resonance signal corresponding to tBu proton (6) of tBA is appeared along with initiator proton (3) at 1.3 ppm. The increase in integrated area corresponding to the signal at 1.3 ppm (compare to the integrated area of the same signal of PE-PCL in Fig. 6.1) is the indication for the formation of block copolymer. The formation of block copolymer is again confirmed by GPC trace (Fig. 6.3). The shifting of GPC trace (Fig. 6.3) for sample D and E towards lower elution time compare to sample A is the manifestation of increase of molecular weight i.e. formation of the block copolymer. Figure 6.1: 1H NMR spectrum of PE-PCL -Br in CDCl3. Figure 6. 2: 1H NMR spectrum of PE-PCL- b-PtBA-Br in CdCl3. 6. 3.4 Synthesis of PE-PCL-b-PtBA-NH2 For synthesizing ammine (-NH2) terminated block copolymer from the bromine (Br) terminated one, we followed the reduction procedure.226 According to the procedure, we first substituted the bromine atoms by azide group (-N3). Finally, azide terminated polymer was reduced to amine using PPh3 and

water. The formation of azide terminated polymer was confirmed by FTIR spectrum (Fig. 6.4). The FTIR spectra of PE-PCL-b-PtBA-Br and its azide terminated form displays no characteristic stretching signal of azide group. But after substituting by sodium azide, it displays a characteristic signature for azide group at 2112 cm-1 which confirmed the successful replacement of bromine with azide group. The 1H NMR spectrum of ammine terminated polymer is displayed in Fig. 6.5. The spectrum displays a signal at 5 ppm (in the inset window) which corresponds to terminal amine protons (-CH-NH2, 7). It also shows additional signals at 4 ppm, 2.3 ppm, 1.6 ppm and 1.3 ppm which are assigned to the main chain protons of PE-PCL-b-PtBA- NH2. 6.3.5 Synthesis of FA attached block copolymer (PE-PCL-b-PtBA-FA) For targeted delivery of drug towards cancer cell, we have decorated the polymer with FA. It has already been reported that tumor cells having folate receptor can easily be targeted by FA.222a Naturally, FA decorated polymer is expected to show higher tendency for selective movement towards tumor cell. Additionally, attachment of FA to the end of polymers or polymeric micelles leads to increase of its biocompatibility as well as circulation time inside the biological environment. 227c Figure 6.3: GPC trace of sample A, D and E Figure 6.4: FTIR spectra of bromine and azide terminated block copolymer. The attachment of FA was accomplished by DCC coupling according to procedure described in the earlier section. Among two (α and γ) -COOH groups, conjugation of FA was shown to occur through 'γ' functionality which leads to retention of its targeting skill towards cancer cell142a The FA attached block copolymer, PE-PCL-b-PtBA-FA displays (Fig. 6.6) a number of resonance signal in its 1H NMR spectrum. The signals those appear at 4 ppm, 2.3 ppm, 1.6 ppm and 1.3 ppm are assigned to the main chain protons as assigned earlier. Another bunch of signals, which are also appeared at the aromatic region i.e. 6-9 ppm are attributed to the FA.207b, 209 The amount of FA attached is about 6.4% (in weight) that was calculated from the intensity of absorption (UV-Vis) of FA attached copolymer and from the calibration curve of free FA in DMF. (Fig. 6.7) Figure 6.5: 1H NMR spectrum of PE-PCL-b-PtBA-NH2 in CDCl3. 6.3.6 Hydrolysis of tBu groups from PE-PCL-b-PtBA-FA and formation of PE-PCL-b- PAA-FA FA attached block copolymer having tBu group at one of its segment (structure 6 in Scheme 6.2) was selectively hydrolyzed by TFA. TFA selectively removed the tBu group not the FA. This is because of the fact that the FA is connected to the polymer through strong amide bond (-CONH- FA). But tBu is connected via comparatively weak ester (-COO-) bond. Hence, TFA selectively breaks the tBu ester bond and removes tBuOH so formed not the FA. The hydrolysis results '- COOH' segmented block copolymer along with the PCL segment which serve the purpose of pH responsiveness. The 1H NMR spectrum of hydrolyzed block copolymer is displayed in Fig. 6.6. Interestingly, the Fig. 6.6 shows a decrease in integrated area (compare to the Fig. 6.5) corresponding to the signal at 1.3 ppm. The decrease in integrated area corresponding to the signal at 1.3 ppm is the indication of detachment of tBu group by TFA. Figure 6.6: 1H NMR spectrum of PE-PCL-b-PAA-FA in d6-DMSO. Figure 6.7: UV-Vis spectra of FA, polymer and FA conjugated polymer. 6.3.7 Water contact angles of the block copolymers In order to check the extent of hydrophilicity, the contact angle (Fig. 6.8) of all synthesized homo and block copolymers were measured using water as a probe liquid. Table 6.1 displays a decreasing trend of contact angle from sample A (77°) to sample E (46°). The decrease in contact angle for

all block copolymers (sample B to E) compared to homo polymer (sample A, 77˘) is attributed to the incorporation of hydrophilic segment (PAA). Though the length of hydrophobic segments for all synthesized polymer are same however, contact angles decrease with incorporation of hydrophilic PAA. Thus, the resulting block copolymers contain both hydrophilic and hydrophobic components together (i.e. amphiphilic in nature) that can be served as promising component for the preparation of stable polymeric micelles. 6.3.8 Characterization of FA conjugated polymeric micelles The synthesized amphiphilic block copolymers form micelle by self-assembly when slowly added into PBS (pH = 7.4) solution as described in earlier section. The HRTEM photomicrograph of polymeric micelle (Fig. 6.9) demonstrates its nearly spherical shape with the average diameter ranging from 40 to 60 nm. Fig. 6.9a and b represent the HRTEM photomicrographs of polymeric micelles prepared from sample C and sample E (Table 6.1), respectively. TEM photomicrographs display nearly spherical micelles having average sizes of 40 nm and 55 nm for sample C and sample E, respectively. But the sizes observed from DLS size profile (with Gaussian fitted curve) are 49 and 70 nm (Fig. 6.9c, d) for sample C and sample E, respectively. The result shows that the size ranges obtained by analyzing TEM photomicrographs are definitely smaller than those obtained by DLS (Table 6.2). This can easily be explained by considering the physical state of analyzing the samples .116, 207 Figure 6.8: The water contact angles of Sample A (770), B (680), C (580), D (530) and E (460) Table 6.2: Different sizes of polymeric micelle and their characteristics Samples CMCa (mg/mL)×10-3 Particle size(nm)b Zeta potential (mV)b a Determinted from fluorescence spectroscopy; bDetermined from DLS Sample B Sample C Sample D Sample E 1. 10 1.14 1.24 2.71 39 49 51 70 -28.20 -33.60 -36.02 -44.40 6.3.9. CMC of polymeric micelles The formation of polymeric micelle was verified by conducting fluorescence spectroscopic analysis (Fig. 6.10) using pyrene as a fluorescence probe.228 The CMC obtained from fluorescence spectroscopy shows increasing trends (Table 6.2) from sample B (1.10 × 10-3 mg/mL) to sample E (2.71 × 10-3 mg/mL). The increase in CMC value from sample B to E is clearly recognized by taking into consideration the composition of synthesized block copolymers. The length of the hydrophobic segments (PE-PCL) for all synthesized block copolymers is same (repeating units: 99; Table 6.1). But the length of hydrophilic segment is gradually increased from sample B (number of repeating unit: 38) to sample E (repeating unit 120; Table 6.1). The increase in hydrophilic chain length results in higher concentration of the block copolymers required to form a PM (i.e. increase in CMC). The zeta potential value (-28.0 to -44.2 mV) of the PM implies its desired colloidal stability in biological systems. Figure 6.9: HRTEM photomicrographs of polymeric micelle (a, b) and their corresponding DLS size profile (c, d) for sample C and sample E (from table 6.1), respectively. 6.3.10. pH sensitivity of polymeric micelles The pH sensitivity of amphiphilic polymeric micelles was checked by DLS study as mentioned in the chapter 2. The variation of hydrodynamic size of the polymeric micelles with varying pH is displayed in Fig. 6.11b (raw data is presented in Fig. 6.11a). The decrease in micelle size with decreasing pH from 7.0 to 3.0 is the manifestation for acid mediated deswelling of the amphiphilic block copolymeric micelle. The acid sensitive deswelling of PM can provide a promising system towards cancer selective drug delivery. Figure 6.10: The fluorescence spectroscopy (a) of pyrene

loaded polymeric micelle with a concentration range of 10-3 to 0.1mg/mL. (b) Evaluation of CMC curve of polymeric micelles prepared from sample D (table 6.1). Figure 6.11: Variation of micelle size with pH for sample E (table 6.1) represented in DLS histogram format (a) and linear format with pH of the solution (b). 6.3.11. DLC and DLE of polymeric micelles The drug loaded block copolymeric micelles were prepared accordingly as elaborated in the earlier section. The amount of DOX (means drug) loaded into the polymeric micelle i.e. DLC and DLE were calculated from Eq. 4.1 and 4.2 and displayed in Table 6.1. The Table 6.1 exhibits the fact that a very high degree of DOX loading (25% for sample B) using the polymeric micelle could be achieved compared to literature reported figures.113, 116, 121 The branch structured block copolymer used for the preparation of PM is the responsible factor for such a high DLC value. During assembling in water, the branch polymers may create large volume of vacant space (compare to linear polymeric analogue) inside the polymeric micelle. These vacant spaces are filled by DOX molecules in the DOX loaded PM. Additionally, the amount of hydrophobic segment (PE-PCL) present in branched polymer is also a controlling factor for high loading content of hydrophobic DOX molecules. The DLC of the block copolymer diminishes from sample B (25%) (Table 6.1) to sample E (6.7%). The decrease in percentage of PE-PCL content from 70% (sample B) to 42% (sample E, Table 6.1) is the responsible factor for this trend in DLC. The encapsulated DOX molecule are holded inside the polymeric micelle by both hydrophobic as well as hydrogen bonding interactions among ammine (-NH2) and acid (-COOH) groups of DOX with acid and ester (-COO-) groups of the block copolymer. The red shifting of absorption wavelength compare to free DOX (Fig. 6.12) is the confirmation for hydrogen bonding interaction between DOX and polymer in DOX loaded polymeric micelle. Figure 6.12: UV-Vis absorbance of free DOX and DOX loaded polymeric micelle. 6.3.12. In vitro cytotoxicity The in vitro cytotoxicity (Fig. 6.13) of the FA conjugated polymeric micelle and DOX loaded polymeric micelles were analyzed by MTT assay on both normal (HaCaT) and cancer (HeLa) cell lines. All the micelles show cell viability above 80% up to a concentration 100 µg/ mL on HaCaT cells that reveals its secured implication in drug delivery. The required doses of free DOX and DOX loaded polymeric micelle for 50% inhibition of HeLa cell growth (IC50) are about 4.28 and 21.24 µg/mL, respectively. Thus for each equivalent dose, free DOX exhibits more cytotoxicity effect compare to DOX loaded polymeric micelle. The time consuming release of DOX from loaded micelles is the responsible factor for its lower cytotoxic effect. The cytotoxic effect of free DOX on HaCaT cell is higher comparing to HeLa cell line as shown in Fig. 6.13. The low proliferation of HaCaT cell compare to HeLa, expedites the free DOX to show more cytotoxic effect on it. In contrary to free DOX, the DOX loaded PM shows low cytotoxic effect on HaCaT cell compare to HeLa, The high cytotoxicity of FA conjugated, DOX loaded PM on HeLa is attributed to its increased uptake on folic receptor positive (+FR) HeLa cell compare to -FR HaCaT cells. Additionally, low pH environment of HeLa cell is also affording the rapid release of bound DOX molecules and showing higher level of cytotoxicity. 6.3.13 Microscopic image The cellular uptake of free DOX and DOX loaded polymeric micelle were studied on HeLa cell line. As DOX itself gives red fluorescence so, only DOX conjugation is enough for studying the cellular uptake. Both the free DOX and DOX loaded polymeric micelle were incubated separately with HeLa cell line. The

fluorescence microscopic image of HeLa cell with DAPI (blue) stained nucleus is displays in Fig. 6.14. Figure 6.13: The cytotoxicity assay of free DOX, DOX loaded micelle and blank micelle on both HeLa (a) and HaCaT (b) cell line. After 1 h of incubation, free DOX shows a very strong fluorescence on nucleus (Fig. 6.15). This result indicates that DOX molecules are uptaken by the cell through passive diffusion mechanism.229 But at the same time, DOX loaded PM shows fluorescence mainly in cytoplasm and very weak fluorescence in nucleus. This result indicates that DOX loaded micelles may be internalized in the cell through endocytic mechanism. With increasing time (from 1 to 4 h) the intensity of the fluorescence gets intensified in cytoplasm. Simultaneously, the acidic environments of HeLa cell stimulate the DOX loaded micelle to release the DOX molecules. The released free DOX are diffused through endocytic compartment to nucleus113, 123a The fluorescence microscopic image displays the effective incorporation of DOX in cytoplasm as well as in nucleus after 4 h of incubation with HeLa cell. 6.3.14. Cell cycle analysis The cell cycle analysis was conducted to evaluate a quantitative analysis of DOX loaded PM mediated cell death using standard PI staining flow cytometry. Many literatures suggest that the DOX prevents cancer cell growth by inducing DNA breakage as well as by cell cycle arrest at G2/M phase. In this study we have attempted to understand the cause of the growth inhibition. Figure 6.14: Fluorescence microscopic image (with a scale bar of 20μm) of DOX loaded polymeric micelle treated HeLa cell. The nuclei are stained by DAPI Cell cycle analysis demonstrated that G2/M phase increased in DOX loaded PM treated cells with respect to the control. Here, the G0/G1 phase decreased from 63.4% in control to 22.4% in sample E and 15.1% in sample D treated groups, respectively. The G2/M phase has increased from 17.7% in control to 55.6% in sample E and 60.0% in sample D treated groups (Fig. 6.16). There was no significant change in 'S' phase. The result suggested that DOX loaded PM retards the growth of HeLa cells by arresting the cell cycle progression at G2/M phase and thereby inhibiting the cell growth. 6.3.15. In vitro drug release study In vitro DOX release from DOX loaded polymeric micelles was carried out through dialysis. In order to investigate the cancer cell specific (pH 5.2) drug release compare to normal cell (pH 7.4), we have carried out the release study in two buffer solution having pH 5.2 and pH 7.4, respectively. The release profile of loaded PM is displayed Fig. 6.17. After 24 h of study, about 68% of DOX was released at pH 5.2 for sample E. In contrary, only about 12.9% of DOX was released at pH 7.4 for the same sample after the same duration of time. Similar trend was also observed for the sample C i.e. 60.3% of DOX releases at pH 5 and 8.5% releases at pH 7.4. Figure 6.15: Fluorescence microscopic image of HeLa cell treated with free DOX at a scale bar of 20μm Figure 6.16: Flow cytometric analysis of cell cycle phase distributions of HeLa cells treated with DOX loaded sample D and sample E. The cell cycle analysis was performed for 24h using propidium iodide. The pH responsive release of DOX molecules can be explained by considering two major parameters namely, (a) hydrogen bonding interaction and (b) higher solubility of DOX in acidic medium.113 The hydrogen bonding interaction between DOX and polymer was supposed to be stronger at pH 7.4 and that becomes weaker at pH 5.2.The weakening of hydrogen bond is attributed to protonation of acid and ammine groups of the DOX molecule. Additionally, the higher solubility of DOX in acidic medium may also be the cause of high release rate at pH 5.2. At pH

5.2, high release rate of drug from PM prepared from sample E compared to sample C can be recognized by considering the presence of acid sensitive PAA segment and lower Tm of the synthesized block copolymer. The higher block length of PAA in sample E (PE-(PCL)99- (PAA)120) compare to sample C (PE-(PCL)99-(PAA)63) leads to higher extent of deswelling as well as high release rate in acidic medium. The possible mechanism of deswelling of four armed polymeric micelle is displays in Fig. 6.18. Again, very low melting temperature (Fig. 6.19: Tm = 38 °C, very near to body temperature) of the sample E compare to sample C (Tm =46 °C, Fig. 6.19) support its high rate of DOX release. Low release rate at pH 7.4 (physiological pH of normal cell) and high release rate at pH 5.2 (physiological pH of cancer cell) accomplish polymeric micelle as a promising material for cancer cell targeted DOX release. Figure 6.17: In vitro DOX releases profile of sample C and sample E in two different buffer solution having pH 5.2 and pH 7.4. Figure 6.18: Schematic presentation of in vitro release of DOX molecule form branched polymeric micelle. Figure 6.19: DSC thermogram of homo and block copolymers 6.4 Conclusions In summary, FA conjugated, pH responsive polymeric micelles were successfully synthesized from branched amphiphilic block copolymer, PE-PCL-b-PAA-FA. Structure of the FA conjugated branched polymer was successfully established by 1H NMR spectrum. UV-Vis absorption spectra reveal that about 6.4% of FA is conjugated with the branched polymer. HRTEM photomicrographs of polymeric micelles, prepared from the branched amphiphilic block copolymer display nearly spherical micelle with an average size of 40-60 nm. The CMC of the block copolymers are in the range of ~0.63 ×10-2 to ~2.01 ×10-2 mg/mL. This low range of CMC of the block copolymer assures it from breaking after large dilution in biological system that leads to its beneficial implication in drug delivery. The DOX loading content of these polymeric micelles is appreciably high (about 25.1%) compare to literature reported polymer micelle prepared from linear amphiphilic polymer analogues. The high DOX loading content may be attributed to branched structure of the micelle forming polymer that accomplish a massive extent of hydrogen bonding interaction between itself and the DOX molecules. The red shifting of UV-Vis absorption maxima of DOX loaded micelle is the signature of polymer-DOX interactions. Polymeric micelles are showing biocompatibility about 80% even at higher concentration but DOX loaded polymeric micelle exhibits very low biocompatibility at the same concentration levels. Selective trafficking of FA leads to enhanced uptake of DOX loaded polymeric micelle as well as their enhanced cytotoxicity against HeLa cell. FACS analysis of DOX loaded PM mediated HeLa provides efficient inhibition of cell growth by arresting the cell cycle in G2/M phase. The acid responsive deswelling of PAA provides significant decrease in miceller size from the pH 7.4 to pH 3. The deswelling of drug loaded micelle, weakening of hydrogen bonding interactions and high solubility of DOX at physiological pH (5.0) of cancer cell furnish a faster release of DOX (i.e. 68% after 24 h). 7 7.1 Introduction In the last decades, delivery of anticancer drug through nano-carrier safely to the cancer cell has received much attention. Different types of nano-carriers e.g. micelle,112 vesicle,113 inorganic or organic nanoparticles,114, 230 polymer modified inorganic nanoparticles40, 115, 231 have been employed for such drug delivery. Among the different nano-carriers, micelles prepared from amphiphilic block copolymers have been extensively exploited for drug delivery.118 On demand release (i.e

externally controlled release) of drug is only possible if drug loaded micelles are sensitive to the external stimuli like sound,132 light,223b magnetic field,233 temperature120c, 234 etc. From application point of view, temperature is one of the simplest external stimuli for drug release. Thermo-responsive PMs are mainly formed by a block copolymer having one hydrophobic block and another thermo-responsive block which is hydrophilic below LCST and turn out to be hydrophobic just above the LCST.235 Thermo-responsive PMs start to collapse just above the LCST.116 The collapsing of the micelle structure promotes the release of loaded drug molecules. A PM prepared from block copolymer poly(γ-2-[2-(2-methoxyethoxy)ethoxy]ethoxy- CL)-b-poly(γ-octyloxy-CL), having LCST 38 ºC affords secure delivery and temperature responsive release of Nile red and DOX.116 The synthesis of thermo- responsive micelle by poly(NIPAM-co-3-(trimethoxysilyl)propylmethacrylate)-b- poly(2-(diethylamino) ethyl methacrylate) block co-polymer has been performed by Chang et al. and these micelles were subsequently used for thermo-responsive release of DOX.128 Self-assembling of diblock copolymer poly(NIPAM)-b-2-(dimethylamino)ethyl acrylate, below their LCST leads to the formation of PMs.130 This micelle favors the fruitful delivery and release of oligo-DNA by self-catalyzed degradation. A series of thermoresponsive water soluble block copolymer poly(ethyleneoxide)-b-trans-N-(2-ethoxy-1,3-dioxan-5-yl) acrylamide (tNEA) has been synthesized by Qiao et al.134 The synthesized block copolymer registers the LCST close to the human body temperature. Thus the formation of PM followed by loading of hydrophobic drug (DOX) at body temperature leads to safe delivery and beneficial therapeutic efficiency. But the delivery of entire drug molecules to the exact pathological site (targeted site) would increase the therapeutic efficiency by reducing the undesired side effect. The attachment of cancer cell targeting moiety (folic acid, biotin, etc.) onto the surface of stimuli responsive PM helps in targeting to 129 Chapter 7 the cancerous cells. The presences of FA receptor, provides efficient trafficking of FA decorated PM to the cancer cell.131 The targeted delivery approach enables carrying most of the drug molecules to the cancer cell and there by reduces the side effects. The shape and characteristics of PM largely depend on architecture of the base polymer. The impressive properties of nonlinear polymers towards solution and in self assembled morphologies promote their application for drug delivery. Nonlinear, branched or hyperbranched polymer have many advantages like wonderful template forming ability, effective solution property and provision of incorporating large number of functional groups leading to improved polymer-drug interaction.101a The entanglement free self-assembly of the branched (i.e. nonlinear) polymer affords micelle higher drug loading as well as its releasing efficiency.101b The goal of this chapter is the preparation of thermoresponsive polymeric micelle for cancer cell targeted thermoresponsive delivery of drug molecules. Thus to construct such a promising micelle having the potential of stimuli-responsive delivery of sufficient amount of anticancer drug, we have synthesized two types of FA conjugate thermo-responsive branched PE-PCL-b-PNIPAM-FA and PE-PCL-b-PNVCL-FA copolymers, respectively. The chemical structure as well as FA conjugation with the synthesized polymers was established by 1H NMR spectrum. FA conjugated polymers was then allowed to form PMs in aqueous medium. The CMCs of the block copolymer were evaluated by fluorescence spectroscopy using pyrene as a

fluorescent probe. The bulk morphology and hydrodynamic diameter of the prepared micelles were studied by HRTEM and DLS. The colloidal stability of the PM was studied by measuring zeta potential. The LCST of each composition of polymer were determined by temperature dependent UV-Vis spectroscopy. The PM was then subjected to loading of DOX. DOX loaded PMs were then incubated with both normal (HaCaT) and cancer (HeLa) cell in order to check the cell viability. The cancer cell specific uptake of the DOX loaded PM was studied by fluorescence microscopy. Cellular interaction of PM was also studied by FACS analysis. The thermoresponsive release of DOX from loaded PMs was studied at above and below the LCST. The in vivo administration of the DOX loaded PM into the sarcoma 180 mice tumor model and the growth of tumor volume with time was studied meticulously. The PM mediated accumulation of DOX into the tumor tissue was also examined by fluorescence microscopy. 130 7.2 Synthesis 7.2.1 Synthesis of four armed PE-PCL Four armed PE-PCL was synthesized by ring opening polymerization of CL. The detailed of the synthesis procedure was described in 3.2.3 section of the chapter 3. 7.2.2 Synthesis of PE-PCL-Br from PE-PCL The synthetic procedure for the preparation of the 'Br' terminated macro initiator is the same as discussed in 6.2.2 section of the chapter 6. 7.2.3 Synthesis of PE-PCL-b-PNIPAM-Br and PE-PCL-b-PNVCL-Br The growth of NIPM or NVCL blocks on bromine terminated PE-PCL was performed by ATRP technique (Scheme 1). Initially, 0.027 g (5 equiv, 1.88×10-4 mol) of copper bromide with magnetic bar was taken in a reaction tube shielded with silicon septum and allowed to purge N2 gas for 15 -20 minutes. After the allowed time, 0.033 g (~5equiv, 1.90×10-4 mol) of PMDETA was taken in to the reaction tube under N2 atmosphere. After the formation of blue color CuBr-ligand complex, 0.5 g (1 equiv, 4.42×10-5 mol) of macro initiator solution in 1 mL of 1,4 dioxane was injected into the reaction mixture. Afterwards, with respect to the macro initiator different molar equivalent of monomer was dissolved in 1,4 dioxane followed by injection into the reaction mixture. The reaction tube was then placed into a pre-heated oil bath at 70 °C. After completion of the reaction, mixture was diluted with 1,4 dioxane and passed through a column encompassed with neutral alumina. 7.2.4 Modification of '-Br' terminated block copolymer to '-N3' terminated one (PE-PCL-b-PNIPAM-N3and PE-PCL-b-PNVCL-N3) The terminal 'Br' atom of the block copolymer was replaced following SN2 reaction using NaN3. In brief, bromine terminated polymers (1 equiv.) was dissolved in dry DMF followed by addition of NaN3 (5 equiv.). The reaction was continued at room temperature for 24 h under N2 atmosphere. In order to separate out the unreacted NaN3 from the reaction mixture, brine solution was added into it. The azide terminated polymer was then extracted with ethyl acetate and concentrated using rotary evaporator. The vacuum dried product was then characterized by FTIR spectroscopy. 7.2.5 The modification of '-N3' terminated block copolymer to '-NH2' terminated one (PE-PCL-b-PNIPAM-NH2 and PE-PCL-b-PNVCL-NH2) The azide terminated block copolymer (1 equiv.) was dissolved in dry THF under N2 atmosphere. Then, required amount of P(Ph3)3 (4 equiv.) was added into it. The reaction was continued overnight at room temperature. The added P(Ph3)3 forms a complex with the azide. After the addition of catalytic amount of water, complex was dissociated into -NH2 terminated block copolymer and phosphine oxide. The amine terminated block copolymer was then extracted from the reaction mixture by using ethyl acetate. The ethyl acetate solution was

dried with MgSO4 and concentrated in a rotary evaporator. The concentrated solution was then precipitated in cooled hexane. The precipitated product was then dried under vacuum at 60 ºC. The vacuum dried product was then characterized by NMR spectroscopy. Scheme 7.1: Synthesis of FA conjugated thermoresponsive block copolymers (PE- PCL-b-PNIPAM-FA and PE-PCL-b-PNVCL-FA) 7.2.6 Conjugation of folic acid (FA) with '-NH2' terminated block copolymer (PE-PCL-b-PNIPAM-FA or PE-PCL-b-PNVCL-FA) The conjugation of FA with '–NH2' terminated polymer was done by DCC coupling reaction.236 The conjugation was achieved in two step procedure, i.e., formation of activated FA ester with N-hydroxysuccinimide (NHS) followed by conjugation with '–NH2' terminated polymer. In brief, 0.5 g (1 equiv., 1.13×10-3 mol) of FA was taken in a reaction tube with 0.22 g (2 equiv., 2.66×10-3 mol) of Et3N and 2 mL anhydrous 132 DMSO. A solution of dry DMSO (3 mL) with 0.26 g of NHS and 0.233 g of DCC was added into the reaction mixture. The reaction was continued at room temperature for 24 h in dark condition. The residue, DCU so far it formed was separated out by filtration. The DMSO and Et3N were evaporated out under vacuum. The vacuum dried FA-NHS (5 equiv.) was dissolved in anhydrous DMSO (2 mL) with Et3N and required amount (1 equiv.) of'-NH2' terminated polymer. The reaction was continued overnight at room temperature. The '-NH2' terminated polymer substitutes the NHS groups followed by forming conjugation with FA. In order to remove the unreacted FA, reaction mixture was dialyzed (3.5 kD cut-off mol. wt.) against fresh DMSO for 24h. Vacuum dried product was then characterized by 1H NMR and UV-Vis spectrum. Table 7.1: The composition of synthesized block copolymers with their characteristic properties Sample Code Polymer (kDa) LCST (°C) Contact Angle (0)b (%) (%) Mna Đa DLCc DLEd A1 PE-P(CL)99-P(NIPAM)35 15.3 1.2 30.0 72 15.8 50.5 Group A2 PE-P(CL)99-P(NIPAM)53 17.3 1.3 31.0 67 16.2 51.8 A A3 PE-P(CL)99-P(NIPAM)91 21.6 1.1 32.0 65 18.1 57.9 A4 PE-P(CL)99-P(NIPAM)98 22.5 1.1 32.0 58 20.3 64.9 B1 PE-P(CL)99-P(NVCL)35 16.2 1.3 38.0 66 16.8 53.7 Group B2 PE-P(CL)99-P(NVCL)57 19.3 1.3 38.5 62 19.5 62.4 B B3 PE-P(CL)99-P(NVCL)83 22.9 1.2 39.0 52 23.2 74.2 B4 PE-P(CL)99-P(NVCL)98 24.9 1.2 39.0 46 24.3 77.7 aDetermined f rom GPC.bDetermined from goniometer using water.c ,d Determined from equation 4.1 and 4.2. 7.2.7 Preparation of DOX encapsulated polymeric micelle The synthesized FA conjugated block copolymer (4 mg) was dissolved in 50 µL of DMSO solution. The DMSO was mixed well with DOX at a concentration level of 25 mg/ mL. The solution of FA conjugated polymer with DOX was sonicated for half an hour in a sonicator bath. The solution was then slowly poured into 4 mL PBS (pH 7.4) under sonication. The colloidal solution was then subjected to dialysis against fresh PBS (pH 7.4) to remove the free DOX and DMSO. The blank PM was also prepared by following the same procedure depicted before. The size, size distribution and morphology of the prepared PMs were characterized by DLS and HRTEM analysis, respectively. The amount of DOX encapsulated into the PM was determined by placing the value of the UV-Vis absorption of DOX encapsulated PM into the pre- 133 evaluated calibration curve. The drug loading content (DLC) and drug loading efficiency (DLE) were determined by Eq. 4.1 and 4.2, respectively. 7 .3 Results and discussion 7 .3.1 Synthesis of four armed PE-PCL-OH The establishment of the four armed structure of the synthesized polymer was discussed in the section 5.3.1 of the chapter 5. 7.3.2 Synthesis of four armed bromine terminated PE-PCL-Br The structure

of the 'Br' terminated macro initiator was already discussed and proved in the section 6.3.2 of the chapter 6. 7.3.3 Synthesis of PE-PCL-b-PNIPAM-Br and PE-PCL-b-PNVCL-Br In order to prepare the thermoresponsive PMs, a series of thermoresponsive block copolymers (table 7.1) were synthesized by growing segments out of the thermoresponsive monomers on bromine terminated macro initiator. A pair of thermoresponsive monomers, NIPAM and NVCL was polymerized from bromine terminated macro initiator exploiting ATRP technique as described in the experimental section. The synthesized block copolymers show a number of resonance signals corresponding to their structural protons. 1H NMR spectrum (Fig. 7.1) of the PE-PCL-b-PNIPAM-Br displays two triplets at 4.0 and 2.3 ppm symbolized by the number 1 and 2. These signals are assigned to methylene proton of the PE-PCL backbone. Two multiplets at 1.6 and 1.3 ppm are attributed to proton signals of PE- PCL and NIPAM parts of backbone symbolized by the number 3 and 4, respectively. The appearance of a signal at 1.1 ppm (5) is the signature of methyl proton of the NIPAM. A weak signal appears at around 4.4 ppm (6) is attributed to the methyne proton of the end group (-CH-Br). Thus from the 1H NMR spectrum, the formation of PE-PCL-b-PNIPAM-Br is confirmed. The shifting of GPC trace (Fig. 7.2) towards lower elution time for sample A2 and A4 compare to the macro initiator PE-PCL also signifies the formation of block copolymer. The growth of hydrophilic block (NIPAM) on hydrophobic initiator (PE-PCL) leads to the reduction of water contact angle from 77° (PE-PCL) to 58° (A4) (table 7.1 Fig. 7.3). The reduction of water contact angle further supports the genesis of block copolymer. The formation of another block copolymer PE-PCL-b-PNVCL-Br is also confirmed by 1H NMR spectrum. The spectrum (Fig. 7.4) displays two triplets at 4.0 (1) and 2.3 (2) ppm for the backbone of the macro initiator (PE-PCL). The spectrum also shows two multiplets at 1.6 (3) and 1.3 (4) ppm which are attributed to the methylene proton of the macro initiator. The signals for NVCL are appeared at 1.6 (-CH2-N-, 7) and 1.3 (- CH2-CH2-N-, 8) ppm along with the methylene proton of the macro initiator. Additionally, another two resonance signals (displayed in inset window) at 3.7 (-CH- N-, 5) and 4.3 (-CH(Br)-N-, 6) ppm are attributed to the main chain and terminal methyne proton, respectively of the NVCL. Again, shifting of GPC trace (Fig. 7.2) of the synthesized block copolymer (B4) towards lower elution time and decrease of water contact angle (table 7.1) from 77° (PE-PCL, Fig. 7.3) to 46° (B4) are further witnesses of the formation of block copolymer. Figure 7.1:1H NMR of PE-PCL-b-PNIPAM-Br in d6 DMSO. 7.3.4 Modification of '-Br' terminated block copolymers to '-NH2' through azide termination The bromine terminated block copolymer is substituted to ammine terminated one through azide modification. The intermediate, azide terminated copolymer was characterized by FTIR spectrum. The replacement of terminal bromine atom by NaN3 is confirmed by appearance of new band at 2114 cm-1 (Fig. 6.4). The azide terminated polymer was then reduced to ammine terminated one by triphenylphophine with water. 1H NMR of the amine terminated polymer displays (Fig. 7.5) two triplets at 4 (-CH2-OH, 1) and 2.3 (-CH2CO-, 2) ppm which are assigned to main chain protons of PE-PCL block. Emergence of two multiplates at 1.6 (4) and 1.3 (3) ppm are attributed to methylene protons of PE-PCL backbone along with main chain proton of NIPAM. The characteristic signal of methyl proton of NIPAM appears at 1.1 ppm (5). The resonance signals corresponding to the protons of the terminal ammine group appears at around 4.8 ppm (-NH2, 6).

Thus, the structure of the ammine terminated polymer can be confirmed by 1H NMR spectrum. The end group of the PE-PCL-b-PNVCL polymer is also modified to ammine terminated one by following the similar procedure (Fig. 7.6). Figure 7.2: GPC trace of the synthesized block copolymers Figure 7.3: Water contact angle of the synthesized block copolymers; 72° (sample A1), 67° (Sample A2), 65° (Sample A3),58° (Sample A4), 66° (Sample B1),62° (Sample B2), 52° (sample B3), 46° (sample B4) and 77° (PE-PCL) at 25°C. Figure 7.4:1H NMR of the PE-PCL-b-PNVCL-Br in CDCl3 7.3.5 Conjugation of FA with '-NH2' terminated block copolymer (PE-PCL-b-PNIPAM-FA and PE-PCL-b-PNVCL- FA) In order to perform cancer cell targeted delivery, the micelle forming polymer should be decorated with targeting moiety e.g. FA, biotin etc.237 Conjugation of FA with ammine terminated polymer is extensively used for cancer cell targeted delivery. The conjugation of FA with ammine terminated polymer was conducted through DCC coupling reaction as described in the earlier section. Out of the two active carboxyl groups (α and γ) of FA, coupling occurs through γ-carboxyl which helps to retain its targeting aptitude towards cancer cell. The FA conjugated PE-PCL-b-PNIPAM-FA displays (Fig. 7.7) a number of resonance signals at 4 ppm (-CH2-OH) and 2.3 ppm (- CH2CO-) which are attributed to methylene proton of the PE-PCL segments, symbolized by the numbers 1 and 2, respectively. The two multiplets at 1.6 and 1.3 ppm are attributed to the methylene protons of PE-PCL back-bone symbolized by the numbers 3 and 4, respectively. The signal corresponding to methyl protons (-CH3, 5) of NIPAM is appeared at 1.1 ppm. Figure 7.5: 1H NMR of the PE-PCL-b-PNIPAM-NH2 in d6 DMSO Figure 7.6: 1H NMR spectra of PE-PCL-b-PNVCL-NH2 Figure 7.7: 1H NMR of the PE-PCL-b-PNIPAM-FA in d6 DMSO The appearance of a number of resonance signal in between 6 to 9 ppm are attributed to the aromatic protons of FA.207b, 209 The attachment of FA with another polymer, PE-PCL-b-PNVCL is also confirmed by spectroscopic methods and displayed in Fig. 7.8. The appearance of FA protons in the FA conjugated polymer confirmed the successful attachment of FA with polymer. Additionally, appearance of UV-Vis absorbance at 288 nm (Fig. 7.9) of the FA conjugated polymer further confirmed the successful attachment of FA. Approximately, 5% (by weight) attachment of FA with block copolymer was evaluated from the predetermined calibration curve and absorption intensity of FA conjugated polymer. 7.3.6 CMC of the block copolymers The formation of PM was confirmed by evaluating the critical micelle concentration (CMC) of the synthesized block copolymers using fluorescence spectroscopy. Pyrene was used as fluorescence probe. The CMC of the synthesized block copolymers (displayed in table 7.2) varies from 0.59×10-2 to 1.31×10-2 mg/mL for group 'A' (A1 to A4) and 0.62×10-2 to 1.52×10-2 mg/mL for group 'B' (B1 to B4) samples at 25 ºC. The lower range of CMC values of the block copolymers for obvious reason helps in constituting a thermodynamically stable micelle. The PMs with such a lower range of CMC's prevent the unwanted release of loaded drug molecules (before reaching to the targeted site) even after large dilution inside the biological system.238 The increase in CMC values from sample A1 to A4 and from sample B1 to B4 is attributed to the increase in hydrophilic (NIPAM for sample A and NVCL for sample B) chain length for a fixed length of hydrophobic (PE-PCL) segment. Figure 7.8: 1H NMR of the PE-PCL-b-PNVCL-FA in d6 DMSO Figure 7.9: UV-Vis spectra of FA conjugated polymeric micelle. 7.3.7 Characterization of polymeric micelles The PMs were

synthesized by slow addition of DMSO dissolved sample with DOX into PBS (pH 7.4) solution according to the procedure described in the earlier section. The size and morphology of the synthesized polymers has been characterized by HRTEM and DLS study. TEM photomicrograph (Fig. 7.10) shows a nearly spherical PM with an average size ranging from 15 to 180 nm (table 7.2). The DLS size profile of the PMs prepared from both the group 'A' and 'B' samples are manifesting slight higher size than those observed from HRTEM photomicrograph as displayed in table 7.2. The relatively large size PMs obtained from 'B' series compared to the 'A' series of sample can be easily understood by taking into account the relative CMC values of both the series. The emergence of higher size of each PM from DLS compare to HRTEM can be presumed by considering the physical state of analysis of both the experiments (HRTEM and DLS). The negative zeta potential values of the PM (shows in table 7.2) support its effective stability in aqueous medium. 7.3.8 Thermoresponsive characteristic of the PMs The lower critical solution temperature (LCST) of each block copolymer was determined by temperature dependent UV-Vis spectroscopy. The variation of transmittance with increasing temperature is displayed in Fig. 7.11. The LCST (table 7.1) of the group 'A' (A1 to A4) and group 'B' (B1 to B4) samples are varying from 30-32 $^{\circ}$C and 38-39 $^{\circ}$C, respectively. The slight variation of LCST for group 'A' samples from the LCST of the NIPAM homo polymer (32 $^{\circ}$C) is attributed to the variation of repeating unites (NIPAM; table 7.1).120c The same phenomenon is also realized for group 'B' samples with different NVCL repeating units (table 7.1). Thermally induced size change of the PM has been studied by DLS. Table 7.2: The CMC, micelle size and zeta potential of the synthesized block copolymers Sample (mg/mL)×10-2 (mV) (nm) Micelle Size (nm)b CMC a Zeta Potential b Micelle size c A1 0.59 -21.8 20 28±2 Group A2 0.98 -21.2 25 32±0.60 A A3 1.23 -28.1 40 65±0.23 A4 1.31 -31.2 45 85±0.41 B1 0.62 -26.5 15 25±0.47 Group B B2 1.12 -29.2 90 96±0.31 B3 1.41 -46.1 150 164±0.44 B4 1.52 -49.6 180 185±0.66 aDetermined from fluorescence microcopy; b Determined from DLS; c Evaluated from HRTEM photomicrograph. Figure 7.10: HRTEM photomicrograph of the polymeric micelle prepared from thermoresponsive block copolymer Figure 7.11: Variation of UV absorbance (at λmax = 520 nm) with increasing temperature scan from 25 to 55°C for sample A4 and sample B4 Figure 7.12: The variation of hydrodynamic size (from DLS) for sample A4 (A) and sample B3 (B) with temperature. There has been a significant decrease in hydrodynamic radius (Fig. 7.12) of the PM on application of heat. Sample A4 shows a change in size from 85 to 58 nm (32% reduction) and sample B3 from 164 to 80nm (51% reduction). The change in nature of the thermoresponsive block (PNIPAM for group 'A' and PNVCL for group 'B') from hydrophilic to hydrophobic followed by shrinking in aqueous medium is the answerable factor for temperature induced decrease of sizes. 7.3.9 DLC and DLE of the PM The loading of hydrophobic drug inside the PM was achieved according to procedure already described in the earlier section. Slow addition of polymeric-DOX mixture into the aqueous medium leads to the creation of a number of vacant spaces by the branched polymers. Figure 7.13: UV-Vis. spectra of the DOX loaded polymeric micelle with free DOX. The DOX molecule can easily fit into that vacant space and appears as DOX loaded PMs. The value of DLC for different composition of polymer is displayed in table 7.1. The DLC values reveal an increasing trend from sample A1 to

A4 and sample B1 to B4. The PMs prepared from branched polymer shows a very high value of DLC (sample B = 24.3%) as compare to those reported in the contemporary literature.113, 116, 239 This higher value of DLC shown by branched polymer can be easily understood by considering the increasing number of vacant spaces created by the branched polymer chains. As well as interaction of functional groups (H-boding) between acid (-COOH), hydroxyl (-OH) and amine (-NH2) group of DOX with ester (-COO-) and secondary amine (-NH-) group of the polymers plays a significant role. The shifting of UV absorption band (Fig. 7.13) of DOX loaded PM compare to the free DOX absorbance is also the evidence for such interactions. 7.3.10 MTT assay Figure 7.14(a) shows cytotoxicity of sample A2, A4, B2 and B4 against HeLa cells. The sample A2, A4, B2 and B4 exhibit no obvious cytotoxicity against HeLa cells. The DOXloaded polymers exhibit (Fig. 7.14(b)) lower cytotoxicity compared with free DOX against HeLa cell at the equivalent dosage. The IC50 values of the free DOX, DOX-loaded PMs i.e. for sample A4 and B4 are 4.68 µg/ml, 8.11µg/ml and 6.79 µg/mL, respectively. The lower cytotoxicity of DOX loaded PM compare to free DOX is mostly due to the slower release and the delayed nuclear uptake of DOX in HeLa cells, as confirmed by both in vitro DOX release (Fig. 7.19) and cellular uptake studies (Fig. 7.18). The cell viability study of free DOX and DOX loaded PM on HaCaT cell line is displayed in Fig. 7.14(c). The IC50 values of free DOX, DOX 142 loaded sample A4 and B4 against HaCaT cell line are 4.37, 190.56 and 98.29 µg/mL, respectively. Like HeLa cell line, HaCaT cell line also respond in a similar way i.e. free DOX exhibits more cytotoxic effect compared to the DOX loaded PM (sample A4 and B4). The higher cytotoxic effect of DOX loaded PM against FA over exposed HeLa cell line compare to HaCaT can easily be recognized by considering the targeting aptitude of FA on HeLa cell line. Figure 7.14: MTT assay of (a) sample A2, A4, B2 and B4 on HeLa cell, (b) free DOX, and DOX loaded sample A4, and B4 on HeLa cell and (c) free DOX, and DOX loaded sample A4, and B4 on HaCaT cell. 7.3.11 In vitro cell uptake study Flow cytometry analysis was performed in order to compare the endocytosis of DOX- loaded PM in HeLa cell line. The fluorescence intensity of cells was recorded at different time intervals and cells without treatment were taken as the negative control. The fluorescence intensity is proportional to the amount of DOX internalized into the cells. Flow cytometry histogram profiles of HeLa cells incubated with DOX-loaded PM for a period of 1h and 4h is shown in Fig. 7.15. The histogram displays a higher amount of DOX uptake by the cells treated with DOX loaded polymer B4 compare to the polymer A4 (Fig. 7.16). The higher uptake of DOX can be easily understood by taking into consideration the DLC of sample B4 (DLC: 24.3 %) and sample A4 (DLC: 20.3%). However, for each sample the amount of DOX uptake at 1h is less compare to that at 4h which proves its time dependency. The red fluorescence of DOX allows us to monitor easily the cellular uptake and intracellular distribution of free DOX and DOX loaded PMs. HeLa cells were incubated with free DOX and DOX loaded PMs, followed by observation under fluorescent microscope after 1h, 2h and 4h of treatment. After 1h of incubation with the free DOX, strong fluorescence was observed mainly in the nuclei (stained as blue) (Fig. 7.17, free DOX). Figure 7.15: Flow cytometric analysis of control and DOX loaded PM (sample A4 and Sample B4) for 1 h (a) and 4 h (b). Figure 7.16: A comparison of fluorescence intensity with time for DOX loaded sample A4 and B4 The rapid nuclear accumulation of DOX indicates it’s uptake

through passive diffusion mechanism.240 However the cell treated with DOX loaded PM displays mild fluorescence in cytoplasm after 1h of incubation. The fluorescence intensity grows stronger with time in the cells and shifts to nuclei (stained by DAPI blue) following 2h and 4h of treatment, as shown in Fig. 7.18. These results suggest that PM might be internalized through the endocytic mechanism into the cells.113, 241 Subsequently the DOX molecules were released and diffused into the nuclei. 7.3.12 In vitro release study The thermoresponsive release of drug from loaded PM was studied at two different ranges of temperature e.g. 30-37 °C (for sample A4) and 37-40 °C (for sample B4). We select the temperature range 30 °C and 37 °C for sample A4 which are the lower and upper temperature with respect to its LCST (32 °C). Similarly, the temperature range 37 °C and 40 °C for sample B4 are the lower and upper temperature with respect to its LCST (39°C). Figure 7.17: Fluorescence microscopic image of HeLa cell treated with free DOX (scale bar of 20μm) The release profile (Fig. 7.19) reveals a slow rate of release at a temperature below the LCST (30°C for sample A4 and 37 °C for sample B4). The sample A4 and B4 are showing only 7.8 % and 9.7 % of drug release after a time span of 24 h. In contrary, above the LCST, sample A4 and B4 show fast rates of DOX release, about 66.1%/24 h and 78.57%/24 h, respectively. A faster rate of DOX release from the sample B4 compared to sample A4 can easily understood by taking into account the extent of temperature induced size decrease. A fast (above the LCST) and slow (below the LCST) rates of DOX release can be explained by taking into account the thermally induced size change of the PMs. Each block copolymer has their characteristic LCST value, above which the phase change (hydrophilic to hydrophobic) of the thermoresponsive segments happens. The phase change leads to shrinking of the thermoresponsive segment that results in the reduction of the effective size of the PMs. Additionally, above the LCST the micelle may start to collapse or at least to start to deform.242 The sudden shrinking of the thermoresponsive block (above LCST) improvises the bound drug molecule to quickly escape out from the loaded micelle giving rise to a faster rate of DOX release. However, below the LCST no such shrinkage occurs but few DOX molecules are diffused through the polymeric shell to the outer solution that offers a slow rate of DOX release. 7.3.13 In vivo antitumor efficacy The average volumes of the tumor (Fig. 7.20b and c) are approached to 3626±574 mm3, 2119±262 mm3 and 1086±361 mm3 for control, free DOX and DOX loaded PM treated groups, respectively at 28 days of post treatment. Both the free DOX and DOX loaded PM show inhibition of tumor volume compare to the control. However, the enhanced efficiency of inhibition of tumor growth is observed by DOX loaded PM compare to the free DOX treated group. The FA guides the PM to selectively reach into the tumor site and leading to the enhanced therapeutic efficiency. Accordingly, an inhibiting of tumor volume about 70.2% was observed with respect to the control. Although, free DOX treated group also inhibited the growth of tumor volume about 32.3% with respect to the control but the systemic toxicity due to the unselective uptake impeded the body weight of mice (Fig. 7.20d). In contrary, the body weight of the mice, treated with DOX loaded PMs and control do not change significantly, demonstrating poor or no systemic toxicity. 7.3.14 In vivo internalization of DOX At 30 minute of post injection, we captured the microscopic images of tissue section for tumor specific DOX accumulation. The intense red

fluorescence of DOX is observed (Fig. 7.21) from the tumor section of DOX loaded PM treated group compare to the free DOX treated one. The targeting aptitude of FA provides significantly high in vivo accumulation of DOX through PM into the tumor compare to free DOX treated group. The in vitro cell uptake (Fig. 7.18) also supports the result. These results are corroborated with effective inhibition of tumor growth by DOX loaded PMs as discussed in earlier section. Figure 7.18: Fluorescence microscopic images of DOX loaded polymeric micelle on HeLa cell line at the time span of 4h. The nuclei were stained with DAPI (blue). Figure 7.19: Release profile of the DOX loaded polymeric micelle Figure 7.20: Control, free DOX and DOX loaded micelle treated (a) mice, (c) tumor size of the mice, (b) variation of tumor volume with time and (d) variation of body weight with time. 7.4 Conclusions The synthesis of two sets of thermoresponsive block copolymers PE-PCL-b-PNIPAM and PE-PCL-b-PNVCL has been successfully preformed by ATRP (NIPAM and NVCL) using the pre-synthesized branched PE-PCL as a template. The bromine terminated block copolymer was then modified to ammine terminated one followed by attachment of FA. The chemical structure of both the synthesized polymers with FA conjugation has been confirmed by 1H NMR spectra. Figure 7.21: In vivo accumulation of DOX into the tumor tissue Approximately 5 wt% attachment of FA has been confirmed by UV-Vis. spectrum. A very low range of CMC (0.59×10-2 to 1.52×10-2 mg/mL) of the synthesized block copolymers has been captured by fluorescence spectroscopy. This low range of CMC would help in reduction of unwanted release of drug molecule after large dilution inside the biological system. The size of the prepared PMs varies from 15 to 180 nm along with the wide range of LCST (from 30 to 39) offers their extensive application in drug delivery. The branched architecture of the polymer helps a high amount of DOX loading (24.3%) by creating a number of vacant spaces inside the PMs. The loading amount is also considerably high as compare to the PMs prepared from linear polymer, reported in the contemporary literature. The slow release and delayed nuclear uptake leads to lower cytotoxic effect by DOX encapsulated PM (sample A4 and B4) compare to the free DOX against HeLa cell line. The comparatively high DLC and rate of DOX release promotes the sample B4 to show higher cytotoxicity compare to the sample A4. The selectively high cytotoxic effect of DOX loaded PMs against HeLa compared to the HaCaT cell line is because of the high targeting aptitude of FA. The FACS analysis reveals the high amount of DOX uptake by the sample B4 which is due to the higher value of DLC compare to the sample A4. The FA receptor promotes the large number of PM to accumulate onto the FA over exposed HeLa which leads to the intense fluorescence that is recognised from HeLa compare to the HaCaT cell line. The change of phase above the LCST followed by shrinkage of thermoresponsive polymer chains leads to the fast release (sample A4; 66% and sample B4; 78% after 24h) of encapsulated DOX. The in vivo intravenous administration of the DOX loaded thermoresponsive PM onto sarcoma 180 tumor mice model enforced selective tumor accumulation and inhibition of tumor growth about 70.2% with respect to the control without any systemic toxicity. 8.1 Summary and Conclusions Novel superparamagnetic and biocompatible polymer grafted nanoparticles have been prepared successfully by grafting four armed PE-PCL onto MNPs (5 and 15 nm) surface. The grafting density of PE-PCL has been found to be significantly high, leading to much lower aggregation

tendency of the MNPs. Although the amount of polymer immobilized onto the surface of 15 nm MNP (i.e. grafting density, determined from TGA) is higher compare to 5 nm MNP, still it displayed associated structure owing to its extremely high magnetic dipole-dipole interaction energy that stems from their superparamagnetic nature. The MAPM has been prepared (from 5nm) successfully and its magnetic activity has been realized in presence of external bar magnet. HRTEM photomicrograph displayed a regular appearance of spherical MAPM having average size of 100 nm which is at par with its hydrodynamic size (116 nm) evaluated from DLS measurement. Biocompatibility test provided information that all MAPM are showing biocompatibility above 90% even at higher concentration (100 µg/ mL). Selective and high cellular uptake of DOX loaded MAPM is observed for magnetically targeted HeLa cell line compared to the untargeted one. Plenty of DOX loaded micelles enter into the cytoplasm of the magnetically targeted cell just after 0.5 h of incubation. The MAPM shows a quick on demand release of about 51.5 % DOX (after 1h) under the influence of HFAMF. Hence, using such novel MAPM we can able to tailor the drug release rate according to the status of the patient (i.e. on demand) by switching 'off' or 'on' HFAMF device. Branched PE-PCL-b-PEC has been successfully synthesized by ring-opening polymerization. FA is attached at its edge by a DCC coupling reaction. The FA-coupled block copolymer forms biocompatible and biodegradable PMs in aqueous media. The spherical micelles with an average size of 80 nm were confirmed by HRTEM, DLS and AFM. PMs produced from branched polymers can encapsulate DOX molecules and show a maximum DLC of 14.7%. The very high cytotoxic effect of FA-conjugated DOX- loaded PM demonstrates the selective targeting aptitude on the HeLa cell line. The high release rate of DOX from the PMs at the physiological pH of cancer cells is responsible for the rapid treatment of cancer by supplying the required amount of DOX. 149 A FA conjugated, pH responsive polymeric micelles were successfully synthesized from branched amphiphilic block copolymer, PE-PCL-b-PAA-FA. UV-Vis absorption spectra reveal that about 6.4% of FA is conjugated with the branched polymer. HRTEM photomicrographs of polymeric micelles, prepared from the branched amphiphilic block copolymer display nearly spherical micelle with an average size of 40-60 nm. The DOX loading content of these PM is appreciably high (about 25.1%) compare to literature reported PM prepared from linear amphiphilic polymer analogues. PMs are showing biocompatibility about 80% even at higher concentration but DOX loaded PM exhibits very low biocompatibility at the same concentration levels. Selective trafficking of FA leads to enhanced uptake of DOX loaded PM as well as their enhanced cytotoxicity against HeLa cell. The deswelling of drug loaded micelle, weakening of hydrogen bonding interactions and high solubility of DOX at physiological pH (5.0) of cancer cell furnish a faster release of DOX (i.e. 68% after 24 h). The synthesis of two sets of thermoresponsive block copolymers PE-PCL-b-PNIPAM and PE-PCL-b-PNVCL has been successfully preformed by ATRP of NIPAM and NVCL, respectively. Approximately 5 wt% attachment of FA has been confirmed by UV-Vis spectrum. The size of the prepared PMs varies from 20 to 180 nm along with the wide range of LCST those (from 30 to 39) offer their extensive application in drug delivery. The branched architecture of the polymer helps in a high amount of DOX loading (24.3%) by creating number of vacant spaces inside the PMs. The loading amount is also considerably high as compare to the PMs prepared from linear

polymer, reported in the contemporary literature. The comparatively high DLC and rate of DOX release promotes the PE-P(CL)99-P(NVCL)98 to show higher cytotoxicity compare to the PE-P(CL)99-P(NIPAM)98. The selectively high cytotoxic effect of DOX loaded PM against HeLa compared to the HaCaT cell line is because of the high targeting aptitude of FA. The change of phase above the LCST followed by shrinkage of thermoresponsive polymer chains leads to the fast release (PE-P(CL)99-P(NIPAM)98; 66% and PE-P(CL)99- P(NVCL)98; 78% after 24h) of encapsulated DOX. The in vivo intravenous administration of the DOX loaded thermoresponsive PM onto sarcoma 180 tumor mice model enforced 150 selective tumor accumulation and inhibition of tumor growth about 70.2% with respect to the control without any systemic toxicity. 8.2 Contribution of the Thesis A number of FA and magnetic targeted nano-carriers based on four armed polycaprolactone are prepared for cancer cell targeted drug delivery. The small size (<200 nm) and highly negative zeta-potential offer the nano-carrier to show effective performance in drug delivery. The branched architecture of the base polymer promotes the nano-carrier to carry high amount of DOX molecules. The nano-carriers displays stimuli (like, magnetic, pH and thermal) responsive release of anti-cancer drug selectively to the cancerous cells (HeLa). The stimuli responsive drug release rates are also quite satisfactory. The DOX loaded nano-carrier shows selective uptake towards cancerous cell that encourages its in vivo administration. The in vivo administration of thermoresponsive nano-carrier exhibits about 70.2% inhibition efficiency towards cancerous cell which further reveals its great potential for in vivo study in the next level of animal models. 8.3 Scope of Future Study Since the branched polymers have many fascinating advantage as mentioned in chapter 1, hence there are wide prospect of branched biocompatible polymers architecture based stimuli responsive nano-carriers. The thesis covers only a small part of such the large prospects. The implementations of some other stimuli responsive moieties to the branched polymer may also offer smart nano-carrier. The crosslinking of the acid functional group containing branched polymer with amine functionalized MNP or FeCl3 may also lead to the formation of stable magnetic nano-gel. These gels may able to carry a significantly large amount of drug molecules with the provision of no premature drug release. The incorporation of glutathione responsive –S-S- linkage into the backbone of the branched polymer may also offer another smart reduction responsive nano-carrier. In order to evaluate the therapeutic performance, these nano-carriers developed in this thesis may be administered in the in vivo system of different hierarchical level. If proven successful in animal model then should be subsequently tested for human trial. 151 Chapter 2 Chapter 2 Chapter 2 Chapter 2 Chapter 2 Chapter 2 Chapter 2 Chapter 2 Chapter 2 Chapter 2 Chapter 3 Chapter 4 Chapter 4 Chapter 4 Chapter 4 Chapter 4 Chapter 4 Chapter 4 Chapter 4 Chapter 4 Chapter 4 Chapter 4 Chapter 4

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