Chapter 1

Introduction

This chapter briefly describes about the various controlled release vectors used for the delivery of therapeutic molecules and their structural characteristics that allow improving the therapeutic efficacy of these bioactive molecules, as well as offers an overview of recent scientific advances in the area chemotherapy and emphasizes on the current challenges in cancer treatment.

1.1 Controlled drug delivery

Drugs have long been used to improve health and extend lives. Multiple types of drugs, including natural products, synthetic medicines, proteins, nucleic acids, and peptides, have been developed and designed for past several decades to combat various diseases [Gershell et al. 2003; Cozzi et al., 2003]. However, the traditional way of taking a drug, such as a pill or injection, often results in plasma drug levels that cycle between too high and too low. When the plasma drug level is above the desired therapeutic range, shows toxic side effects and become ineffective when it is below the optimal range. **Figure 1.1** exhibits the typical profiles of plasma drug concentration as a function of time. In case of conventional drug formulations, plasma drug level reaches above the maximum safe concentration level (MSC) within a very brief period of time after drug administration, then peaks and declines very steeply below the minimum effective concentration (MEC) level. Hence, these formulations are unable to maintain the therapeutic dose for desired periods of time and require multiple administrations to obtain therapeutic effect. This leads to poor patient compliance. The fluctuating drug levels may cause undesired side effects or loss of therapeutic advantage to the patient. Therefore, an efficient drug delivery system (DDS) is necessary to overcome the drawbacks of conventional drug delivery systems and improve the clinical therapy by maintaining drug concentration within the therapeutic window after controlling the drug release rate and reducing adverse side effects by targeting diseased tissues. Sustained release and controlled release drug delivery systems can potentially reduce the undesired fluctuations of drug levels, thus reduce the frequency of drug administration and thereby diminishing the adverse side effects while improving the therapeutic index of the drug. Although, the terms sustained release and controlled release are often used interchangeably, they actually refer to two different types of drug delivery systems. Sustained release vehicles are the

drug delivery systems which prolonged the duration of action by slowing down the drug release rate, thereby also delays the start of the therapeutic action. Controlled drug

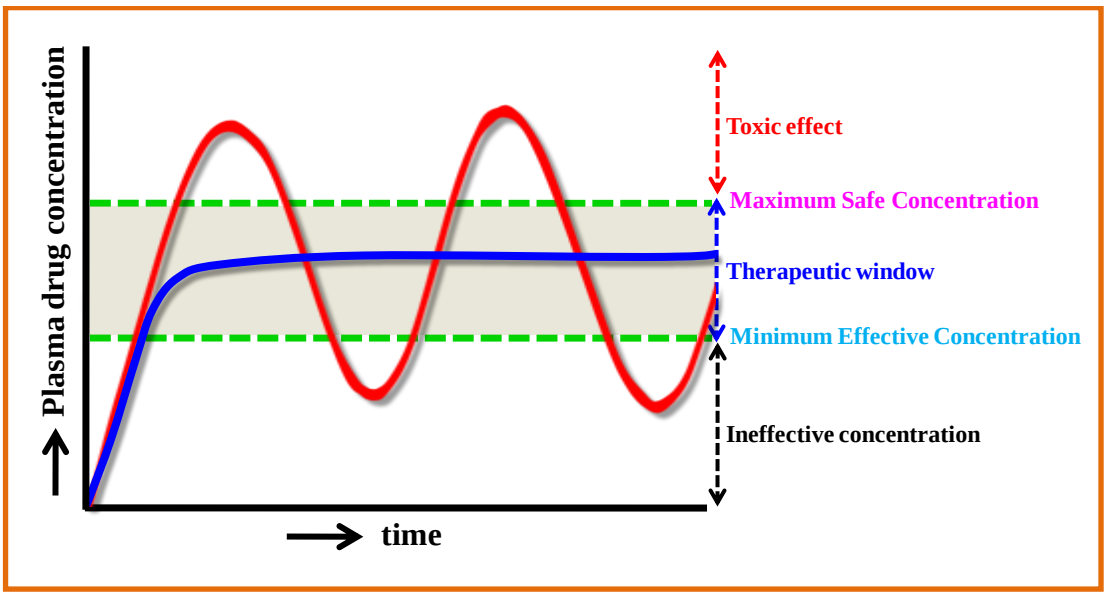

Figure 1.1: Representation of plasma drug concentration versus time profile after administration of conventional drug (red line) and controlled drug release formulation (blue line)

delivery systems are more sophisticated than just simply delaying the drug release rate and are designed to deliver the drug at specific release rates within a predetermined time period. Controlled drug delivery systems were designed to overcome the shortcoming comings of conventional drug delivery systems and thereby revolutionize the drug release system and offer several therapeutic benefits. Controlled drug delivery system is capable of maintaining the concentration of the drug in the plasma within the therapeutic window for prolonged duration that avoid frequent doses of administration and thereby reduce the side-effects and, therefore, improve the patient compliance. Thus, it is evident that these two systems represent two different kinds of drug delivery systems, and we cannot consider all sustained release systems as controlled release systems. They are primarily developed to improve patient compliance and to enhance therapeutic efficacy

of drugs. **Table 1.1** gives examples of problems exhibited by free drugs that can be ameliorated by the use of controlled DDS.

Table 1.1: Limitations of conventional drug delivery and their therapeutic improvement by using controlled drug delivery systems

Problem	Implication	Improvement using Controlled DDS
Poor water solubility and bioavailability	Concentration of the drug is difficult to achieve within the therapeutic window, as hydrophobic drugs may precipitate in aqueous media.	Controlled DDSs can enhance drug solubility and bioavailability by encapsulating the drugs into its matrix.
Poor biodistribution	Free drugs that have widespread distribution in the body may highly accumulate in normal tissues, resulting in adverse side effects.	It has the potential to lower the volume of distribution and thereby reduces undesired side effects in untargeted tissues.
Unfavorable pharmacokinetics	Plasma drug concentration levels fluctuate very rapidly and cleared from the body quickly, requiring continuous drug administration.	Maintain the plasma drug levels within therapeutic range for desired period of time.
Poor patient compliance	Frequent drug administration resulting in poor patient compliances.	Improve patient compliances by reducing frequency of administration.
Lack of selectivity for target tissues	Lack of selectivity and widespread distribution of conventional drugs give rise to increased toxicity and reduced efficacy.	Controlled DDSs can increase drug concentration in the targeted tissues through in active or passing targeting
Multidrug resistance	Efflux pump such as P-glycoprotein (P-gp) in the cancer cell membrane facilitates transports drug molecules out of cancer cells.	Application of controlled DDSs can effectively overcome the limitations of multidrug resistance of cancer cells.

In the past 30 years, controlled drug delivery technology has emerged as one of the most rapidly advancing research areas [Rodríguez et al., 1998; Uhrich et al., 1999; Singh et al., 2000; Santini et al., 2000; Lee et al., 2001; Yoo 2011; Park 2014]. These controlled drug delivery systems offer numerous advantages compared to conventional dosage forms as listed below:

(1) Enhance bioavailability of the drug
(2) Reduce frequency of doses
(3) Reduce adverse side effects resulting from widespread distribution of the free drug to other body parts

(4) Maintain plasma drug concentration level within therapeutic range for prolonged period of time

(5) It overcomes patient compliance problems

One of the main applications for the controlled drug delivery carriers has been the delivery of anticancer drugs and plasmids for cancer treatments.

1.2 Cancer and Chemotherapy

Cancer is a group of diseases in which abnormal cells proliferate in an uncontrolled way with the potential to invade or spread to other parts of the body. There are more than 100 types of cancer, including breast cancer, skin cancer, lung cancer, colon cancer, prostate cancer, and lymphoma. According to the World Health Organization (WHO) estimation, 8.8 million deaths worldwide in 2015 were due to cancer, which is nearly one in six global deaths [Laviano et al. 2017]. Cancer-related deaths are projected to increase in the near future, with the estimation of International Agency for Research on Cancer (IARC) about 13.1 million cancer-related deaths by the year 2030 worldwide [Boyle et al., 2008]. However, mortality rate has decreased in the last few years owing to better understanding of tumor biology and with the advancement of diagnostic devices and treatments.

Current cancer treatment options include surgical intervention, radiation therapy and chemotherapy or combination of them. Most of the chemotherapeutic drugs are DNA-damaging agents that are designed to kill or inhibit rapidly dividing cells, often also kill healthy cells and cause toxicity to the patient *e.g.* loss of appetite, nausea, myelosuppression and alopecia. Moreover, the chemotherapeutic drugs often have poor water solubility, which limits their bio-accessibility to tumor tissues and as a consequence higher doses are required that elevate toxicity for normal cells along with an increased incidence of multiple drug resistance. It would therefore be desirable to

develop novel controlled drug delivery systems that can either passively or actively target cancerous cells and thereby reducing the adverse side effects while improving the therapeutic efficacy.

1.2.1 Strategies behind chemotherapy treatments

Cancer tissue is composed of noncellular (i.e., vascular and interstitial) and cellular compartments that are remarkably different in nature compared with the surrounding normal tissues. Each of these compartments introduces challenge for the delivery of chemo drugs to tumor cells. General features of tumors include leaky blood vessels and poor lymphatic drainage. Whereas free drugs diffuse nonspecifically to reach tumor cells, controlled drug delivery carriers can selectively dispose the loaded drugs into the tumor tissues via passive or active targeting pathway.

1.2.1.1 Passive and active targeting

Passive targeting exploits the anatomical and physiopathological characteristics of tumors, such as leaky blood vessels and poor lymphatic drainage. In contrast to endothelial cells of normal blood vessels with tight endothelial junctions, tumor endothelial cells have leaky vascular openings of 300-700 nm [Allen et al., 2004; Brigger et al., 2002]. These fenestrations allow the entry of controlled drug delivery carriers with size below the above range into the tumor tissue. This process is termed as enhanced permeability and retention (EPR) effect [Maeda, 2001; Greish et al., 2010] Again, the poor lymphatic drainage system in tumors is also entrapping drug carrier particles, delaying their clearance and allow them to release drugs into the vicinity of the cancer cells [Fang et al., 2001; Maeda et al., 2013]. This leads to passive targeting of controlled drug delivery carriers and improves the bioavailability of loaded chemotherapeutic drugs to tumor cells with minimum drug toxicity to normal cells.

Active targeting consists of conjugation of a ligand to the surface of nanocarriers that can interact with its complementary receptor present at the target cell site. For active targeting of cancer cells, targeting ligand are chosen on the basis of their selectivity and affinity to the appropriate receptors that overexpressed on cancer cells. Active targeting ligands which can be coupled to the surface of the nanocarriers include proteins, peptides, carbohydrates, nucleic acids, folic acid, aptamers etc [Peer et al., 2007; Egusquiaguirre et al., 2012; Xu et al., 2015]. By actively targeting cancer cells, nanocarriers are able to increase the therapeutic index of the drug to target cells while concurrently reducing the cytotoxicity of the free drug to non-targeted tissues.

Thus, by employing both passive and active targeting strategies, a drug-loaded carrier can be able to circulate throughout the body for a prolonged period of time until it is successfully reached to its target through the use of EPR effects or cell-specific ligands. Because of these advancements, adverse side effects from conventional drugs will be markedly reduced as a result of the drug-loaded vehicle affecting only diseased tissue.

1.2.2 Gene therapy for cancer treatment

Gene therapy implies any approach primarily focused to treat or alleviate a genetic disease by employing therapeutic genes. Approaches to cancer gene therapy include three main strategies: insertion of a normal gene into cancer cells to replace the mutated gene, silencing a mutated gene through genetic modification, and genetic approaches to directly kill the cancer cells. A variety of therapeutic genes encoded with cellular proteins that are involved in apoptosis and/or antiproliferation of cancer cells, such as p53, p202, E1A, BAX, Bik, and PEA3 can be used for direct antitumor effects [Lo et al., 2005; Huang et al., 2010]. Such therapy requires efficient gene delivery vehicle to reach the targeted cells because naked nucleic acids alone are not able to get across cell membranes. This

delivery problem is addressed by the development of both viral and non-viral vectors. Viral vectors, which exploit the infection mechanism of natural viruses, evidently are the most effective system but viral vector induced side effects such as systemic toxicity, inflammatory response critically and the possibility of gene recombination critically impede their clinical translations [Thomas et al., 2003; Giacca et al., 2012; Cheng et al., 2016]. As an alternative to viral vectors, nonviral gene delivery method based on cationic polymers, cationic lipids or polymeric or inorganic nanoparticles provide much safer features and high gene-carrying ability [Panyamet al., 2003; De Smedt et al., 2000; Yin et al., 2014].

1. 3 Delivery vehicles:

Over the past few decades, several innovative drug and gene delivery strategies were studied for their use in cancer treatment. A wide range of compounds based on polymers, lipid, protein, organic and inorganic particles have been developed to enhance the pharmacological properties and therapeutic index of a myriad of drugs or to serve as efficient gene delivery vehicles [Sinha et al., 2006; Cho et al., 2008; Chow et al., 2013; Fan et al., 2013; Liang et al., 2014].

To date, various types of nanoscale carriers have been developed with varying sizes, architecture, surface physicochemical properties with targeting strategies for the treatment of cancer (**Figure 1.2**).

1.3.1 Liposomes

Liposomes are small, spherical, self-closed, synthetic vesicles having at least one concentric lipid bilayer with an encapsulated aqueous phase in the center. They have been widely used as a delivery vehicle for therapeutic molecules since Bargham et al.

discovered them in 1965. Its biocompatible and biodegradable nature, as well as their unique ability to encapsulate hydrophilic agents (hydrophilic drugs, DNA, RNA, etc.) in

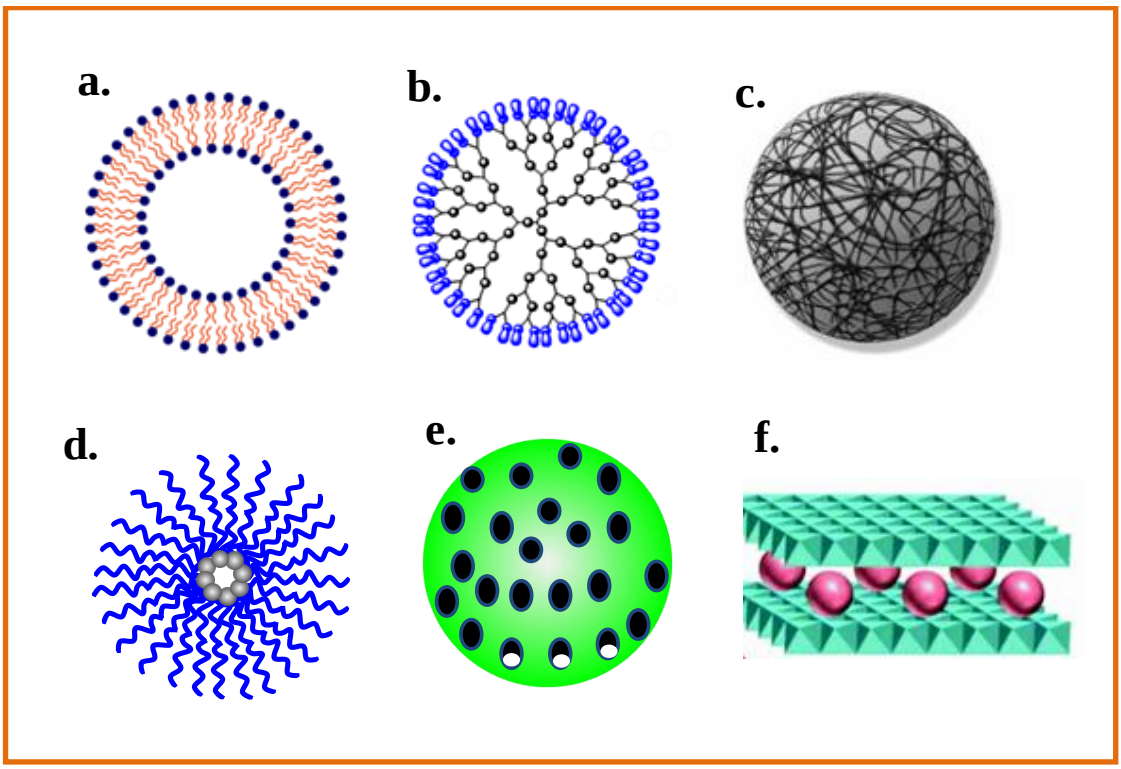

Figure 1.2: Types of nanocarriers currently in use for the delivery of anticancer agents. (a) Liposomes are self-assembling structures composed of phospholipid bilayers mimicking the structure of cell membrane. (b) Dendrimers are highly branched synthetic polymer with nanometer scale dimension. (c) Polymeric nanoparticles are nano sized solid polymeric matrices. (d) Polymeric micelles are composed of amphiphilic block copolymers forming nanosized core/shell structure in aqueous solution. (e) Mesoporous silica nanoparticles and (f) Layered double hydroxide nanoparticles.

their inner aqueous core and hydrophobic agents/drugs within their lamellae, make liposomes excellent therapeutic carriers. Again, amphiphilic drugs can also be loaded into the liposome inner aqueous core using remote loading methods like the ammonium sulphate method for doxorubicin [Bolotin et al., 1994] or the pH gradient method for vincristine [Boman et al., 1994]. However, the practical use of conventional liposomes is restricted by its rapid clearance from blood stream. The development of Stealth®

liposomes, which utilize a surface coating of a hydrophilic polymer, usually a lipid derivative of polyethylene glycol (PEG) prolongs the circulation half-life of liposomes from less than a few minutes (conventional liposomes) to several hours (Stealth liposomes). [Sapra et al., 2003]

Liposome has the potential to target specific cells through both active and passive targeting strategies. PEGylated liposome was found to be more effective for passive targeting cancer cells both *in vitro* and *in vivo* compared to the conventional liposome and moreover, PEGylated liposomes were exhibited a high degree of nuclear transfection

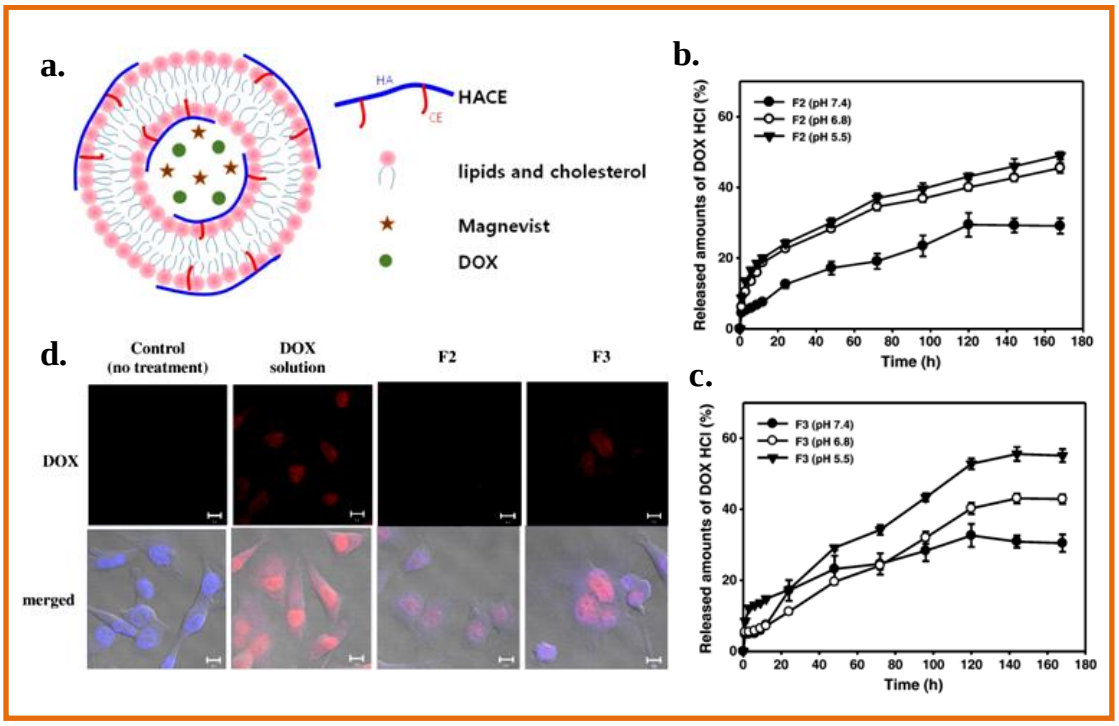

Figure1.3: (a) Schematic illustration of MR-visible hyaluronic acid–ceramide (HACE)-coated nanohybrid liposomes containing DOX. *In vitro* drug release profiles of the developed formulations; **(b)** F2 and **(c)** F3. DOX release was determined at pH 5.5, 6.8, and 7.4. **(d)** Cellular uptake analysis using MDA-MB-231 cells as visualized by CLSM. DOX solution, F2 and F3 (all 50 μg/ml DOX) were incubated for 2 h. Red and blue colors indicate DOX and DAPI staining, respectively. The length of the scale bar is 10 μm [Park et al., 2014].

and liposomal antisense oligonucleotides (ASO) was found to be effective for inhibition of pump and nonpump resistance of multidrug resistant tumors [Pakunlu et al., 2006]. Ligand-targeted liposomes were found to promote internalization of the liposome-drug conjugate into the target specific cell both *in vitro - in vivo*, and the vector can be designed to release their contents in the enzyme rich, low pH environment of endosome and lysosomes through the use of pH-triggered approaches [Jiang et al., 2013; Guo et al., 2001; Ishida et al., 2001]. A nanohybrid liposome coated with amphiphilic hyaluronic acid–ceramide (HACE) has been developed for tumor-targeted delivery of doxorubicin (DOX) and MR imaging of cancer [Park et al., 2014]. It has been found that DOX release from the developed nanohybrid liposome formulation (F3) was improved at acidic pH (pH 5.5 and 6.8) versus physiological pH (pH 7.4). Cellular uptake of DOX from F3 was higher than that from conventional liposome (F2). Targated zwitterionic oligopeptide liposomes exhibited enhanced tumor cellular uptake, improved cytoplasmic distribution, and enhanced mitochondrial targeting [Mo et al., 2012]. A pH-sensitive stearoyl-PEG-poly(methacryloyl sulfadimethoxine)-decorated liposome system has been developed that efficiently associates with and delivers the protein payload to cancer cells and macrophages under conditions mimicking the bladder environment [Vila-Caballer et al., 2016].

1.1.3.2 Dendrimers

Dendrimers are highly branched, 3D globular macromolecules with high degree of surface functionality and versatility. Since their introduction in the mid-1980s, this promising polymeric materials has attracted considerable attention because of their unique properties such as uniform size, water solubility, multivalency, narrow polydispersity, well-defined molecular weight, nanoscale size and controllable internal cavities bearing specific species for the encapsulation of guest drug molecules and

external periphery with multiple functional moieties for solubilization, conjugation of bioactive compounds and targeting molecules, and recognition purposes make them attractive for biological and drug-delivery applications [Nanjwade et al., 2009; Mignani et al., 2013]. Two distinct synthetic strategies exist for the preparation of dendrimers: the divergent approach [Tomalia et al. 1985; Newkome et al. 1985; Vögtle et al., 1978] in which the construction starts from a core and progresses to the periphery with successive formation of new generations and the convergent approach developed by Hawker and Fréchet [Hawker et al., 1990], it involves the prior construction of branches of a dendrimer, the so called dendrons, which are then attached to the central core. Although, various types of dendrimers including polyamidoamine (PAMAM), polypropyleneimine (PPI), melamine, poly(glycerol-co-succinic acid), poly(glycerol), poly-L-lysine (PLL), triazine, poly[2,2-bis(hydroxymethyl)-propionic acid] and poly(ethylene glycol) (PEG), as well as carbohydrate-based and citric-acid-based ones, have been developed for drug delivery, PAMAM- and PPI-based dendrimers have been the most widely investigated vectors that have gained tremendous attention [Kesharwani et al., 2015]. Due to compact, globular structure and availability of interior cavity spaces and multiple surface functional groups, drug molecules can be encapsulated both in the interior of the dendrimers (physical encapsulation) as well as attached to the surface functional groups (covalent conjugations) [Singh et al., 2016]. Internal cavities of dendrimers are hydrophobic in nature; hence, poorly water soluble drugs can be encapsulated through hydrophobic interactions [Liu et al., 1999]. Morgan et al. encapsulated two hydrophobic antitumor camptothecins, 10-hydroxycamptothecin and 7-butyl-10-aminocamptothecin within the cavity of a polyester dendrimer (composed of the natural metabolites, glycerol and succinic acid) using the solvent evaporation method [Morgan et al., 2006]. These dendrimers encapsulated camptothecins exhibited better anti-tumor activity compared to

free camptothecin against human cancer cell lines. Cellular uptake and efflux measurements in MCF-7 cells found to an increment of 16-fold for cellular uptake and an increase in drug retention within the cell when using the dendrimer vehicle. Anticancer drug cisplatin has been encapsulated through conjugation with carboxylated PAMAM dendrimers and this PMMA-drug conjugate has been found to exhibit sustained drug release, enhance anti-tumor efficacy, and lower toxicity compared to free cisplatin [Nguyen et al., 2015].

1.1.3.3 Polymeric nanoparticles

Polymers are the most widely explored materials used for the development of drug/gene delivery vehicle to treat cancer. These polymeric systems are engineered from biocompatible and biodegradable polymers. Polymeric nanoparticles can be made from synthetic polymers, including poly(ε-caprolactone) (PCL), poly(lactic acid) (PLA), poly(lactic co-glycolic acid), poly(styrene-maleic anhydride) etc. or from natural polymers such as gelatin, dextran, chitosan collagen etc. and may be used for encapsulating drugs/gene without any chemical modification. The anticancer agents can be released in a controlled way through surface or bulk erosion, swelling followed by diffusion, diffusion through the polymer matrix, or in response to the local environment. The degradation rate and accordingly, the release rate can be tailored by varying the polymer composition and architecture. The chemistry involved for the synthesis and preparation of polymeric nanoparticles can be easily manipulated to allow desired properties to be built into the nanoparticle, such as surface modifications to improve biodistribution profiles, pharmacokinetic characteristics, and entrapment of therapeutic agents. Studies have shown that conjugation of doxorubicin with dextran and then encapsulation this drug conjugate in hydrogel nanoparticles using reverse microemulsion technique can minimize the adverse cytotoxic effects of the anti-cancer drugs [Mitra et al.

2001]. Again, *in vivo* antitumor investigation illustrated that encapsulation of the conjugate in nanoparticles not only reduces the side effects, but also improves its therapeutic efficacy in the treatment of solid tumors. Multifunctional Taxol-loaded PLGA nanoparticles illustrated chemotherapeutic and near-infrared photothermal destruction of cancer cells *in vitro* and *in vivo* [Cheng et al. 2010]. Tamoxifen loaded PLGA nanoparticles, prepared using emulsified nanoprecipitation method, exhibit DNA cleavage potential and greater *in-vitro* anti-cancer activity as compared to pure drug [Pandey et al., 2016]. Cationic polymer based gene delivery carriers have shown promise as a safe, biodegradable and nontoxic alternative to viral gene therapy. Various types of polymers have therefore been investigated as a gene delivery vehicle, such as chitosan, PEI, polylysine, polyamino ester and so on [Choi et al., 2004; Jin et al., 2014; Hwang et al., 2001; Kean et al., 2005].

Chitosan is a biocompatible and biodegradable linear aminopolysaccharide, can easily form stable, nanoscale range complex with pDNA and its cationic polyelectrolyte nature facilitates strong electrostatic interaction with mucus, negatively charged mucosal surfaces and other biomacromolecules such as DNA [Morille et al., 2008; Hejazi et al., 2003]. This cationic polymer has the potential to protect against DNase degradation that is comparable to PEI's one, and exhibits a significantly better biocompatibility [Köping-Höggård et al. 2001]. Several groups have investigated chitosan/ DNA nanoparticles, including use of galactosylated chitosan [Erbacher et al., 1998], trimethylated chitosan oligomers [Thanou et al., 2002], deoxycholic acid modified chitosan [Kim et al., 2001], N-dodecylated chitosan [Li et al., 2002], or ligand decorated chitosans for targeting cell membrane receptors [Sato et al., 2001]. Polyethylenimine (PEI) and its variants are considered the gold standard for gene delivery because of its numerous amine groups, which facilitate enhanced cellular uptake and better endosomal escape under mildly

acidic conditions [Fu et al., 2012; Kircheis et al., 2001]. Poly(L‑lysine) (PLL) is a homopolypeptide of the basic amino acid lysine and because of its biodegradable nature is advantageous for *in vivo* use over PEI based carriers. The PLL/DNA complexes are internalized into cells a way similar to PEI/DNA complexes, but their transfection efficiency is lower [Merdan et al., 2002]. However, a leptin derived 30-amino-acid peptide modified pegylated poly-L-lysine dendrigraft (DGL-PEG-Leptin30), exhibited significantly higher transfection efficiency than that of pure PLL, while no measurable cytotoxicity was detected [Liu et al., 2010].

1.1.3.4 Micelles

Micelles are colloidal nanoscale systems that arrange themselves in a spherical or globular form by self assembly of amphiphilic block copolymers in aqueous solution forming a hydrophobic core and a hydrophilic shell. In aqueous solution, the block co-polymers aggregate to form entropically favored, supra-molecular assembly under certain concentrations (critical micelle concentration; CMC) and temperatures. The hydrophobic core serves as a reservoir for variety of hydrophobic molecules, such as therapeutics and imaging agents, thus, thereby improving the solubility and stability in the biological system, whereas the hydrophilic shell stabilizes the hydrophobic core and protects the loaded drugs from interactions with the blood components, making it an appropriate vehicle for i.v. administration. The therapeutic molecules can be incorporated into a polymeric micelle by physical, chemical or electrostatic interactions [Park et al., 2008]. The first micelle formulation of paclitaxel, Genexol-PM (PEG-poly(D,L-lactide)-paclitaxel), which was safely administered without hypersensitivity reactions (HSRs) and showed a favorable toxicity profile [Kim et al., 2004]. Multifunctional star-shaped polymeric micelles based on four-arm disulfide linked poly(ε-caprolactone)-poly(ethylene glycol) amphiphilic copolymer decorated with folate

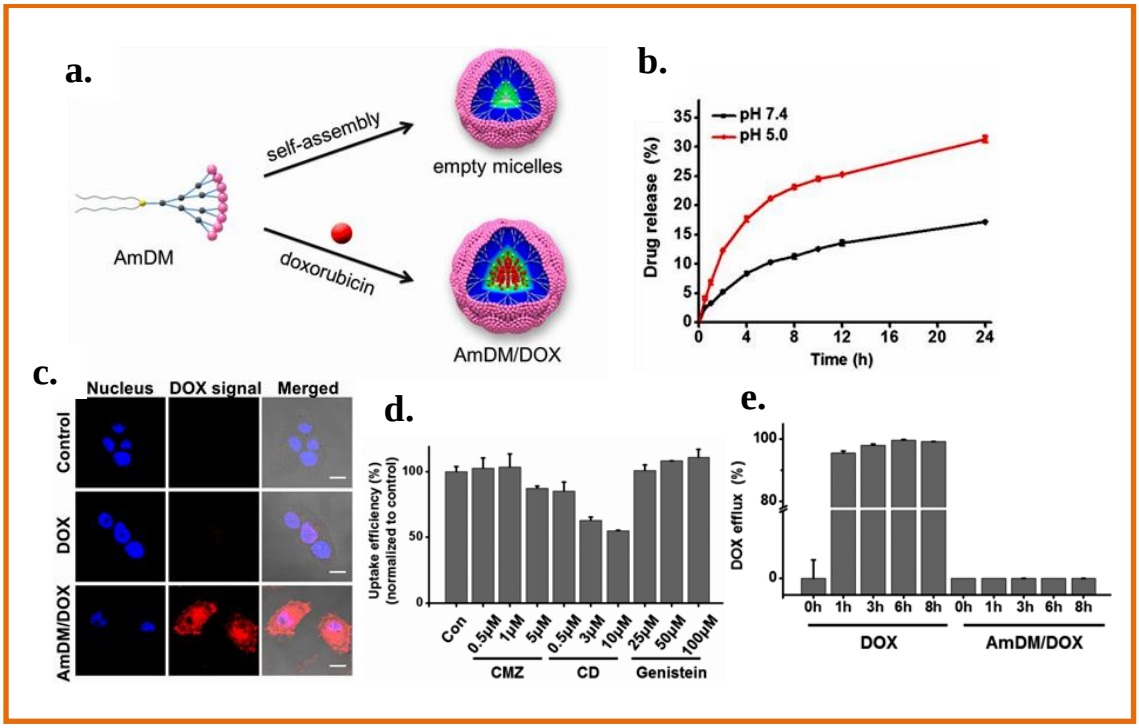

Figure 1.4: (a) Formation of empty amphiphilic dendrimers (AmDM) nanomicelles and DOX-encapsulated mDM/DOX nanomicelles. (b) *In vitro* DOX release behaviour from AmDM/DOX micelles at pH 5.0 and pH 7.4 at 37 °C. (c**)** The cellular uptake was imaged using confocal microscope following treatment with free DOX and AmDM/DOX in MCF-7R cells. (d) Inhibition of the uptake of AmDM/DOX micelles on MCF-7R cells using specific endocytosis inhibitors. CD: inhibitor of macropinocytosis; genistein: inhibitor of caveolae-mediated endocytosis; CMZ: inhibitor of clathrin-mediated endocytosis. (e) Inhibition of doxorubicin efflux by AmDM/DOX nanomicelles was determined in MCF-7R cells [Wei et al., 2015].

ligands has been developed with high stability, high drug loading capacity and sustained release profile in physiological environment while prompt release exhibited in acidic environment [Shi et al., 2014]. Supramolecular nanomicellar system based on the amphiphilic dendrimer (AmDM) has been developed to effectively deliver the clinical anticancer drug DOX, enhance anticancer activity, and combat drug resistance while obviating systemic toxicity [Wei et al., 2015]. The use of lipid- and polyion complex-

based micelles for rapid generation of multivalent agonists targeting necrosis factor receptor (TNFR) exhibiting promising therapeutic potential has been reported recently [Gilbreth et al., 2016].

1.1.3.5 Mesoporous silica nanoparticles

Mesoporous silica nanoparticle (MSNP) has emerged as promising biomaterials for the delivery of drug, DNA, or siRNA molecules as a result of their unique properties, e.g., facile synthesis, biodegradable nature, biocompatibility, optical transparency, water dispersibility, physiochemical stability, adjustable and ordered porous channel, tuneable pore size, and easy functionalization chemistry for bioconjugation [Trewyn et al., 2007; Liong et al., 2008; Slowing et al., 2008; Meng et al., 2010; Wang et al., 2015].Various research groups have demonstrated that MSNPs system is an efficient carrier for the delivery of anticancer drugs, including paclitaxel, camptothecin, and doxorubicin in a targeted fashion and release them on demand to enhance their cellular internalization without any premature release prior to reaching the target tissue [Lai et al., 2003; Liong et al., 2008; Kim et al., 2008; , Vivero-Escoto et al., 2009; He et al., 2011]. MSNPs can target a tumor cell via both passive and active targeting pathway. In passive targeting strategy, MSNs with a particle size in the nanoscale range can accumulate in tumor tissues via the enhanced permeation and retention (EPR) effect and site specific delivery can be achieved via active targeting pathway by functionalizing MSNs with targeting ligands such as folate (FA) or EGF [Zhu et al., 2010; Mamaeva et al., 2011, Luo et al., 2011; Mackowiak et al., 2013]. Antibodies, peptides, and magnetic nanoparticles can also be functionalized with MSNs, thereby acting as homing devices.

1.1.3.6 Layered Double Hydroxides (LDH)

In recent years inorganic nanocarriers have emerged as promising materials for the delivery drugs, gene and imaging agents at the target sites due to their great advantages, such as large surface area, better loading capacity of drug, better bioavailability, controlled release of drug and lower toxic side effects and unlike polymer-based nanoparticles they can tolerate most organic solvents. Among the inorganic nanocarriers, layered double hydroxides (LDHs), also known as hydrotalcite-like material, are gaining increasing attention recently for their potential as delivery carriers because of their excellent biocompatibility, excellent anion exchange capacity, high drug/gene loading efficacy, full protection for the loaded therapeutics, pH responsive release, facile preparation method, low cost, can easily and efficiently penetrate the cell membrane and deliver most of the intercalated molecules into cells, excellent endosomal escape, biodegraded in the cellular cytoplasm (pH = 4–6) and furthermore drug release rate can be tailored through structural modifications [Miyata 1983; Xu et al., 2006; Ladewig et al., 2009; Wang et al., 2012]. LDHs are a class of natural/synthetic two-dimensional (2D) nanostructured anionic clays whose structure can be described as containing brucite-like layers LDH two dimensional (2-D) structure can be described as containing brucite-like layers, where a fraction of the divalent cations (e.g., Mg^{2+}, Ca^{2+}, Ni^{2+}, Zn^{2+}, etc.,) coordinated octahedrally by hydroxyl groups have been replaced isomorphously by trivalent cations (e.g., Al^{3+}, Fe^{3+}, Co^{3+} etc to give the layers a net positive charge (**Figure 1.5**) [Evans et al., 2006; Leroux et al., 2005]. This charge is balanced by interlayer hydrated anions, resulting in a multilayer of alternating host layers and with exchangeable gallery anions, such as Cl^{-}, NO_3^{-}, CO_3^{2-}, etc.

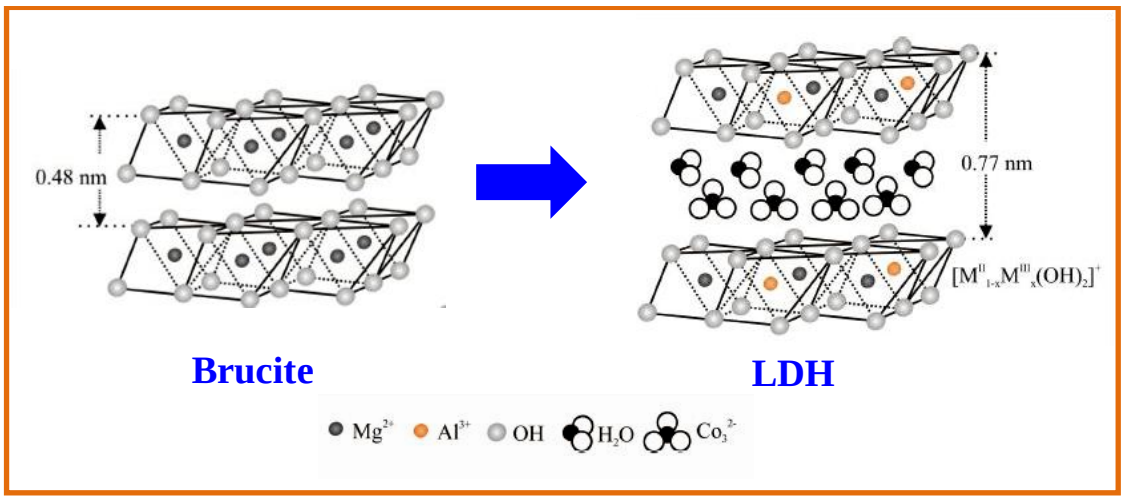

Figure 1.5: Schematic diagram showing formation of LDH structures by isomorphic substitution of Mg^{2+} of brucite by Al^{3+} [Basu et al. 2014].

LDHs can be described by the general formula:

$$[M_{1-x}^{z+} M_x^{3+} (OH)_2]^{x+}[A^{n-}]_{x/n} \cdot mH_2O$$

Where M^{z+} and M^{3+} are the uni or divalent and trivalent layer cations respectively, $0.2 < x < 0.33$ and A^{n-} is the exchangeable anion. **Table 2** summarizes some the commonly reported LDHs having varied cations and interlayer anions.

There are a number of approaches to the preparation of LDHs: (I) coprecipitation methods [Liu et al., 2004; Seftel et al., 2008]; (II) the hydrothermal urea method [Arai et al., 2009; Lv et al., 2006]; (III) the sol gel method [Prinetto et al., 2000], (IV) the microwave irradiation method [Hussein et al., 2000; Benito et al., 2006] and (V) the alkali metal method [He et al., 2006; Othman et al., 2009]. Furthermore, some other approaches, such as modified sol-gel synthesis using ethanol and acetone solvents [Aramendí et al., 2002], and a fast nucleation process followed by a separate aging step at elevated temperatures [Zhao et al., 2002], have also been reported. The co-precipitation technique is the facile and most commonly used of all methods for the synthesis of LDHs

and I have also employed this technique to synthesize various LDHs during my PhD work.

Table 1.2: Summary of the commonly reported LDHs:

M^+/M^{2+}	M^{3+}	Interlayer anion (A^{n-})	Chemical composition	Ref.
Li	Al	OH	$[Li_{0.33}Al_{0.66}(OH)_2](OH)_{0.33}.nH_2O$	[Fogg et al., 1999]
Li	Al	NO_3	$[Li_{0.33}Al_{0.66}(OH)_2](NO_3)_{0.33}.nH_2O$	[Meyn et al., 1990]
Li	Al	Cl	$[Li_{0.33}Al_{0.66}(OH)_2](Cl)_{0.33}.nH_2O$	[Chisem et al., 1994]
Li	Al	Br	$[Li_{0.33}Cl_{0.66}(OH)_2](Br)_{0.33}.nH_2O$	[Besserguenev et al. 1997]
Ca	Al	NO_3	$[Ca_{0.66}Al_{0.33}(OH)_2](NO_3)_{0.33}\cdot 0.66H_2O$	[Millange et al., 2000]
Ca	Al	CO_3	$[Ca_{0.66}Al_{0.33}(OH)_2](CO_3)_{0.17}\cdot nH_2O$	[Millange et al., 2000]
Ca	Al	OH	$[Ca_{0.66}Al_{0.33}(OH)_2](OH)_{0.33}\cdot n$H2O	[Leroux et al. 2001]
Co	Al	Cl	$[Co_{0.66}Al_{0.33}(OH)_2](Cl)_{0.33}\cdot nH_2O$	[Prevot et al., 2001]
Cu	Cr	Cl	$[Cu_{0.69}Cr_{0.31}(OH)_2]Cl_{0.31}\cdot 0.61H_2O$	[Rousse et al., 2000]
Cu	Al	$[Fe(CN)_6]$	$[Cu_{0.88}Al_{0.5}(OH)_2][Fe(CN)_6]_{0.31}\cdot 1.25H_2O$	[Challier et al., 1994]
Mg	Al	Cl	$[Mg_{0.66}Al_{0.33}(OH)_2](Cl)_{0.33}\cdot nH_2O$	[Borja et a., 1992]
Mg	Al	NO_3	$[Mg_{0.66}Al_{0.33}(OH)_2](NO_3)_{0.33}\cdot nH_2O$	[Choy et al., 2000]
Mg	Al	CO_3	$[Mg_{0.66}Al_{0.33}(OH)_2](CO_3)_{0.17}\cdot nH_2O$	[Brindley et al., 1979]
Mg	Cr	CO_3	$[Mg_{0.71}Cr_{0.29}(OH)_2](CO_3)_{0.145}\cdot nH_2O$	[Prakash et al., 2001]
Mg	Fe	CO_3	$[Mg_{0.75}Cr_{0.25}(OH)_2](CO_3)_{0.125}\cdot nH_2O$	[Raki et al., 1995]
Ni	Al	NO_3	$[Ni_{0.67}Al_{0.33}(OH)_2]\ (NO_3)_{0.20}\cdot 0.7H_2O$	[Caravaggio et al., 2001]
Ni	Fe	CO_3	$[Ni_{0.75}Fe_{0.25}(OH)_2](CO_3)_{0.125}\cdot 0.38H_2O$	[Kagunya et al., 1996]
Zn	Al	Cl	$[Zn_{0.66}Al_{0.33}(OH)_2](Cl)_{0.33}\cdot 0.66H_2O$	[Depege et al. 1996]
Zn	Al	NO_3	$[Zn_{0.66}Al_{0.33}(OH)_2](NO_3)_{0.33}\cdot 0.66H_2O$	[Meyn et al., 1990]
Zn	Al	CO_3	$[Zn_{0.75}Al_{0.25}(OH)_2](CO_3)_{0.13}\cdot nH_2O$	[Miyata et al., 1973]

Anionic therapeutic agents (including drugs, genetic materials, peptides, proteins, imaging agents etc.) can be easily intercalated into the interlayer host gallery through direct synthesis, coprecipitation, anion exchange techniques and are thus can be protected against enzymatic degradation [Rives et al., 2014]. Moreover, their internal and/or

external surfaces can be easily modified and functionalized to facilitate site specific targeting function, provide high specific surface area and improve chemical stability, emerged them as an attractive candidate for diverse applications. Several investigations demonstrated that LDHs can intercalate diverse important anionic bioactive molecules, such as DNA, siRNA, nucleotides and anticancer drugs, exhibiting controlled delivery with enhanced therapeutic efficiency and bioactivity as well. The first LDH–porphyrin intercalation compound was developed by stirring a suspension of Mg-Al LDH in a solution of 5,10,15,20-Tetra(4-sulfanatophenyl) porphyrin (TSPP) at 60 °C for one week [Park et al 1989]. The interlayer gallery spacing of the intercalated material has been found to be 22.4 Å which indicates that the porphyrin intercalated with the molecular plane perpendicular to the hydroxide layers. From elemental analysis they confirmed that 81% of the Cl^- ions of precursor LDH had been replaced and further they used infrared spectroscopy to prove that the porphyrin intercalated intact. Intercalation of vitamin A (retinoic acid), vitamin C (ascorbic acid) and vitamin E (tocopherol) into Zn-Al LDHs has been obtained by the coprecipitation method [Hwang et al., 2001]. These vitamins are normally all sensitive to light, heat and oxygen in solutions and after incorporating these molecules into a layered inorganic lattice proposing that this may lead to their stabilization, resulting in a wider range of potential applications.

Anionic drug molecules have also been intercalated into various LDHs, for the storage, transport and ultimately controlled release of the drug [Ambrogi et al., 2001; Khan et al., 2001; Del Arco et al., 2004; Chakraborty et al., 2011]. The *in vitro* release behaviors of the drugs have been investigated by adding their intercalation compounds to samples of simulated gastrointestinal and intestinal fluid (**Figure 1.6**). It has been reported that LDHs can also serve as hosts for the controlled release of a plant growth regulator, a-naphthaleneacetate [Hussein et al., 2002]. For the delivery of non-ionic, insoluble drugs,

a unique strategy has been employed by intercalating the water insoluble drugs into micelles and then intercalating the drug embedded micelle into LDHs host layer [Tyner et al. 2004].

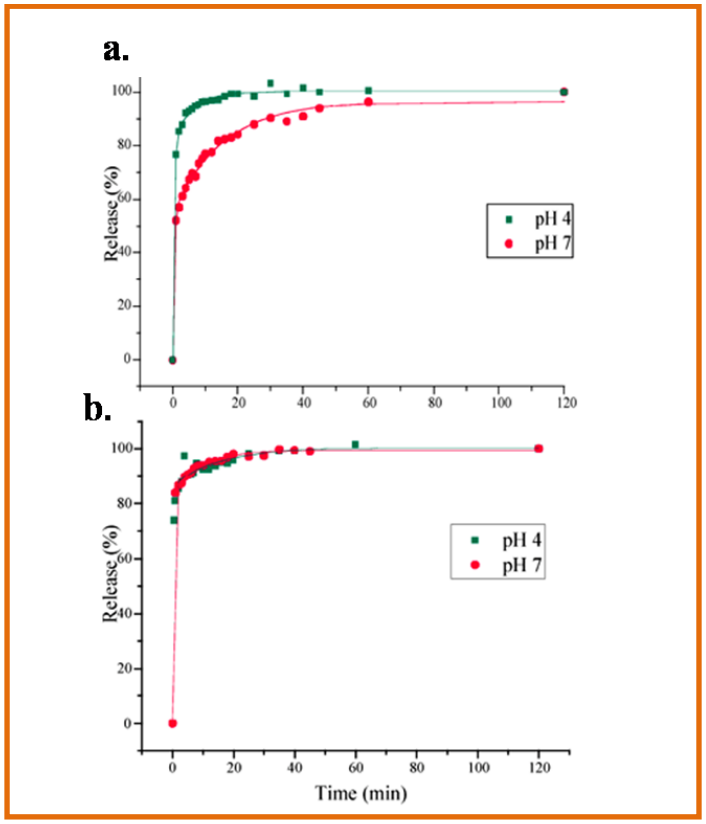

Figure 1.6: *In vitro* drug release profiles for $[Li_xAl_2(OH)_6][drug]_x \cdot yH_2O$ LDHs: (a) release of diclofenac at pH 4 and pH 7 and (b) release of gemfibrozil at pH 4 and pH 7 [Khan et al., 2001].

Large biological molecules such as DNA, ATP and nucleosides have been successfully interacted into the interlayer galleries of Mg-Al LDHs [Choy et al., 1999, 2000, 2001], where the host layers may protect biomolecules from degradation and also help their transport to specific targeted sites. This LDH-DNA complex has the advantage that it overcomes the repulsive interactions between the negatively charged cell membrane and the anionic biofunctional molecules during transfer into mammalian cells via endocytosis [Davis 1997; Ledley 1995]. The use of LDHs as gene delivery vehicles can not only enhance the thermal stability of DNA but also improve its cellular uptake efficiency.

Cellular uptake experiments have been performed in order to investigate the delivery potential of these hybrids. Li et al. monitored the uptake of FITC labelled Mg-Al LDH by NSC 34 cells preliminary to study of their potential for possible gene delivery applications [Li et al., 2013].

1.2 Scope of the present work

The prime target of ongoing research in cancer therapy is to achieve clinical efficacy with minimal adverse side effects to normal healthy tissues. Current drug/gene delivery strategies for cancer treatment are mainly based on chemical methods involve cationic compounds, recombinant proteins, polymeric or inorganic nanoparticles. Usually the cationic compounds are highly toxic in nature, while preparation cost of recombinant proteins are very high. Again, the polymeric systems are generally hydrophobic in nature, leading to weak cell-material interactions and as a consequence have poor cell adhesion and cellular uptake properties. Hence they require surface modification with positive moieties involving poly(L-lysine) (PLL) and polyethylenimine (PEI), to enhance their therapeutic efficacy. However, these cationic moieties are highly cytotoxic in nature. In recent years, inorganic based nanocarriers are becoming strong competitors due to their great advantages, such as large surface area, better loading capacity of drug, better bioavailability, lower toxic side effects, controlled release of drug and can be used in injectable form. Amongst them, positively charged layered double hydroxides recently emerge as one of the most promising inorganic nanocarriers that can easily penetrate the cellular membrane. The lacuna of present drug delivery systems motivates us to design and develop LDH based carriers for cancer treatment:

- Polymeric systems and mesoporous silica nanoparticle based carriers has been extensively investigated as a delivery vehicle for anticancer agents, while LDH remains unexplored although it possesses excellent profile of a delivery vehicle.

- Tailoring of drug release rate through structural variations of LDH has not been yet reported.
- *In vivo* tumor suppression study using LDH has not been investigated in details.
- *In vivo* biocompatibility of LDHs has not studied.
- Therapeutic efficacy of LDH-polymer nanocomposites as an anticancer drug delivery carrier has not been investigated.
- Transfection efficiency of LDH based system has not been studied.
- Potential of LDH as suicide gene therapy vehicle has not been investigated.

1.3 Objective of the present thesis work

The objective of this thesis research work is to design and develop LDH based nanocarriers for the controlled and safe delivery of anticancer drugs and plasmids for cancer treatments. To achieve this, a systematic design and evaluation strategies were employed as follows:

a. Synthesis and evaluation of Mg-Al based LDHs and tailoring of drug release rate through its structural variations for effective cancer treatment

- Synthesis and characterization of the developed Mg-Al based LDHs with varying interlayer anions
- Intercalation of a model anticancer drug intercalated into interlayer space of LDHs
- Physicochemical characterizations of LDHs and drug intercalated LDHs by using various analytical techniques
- Investigation and understanding of *in vitro* release behavior of the drug intercalated LDHs
- Evaluation of biocompatibility profiles of the developed LDHs
- Evaluation of *in vitro* therapeutic efficacy drug intercalated LDHs
- Investigation of tumor suppression efficiency of various drug intercalated LDHs in animal model
- Evaluation of the effect of controlled release on different organs

b. Development of layered double hydroxides-polymer nanoconjugate for enhanced cellular uptake and controlled delivery of hydrophobic anticancer drug

- Intercalation of hydrophobic anti-cancer drug, raloxifene hydrochloride (RH), into a series of zinc iron LDHs with varying anion charge densities
- Embedment of the drug intercalated LDH into polymer matrix to achieve *in vivo* sustained drug release profile
- *In vitro* cellular uptake studies
- Comparative evaluation of *in vitro* antitumor efficacy of the developed materials
- Investigation of *in vivo* drug release behaviors
- Evaluation of effect of controlled release on different organs.

c. Development of layered double hydroxide based gene delivery vehicle for cancer treatment

- Synthesis and characterization of the developed Li-Al based LDH
- Intercalation of DNA into the interlayer gallery of LDH
- Analysis of DNase protection assay
- Analysis of thermal protection assay
- Evaluation of cellular uptake efficacy of the developed vehicle
- Investigation of transfection efficacy of the developed vehicle
- Evaluation of the potential of the developed LDH based vehicle to induce p53 mediated apoptosis in mammalian cancer cell