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Trigger responsive polymeric nanocarriers for cancer therapy

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Abstract

Conventional chemotherapy for the treatment of cancer has limited specificity when administered systemically and is often associated with toxicity issues. Enhanced accumulation of polymeric nanocarriers at tumor site may be achieved by passive and active targeting. Incorporation of trigger responsiveness to these polymeric nanocarriers improves anticancer efficacy of such systems by modulating the release of drug according to the tumor environment. Triggers used for tumor targeting include internal triggers such as pH, redox and enzymes and external triggers such as temperature, magnetic field, ultrasound and light. While internal triggers are specific cues of tumor microenvironment, external triggers are those which are applied externally to control the release. This review highlights various strategies employed for preparation of such trigger responsive polymeric nanocarriers for cancer therapy and provides an overview of the state of the art in this field.

1. Introduction

Cancer has been a leading cause of death with ever increasing worldwide rate of incidence with 14 million new cases and 8.2 million cancer related deaths in 2012, according to WHO statistics. Despite decades of research in the field of cancer therapeutics, complete cure of cancer is yet not established and in late stages of cancer, patients are mostly given palliative care. This is due to the smart and complex nature of the disease; multiple metabolic pathways involved in its development and progression, its tendency to metastasize to distant tissues due to the presence of circulating tumor cells, ability to acquire multi-drug resistance through mutations and lastly the challenges faced in early detection of cancer. These factors contribute to limitations in cancer therapy despite several clinical advances. Conventional therapies to treat cancer mainly include surgery, chemotherapy and radiotherapy, with each of them having different advantages and limitations for varying types and stages of cancers. Chemotherapy is commonly employed treatment for most of the cancers, either alone or after

surgical resection of the tumor. Even after using potent chemotherapeutic drugs, there are limitations due to associated systemic side effects of cytotoxic drugs. When administered systemically, these highly potent cytotoxic drugs lack targeted action on tumors, causing damage to healthy tissues and hence related toxicity. Apart from the non-specificity, poor pharmacokinetics and biodistribution are the other major limitations of conventional chemotherapy. Along with it some of these highly potent drugs have poor solubility and hence they have to be given along with surfactant and solubilisers which sometimes are an additional source of toxicity.

1.1. Evolution of drug delivery systems

The concept of drug delivery systems as improvement to conventional therapy was given by Paul Ehrlich in 1906 and the term "magic bullets" was coined. Nanoparticulate carriers have been proposed and evaluated for drug delivery application to overcome the limitations of conventional therapy for more than three decades.[1] Along with overcoming the limitations of the conventional chemotherapy, nanocarriers also provide advantages of controlled and targeted drug release, increased encapsulation of highly hydrophobic drugs, as well as prevention of sensitive therapeutic agents like peptides, siRNA from degradation in the body while in circulation. Different type of nanocarrier systems have been proposed for cancer therapy, which can be broadly classified as lipid based nanocarriers which mostly include liposomes and solid lipid nanoparticles and polymer based nanocarriers which mainly include polymeric micelles and nanoparticles.

1.1.1. Polymeric nanocarriers

The concept of using polymers for delivery of therapeutically active was introduced in 1975.[2] Since then, different types of polymeric nanocarriers have been used for drug/gene delivery for cancer therapy, which include polymeric micelles, nanocapsules, dendrimers, polymersomes, polymeric nanogels and polymer drug conjugates. The advantage of using polymeric nanoparticles is defined control over their synthesis and modification. The first generation polymeric nanocarriers were prepared just to entrap the agent which was released non-specifically over time. To obtain the desired effect of the therapeutic agent, the second generation polymeric nanocarriers were modified to show targeted release of the drug in presence of environmental cues specific to the target site. Higher accumulation of nanocarriers at the tumor site can be achieved through passive and active targeting to tumor tissues. Passive targeting of nanosized carriers to reach solid tumors is inspired by the compromised tumor vasculature and poor lymphatic drainage, which allows the nanoparticles

to specifically permeate and accumulate in the tumor tissue.[3,4] This phenomenon is known as Enhanced Permeability and Retention (EPR) effect, resulting in selective extravasation and prolonged retention of circulating nanoparticles into the perivascular space of the tumor region by convective transport through endothelium.[5,6] Active targeting involves further modifications of the particles, so that they get recognized by cells at the tumor site which leads to their increased accumulation and uptake by target cells. To meet their nutritional demands, rapidly proliferating tumor cells over express certain receptors for uptake of nutrients like folic acid, sugars and vitamins. Different type of moieties may be used for targeting, like ligands for various cell surface receptors overexpressed in cancer cells, aptamers, or antibodies recognized by cancer specific surface antigens. Ligand targeting is an attractive approach for specific targeting of the drug but designing of such nano-vehicles becomes difficult as the ligands on the surface may lead to rapid uptake by RES or unwanted host immune response. Along with targeting of the nanocarriers to the tumor site, increased circulation time in body without loss of the encapsulated therapeutic agent is also important to maintain its required concentration for long time in the body. For prolonging the circulation time, the nanoparticle should minimize the binding of plasma proteins on its surface which may aggravate opsonization and clearance through RES system. The concept of stealth liposomes having hydrophilic surface, either a glycolipid or hydrophilic polymer on the surface have been proposed to increase circulation time and prevent RES clearance.[7] This concept was widely accepted and has been successfully exploited for polymeric particles with polyethylene glycol (PEG) chains on the surface, for more than a decade.[8] By far, PEG is the most popular choice to increase the longevity of the nanocarriers in circulation and various PEG based block polymers have been used in the literature.[9]

1.1.2. Targeting using triggers

Active and passive targeting strategies help in accumulation of nanosized particles at the tumor site but their anti-cancer efficacy can still be limited by insufficient drug release at the tumor site, which may be modulated by incorporating trigger responsiveness. Trigger refers to a specific stimulus at the tumor site which may be exploited to release the encapsulated therapeutic agent either in the tumor extracellular space or intracellular regions. Triggers used for tumor targeting of nanocarriers can be fundamentally categorized into two classes, internal and external triggers (Fig. 1). Internal triggers exploit the inherent characteristics of tumor cells and microenvironment which differ from normal tissue physiology, while external triggers involve the use of external source to cause drug release at tumor site causing preferential killing of tumor cells. Internal triggers mainly include pH,

redox and enzyme triggers as tumor microenvironment (TME) has relatively acidic pH, overexpression of certain enzymes, reductive and hypoxic conditions as compared to normal tissue environment. External triggers include the use of ultrasound, magnetic field, and light triggers which cause focused drug release from the nanocarriers or cause local hyperthermia (increase in temperature) of tumor tissues resulting in heat induced damage to tumors. The advantage of using trigger responsive nanocarriers is their ability to prevent non-specific drug leakage as they are designed to release the drug in the presence of the specific trigger, which imparts spatial and temporal control over drug release. Trigger responsive nanocarriers which respond to internal triggers, are in fact bioinspired systems as they respond to the biological cues in the body at the diseased site. In this review, we discuss trigger responsive polymeric nanocarriers for cancer therapy using different internal and external triggers, focusing on the approaches that have been used to make these responsive systems.

2. Design criteria for an ideal trigger responsive nanocarrier

An ideal trigger responsive polymeric nanocarrier should meet following criteria for cancer therapy,

i. High sensitivity to trigger at tumor site *in vivo*
ii. High penetration into tumor
iii. Negligible drug release in absence of trigger
iv. High accumulation at tumor site
v. Enhanced active uptake by tumor cells
vi. Reduced therapeutic dose
vii. Biodegradability of nanocarriers
viii. High stability in circulation with increased circulation time
ix. Reduced systemic toxicity - Prevention of non-specific drug release in circulation
x. Feasible for patient compliant routes of administration
xi. Ease of preparation and scale-up
xii. Cost effective

3. Internal triggers

TME plays an important role in tumor growth and metastasis, and has physiological conditions which differ from the normal cell environment. Tumor microenvironment has slightly acidic pH (6.8-7.2),[10] and overexpression of certain enzymes (cancer associated proteases);[11] tumor tissue also exhibits reductive environment as compared to normal

tissues[12] and in some tumors cells might be under oxidative stress producing more reactive oxygen species as compared to normal cells.[13] Most of these features can be attributed to the active metabolism of tumor cells owing to their rapid growth. Furthermore, these can be exploited as triggers for on-site delivery of drugs using responsive nanomaterials. In this section we discuss widely exploited internal trigger responsive polymeric nanomaterials for drug release in cancerous tissues.

3.1. pH responsive polymeric nanosystems

Tumor cells generate most of the energy through high rate of glycolysis by Warburg effect which results in accumulation of lactic acid in cytosol instead of oxidative phosphorylation as is carried out in normal cells.[10,14] Due to this increased fermentative metabolism and poor perfusion, the TME has an acidic pH (pH 6.5-7.2) compared to the normal tissue under physiological conditions (pH 7.2-7.4).[15] Other than the external pH of TME, there exists a pH gradient from early endosome (pH 6-6.5), late endosome (pH 5-6) to lysosomes (pH 4.5-5) ranging from pH 6.5 – 4.5, which may be exploited for intracellular delivery of cargo given that endocytosis is one of the important phenomena through which nanoparticles are taken up by tumor cells. Taking advantage of this natural cue, pH responsive drug delivery nanosystems have been designed to carry and protect drug molecules at physiological pH, specifically accelerate its release at the tumor site or to the specific organelles within the tumor cells and maintain the therapeutic drug concentration within the cells having acidic pH environment. Due to their specific advantage of delivering cargo to cytoplasm, pH responsive polymeric carriers have also been widely and successfully studied for gene delivery to cancer cells.

Researchers have employed variety of strategies to achieve pH responsiveness of drug delivery nanosystems (Fig. 2).[16,17 ,18] One such approach is use of polymers having ionizable functional groups (anionic and cationic polymers) which either accept or donate protons leading to different physical characteristics in response to pH changes. Insertion of acid labile linkages either within the polymer stretch or between the polymer and drug molecules is yet another approach for pH responsive nanoparticles. These linkages are stable at physiological pH, but undergo cleavage at acidic pH, thus ensuring a triggered release in the acidic TME. Acid labile linkages commonly used include imine, hydrazone, acetal, orthoester and cis-acotinyl bonds.[19] Each strategy is discussed in detail in the following subsections.

3.1.1. Polymeric nanosystems with ionizable functional groups

3.1.1.1. Anionic polymer based nanoparticles

Polymers having ionizable acidic functional groups such as carboxylic and sulfonic groups undergo hydrophobic/hydrophilic transition in response to pH changes. The pH at which acidic groups of polymers undergo protonation or deprotonation depends on the pKa values (negative logarithm to the base 10 of the acid dissociation constant) of the polymers, which is in turn controlled by the polymer composition and molecular weight. Below pKa value these anionic polymers are in deionized (protonated) form and are hydrophobic in nature and above pKa value acidic groups of these polymers undergo ionization (deprotonation) and become hydrophilic.[20] Polymers having pKa value in the acidic pH range have been found useful for the preparation of pH sensitive nanoparticles for oral delivery of drug molecules as they can retain their structure in the acidic environment in the stomach but release the encapsulated drug after absorption into small intestine due to ionization and swelling of polymers. Poly(acrylic acid) (PAA), poly(methacrylic acid) (PMA), poly(ethyl acrylic acid) (PEAA), poly(propyl acrylic acid) (PPAA), poly(butyl acrylic acid) (PBAA) and poly(glycolic acid) (PGA) are commonly used anionic polymers having carboxylic groups. PAA has been used in combination with hydrotropic polymers to make pH sensitive micelles for the oral delivery of PTX (paclitaxel).[21] Another widely explored application of these anionic polymers is for the delivery of cationic drugs such as doxorubicin hydrochloride (DOX). For example, complexation of positively charged ammonium group in the daunosamine part of DOX with negatively charged poly(ethylene oxide)-*b*-PMA (PEO-*b*-PMA) block copolymer based micelles at physiological pH has exhibited pH-responsive behavior with accelerated release of DOX in acidic environment due to the protonation of carboxylic groups in the cores of the micelles.[22] Similar approaches have been tried with Pluronic-PAA copolymeric micelles and microgel particles.[23,24] Dextran modified with succinic acid to introduce carboxylic acid groups so as to complex with DOX and further cross-linking with cisplatin *via* electrostatic and chelation reaction demonstrated controlled and pH dependent release of DOX. The systemic i.v. administration of these crosslinked nanoparticles carrying DOX have been shown to significantly inhibit tumor growth in human lung adenocarcinoma (A549) xenograft murine model due to the prolonged circulation, enhanced drug accumulation and facilitated intracellular release in the tumor cells.[25] pH-induced release of liposomal contents from PEAA incorporated liposomes was shown to be dependent on polymeric molecular weight. The pH at which PEAA conformation collapses from an expanded hydrophilic form to a globular hydrophobic coil due to protonization and starts to induce release of liposomal contents was found to increase with increase in the molecular weight of the polymer.[26]

Another category of pH sensitive anionic polymers are those with sulfonic groups. In polymers having sulfonamide groups, due to electron withdrawing property of oxygen atoms in $–SO_2NH$, there is movement of the N–H electronic cloud toward the nitrogen atom which results in ionization such that the polymers becomes hydrophilic above their pKa. pKa of these polymers vary according to the substituents on the sulfonamide groups.[27] As ionization of acidic protons of sulfonamide groups increases with increase in pH, the behavior of these polymers can be controlled over a narrower pH range close to physiological conditions, making them more sensitive than conventional carboxylic acid polymers. While the carboxylic acid based polymers show broad transitions in about one pH unit, much below the physiological and tumor pH range, sulfonamide polymers show much sharper transition within 0.2 pH units between the physiological and tumor pH. Conjugate of a sulfonamide derivative, sulfadimethoxine (SDM) with pullulan acetate demonstrated self assembly into nanoparticles of diameter 50–60 nm with a low critical aggregation concentration of 3.16×10^{-3} mg/ml at pH 7.4. These nanoparticles shrunk and self-aggregated at pH below 7, thus enhancing the release rate of encapsulated adriamycin (ADR).[28] At pH 6.8 these ADR nanoparticles showed improved cytotoxity and internalization in MCF-7 breast cancer cell lines compared to that at normal pH showing that SDM containing polymeric nanoparticles can enhance efficiency of tumor targeting using acidic TME pH as a trigger.[29] It has been demonstrated that under acidic conditions, detachment of sulfonamide occurs from the neutral nanoparticle complexes formed by electrostatic interactions between plasmid DNA, polyethylenimine (PEI), and poly(methacyloylsulfadimethoxine) (PSD), exposing plasmid DNA condensed with cell transfecting PEI, to act on cancer cells.[30] Deshielding of TAT-cell penetrating peptides at acidic pH has also been demonstrated using polymeric micelles having SDM. Using a two component polymeric micelle made by complexation of cationic TAT (TAT cell penetrating peptide)-micelle conjugate of poly(L-lactic acid)-b-PEG (PLLA-b-PEG) and a pH-sensitive anionic diblock copolymer (poly(L-cystinebisamide-g-sulfadiazine))-b-PEG (PCBS-b-PEG), TAT-micelles were exposed and its improved endocytosis was observed at pH 6.6.[31] Authors have not reported the efficacy of such systems in animal cancer models. Though in terms of sensitivity this system is superior to carboxylic group systems, non-degradability and toxicity of aggregates formed are issues of concern. Further this can be employed only for tumors having only slight change in pH from normal range.

3.1.1.2. Cationic polymer based nanoparticles

These are nanoparticles based on polymers having basic functional groups such as primary, secondary and tertiary amines which are ionized/protonated, acquires positive charge at or below pKa. Nanoparticles made of these polymers swell due to electrostatic repulsion between the positively charged groups and release the loaded drug molecules into the surrounding medium. These polymers undergo deprotonation/deionization above pKa values. Modification of polymers has been done to make the pH transition of these cationic polymers near to the physiological pH so as to exploit it for biomedical applications. Examples include poly(N,N′-dimethylaminoethyl methacrylate) (PDEAEM), poly(β-amino ester) (PbAE), poly(L-histidine) (PHIS) etc.

PDEAEM which was once widely used for transfection of cells due to its cationic nature are being explored for the development of pH responsive nanosystems. Copolymers of PDEAEM have been utilized for preparation of pH sensitive nanoparticles for delivery of cisplatin to cytoplasm. Cisplatin-loaded nanoparticles with pH-responsive PDEAEM cores synthesized using PDEAEM-PEG copolymer demonstrated dissolution at pH < 6 and rapid internalization and transfer to lysosomes. These nanoparticles showed enhanced cytotoxity compared to equivalent dose of free cisplatin. Further upon intraperitoneal administration, cisplatin released induced cellular pyknosis and reduced the tumor burdens of mice having intestinal/mesentery tumors, than the slow-release nanoparticles.[32] Monodispersedpoly(styrene-co-N,N'-dimethylaminoethyl methacrylate) nanoparticles having size of 100 nm showed enhanced release of encapsulated coumarin-6 at acidic pH.[33] Limitation of such systems is their non-biodegradable nature.

PbAE is yet another cationic polymer used for preparing pH sensitive nanosystems as it undergoes hydrophobic to hydrophilic transition and solubilization upon reducing the pH below its pKa. These are a class of cationic biodegradable step growth polymers synthesized by Michael addition polymerization reaction between amine and diacrylate monomers. Though PbAE containing tertiary amine, was first reported by in 1970,[34] Langer group has synthesized a library of over 5000 PbAEs by reacting diol-diacrylates with various types of primary and secondary amines and evaluated for cellular toxicity and transfection potential.[35,36,37] Being cationic, PbAE can condense and protect negatively charged stranded DNA and has been initially used as transfection vectors like any other cationic polymers. Their high buffer capacity helps escape of polycation/DNA complexes from an endolysomal system *via* the “proton sponge” effect.[38] In terms of biocompatibility, this has been found to be superior to other cationic amine containing polymers such as poly(lysine) and PEI.[39] Since PbAE is insoluble at physiological pH (pH 7.4) and undergoes rapid dissolution under acidic

pH (pH <6.5), this has been used to make pH responsive nanoparticles, which can at once release its contents within the acidic TME and endo/lysosome compartments of cells.[40]

To modulate the apoptotic threshold in tumor MDR, single PLGA/PbAE (70:30 by weight) blend nanoparticles have been designed for delivery of combination of PTX and CER (ceramide) in such a way that there is rapid release of PTX at acidic pH by the dissolution of PbAE, retaining slow release of incorporated CER which is associated with PLGA. Blend particles modified with PEO have demonstrated preferential accumulation in tumors with reduced clearance of PTX from the systemic circulation and tumor mass upon systemic administration in orthotopic (drug sensitive) MCF-7 and multidrug resistant MCF-7TR human breast adenocarcinoma models.[41] Ko *et al.* has synthesized an amphiphilic mPEG-PbAE block copolymer which formed nano-sized self-assembled micelles under aqueous conditions and loaded with DOX (74.5%) using a solvent evaporation method. These micelles have demonstrated demicellization at acidic pH (6.4) leading to fast release and enhanced cellular uptake of DOX. Tail vein injection of these micelles on B16F10 tumor-bearing mice remarkably suppressed tumor growth and prolonged survival as compared to mice treated with free DOX.[42] As an improvement to this system in terms of pH at which dissolution occurs, degradable amphiphilic PbAE copolymers with mPEG having low toxicity have been synthesized to form micelles with a core soluble at about pH 6 which carry drugs into endosomes/lysosomes and quickly disrupt their membranes to release the drug into the cytosol.[43] Similarly amphiphilic triblock copolymer PbAE*g*-mPEG-chol micelles encapsulated with DOX exhibited high cytotoxicity in human hepatocellular carcinoma (Hep G2) cells, whereas the copolymer showed low toxicity.[44]

Min *et al*. have prepared mPEG-b-PbAE copolymer micelle having a sharp pH-dependant micellization/demicellization transition at the tumoral acidic pH value (pH 6.4) for tumor imaging and therapy *in vivo* by encapsulating a fluorescent dye tetramethylrhodamineisothiocyanate (TRITC) and an anti-cancer drug campothecin (CPT) with an efficiency of 80%. These pH-responsive micelles released TRITC efficiently to the tumor tissue (MDA-MB-231 human breast tumor), with 11-fold higher targeting ability than that of mPEG-PLLA micelles without pH-responsiveness. Moreover, CPT encapsulated micelles exhibited significantly increased therapeutic efficacy with minimum side effects, compared to free CPT and CPT encapsulated PEG-PLLA micelles.[45] Gao *et al.* had prepared mixed shell micelles using poly(caprolactone)-b-PEG (PCL-b-PEG) and PCL-b-PbAE-c(RGDFK), such that targeting ligand c(RGDFK) remains hidden during circulation at pH 7.4 along with hydrophobic PbAE and only PEG is exposed on the surface prolonging the

circulation time in blood. On reaching tumor environment PbAE becomes hydrophilic and targeting ligand is exposed on the surface of the micelles enhancing the uptake of micelles by tumor cells. Significant difference was found in the IC_{50} of the micelles at pH 7.4 and pH 6.4, indicating pH dependent enhanced uptake. Also, these targeted micelles have shown high anti-tumor efficacy, 5 fold higher than non-targeted micelles and 10 fold higher than PEG micelles in Hep G2 xenograft model after one month of treatment.[46] Bioreducible PbAEs were prepared by introducing disulfide groups in each repeating unit of PbAEs by Michael addition polymerization of 2,2′-dithiodiethanol diacrylate, 4,4′-trimethylene dipiperidine, and methoxy-PEG-NH_2. The resultant polymer was used for preparation of micelles (size, 100 nm) having core–shell structure under physiological conditions, but quickly release the loaded drugs responding to acidic and reductive stimuli. DOX-loaded micelles showed higher cytotoxicity for Hep G2 tumor cells than free DOX.[47]

PHIS are another group of pH sensitive polymers having imidazole ring with an electron lone pair on the unsaturated nitrogen that have the ability to acquire a cationic charge below pKa value (pH 6) and induce destabilization of micelle. There are also some reports to show the fusogenic activity of PHIS by which it can disrupt the acidic enveloped membrane of subcellular compartments such as endosomes and allow drug/nucleic acids to reach the cytosol, enhancing the delivery efficiency.[48] Bae's research group have developed DOX-loaded micelle systems using mixture of 75wt% PHIS-PEG, and 25 wt% PLLA-*b*-PEG that showed destabilization of micelles at pH 6.8 and enhanced anticancer efficacy. Furthermore, they have modified micelles with folate to improve cytotoxicity against MDR tumor cells *in vitro* and *in vivo*. These folate modified micelles demonstrated superior drug distribution in the cytoplasm compared to free DOX and a control sample of DOX loaded pH-insensitive copolymer micelle having no histidine block. These pH-sensitive mixed micelle systems demonstrated effective antitumor efficacy against the multidrug resistant ovarian tumor A2780/DOX^R.[49,50,51] Liu *et al.* prepared DOX encapsulated pH-sensitive nanoparticles using PEG-PHIS-PLLA triblock copolymers for anticancer drug delivery. These nanoparticles (size 100 nm) were having an inner hydrophobic PLLA segment layer, middle pH-responsive PHIS blocks and the outer hydrophilic PEG chain layer. Release rate of DOX was much faster in pH 5.0 than that in pH 7.4 and the nanoparticles were efficiently internalized in Hep G2 cells.[52] Recently Qiu *et al.* conjugated PHIS to biopolymer hyaluronate (HA) to make pH-responsive and active tumor-targeted, HA-PHIS micelles, for use as a carrier for DOX. Authors have reported that those micelles having a low PHIS degree of substitution demonstrated highest cytotoxicity and cellular internalization towards CD44 receptors over

expressed MCF-7 showing that endocytosis was mediated *via* CD44 receptors. Clathrin-mediated endocytosis was revealed by endocytosis inhibition studies and then transported to lysosomes, where DOX was released from the micelles and finally made a way into the nuclei.[53] In another study by Hu *et al.*, PTX loaded cationic micelles of PHIS-block-short branched PEI were prepared and shielded by electrostatically complexing with a negatively charged mPEG-b-PSDM (Polysulfadimethoxin) at pH 7.4. PSDM being pH sensitive anionic block, prolong and protects the cationic micelles during circulation but once it reaches the tumor environment deshielding of the micelles occurs as the anionic block becomes neutral. Due to its positive charge, the cationic micelle is taken up by tumor cells and drug release occurs at endosomal pH, as a result of protonation of imidazole group of PHIS leading to dissociation of micelles. Thus the system is prepared to have pH responsiveness at tumor extracellular pH as well as endosomal pH. Cellular uptake of unshielded micelles (US-M) at pH 7.4 is similar to that of shielded micelles (S-M) at pH 6.6 confirming that the deshielding occurs at tumoral extracellular pH (Fig. 3). Similar pattern was observed for *in vitro* studies in MCF-7 cell line, IC_{50} of the unshielded micelles was lower than that of shielded micelles at pH 7.4 while IC_{50} values were similar for unshielded and shielded micelles at pH 6.6. *In vivo* studies were carried out in human breast cancer xenograft model, tumor volume reduction was found to be highest in case of shielded micelles, which was 3 fold higher than in unshielded micelles.[54]

3.1.2. Polymeric nanosystems with acid labile linkages

Acid-labile chemical bonds have received widespread interest to make pH responsive nano based drug delivery systems for cancer therapy. Nanoparticles with acid-labile linkers within the polymeric structure, either in the backbone in the junctions of block copolymers or in the side chain and between drug molecules and polymer, are designed to remain stable at physiological pH, but degrade quickly in the mildly acidic environment of lysosomes, endosomes, or tumor tissues, leading to rapid drug release. Acid labile linkages widely exploited to achieve this mission are acetal, orthoester, imine, hydrazone, oxime and cis acotinyl bonds which rapidly hydrolyze in the endosomal compartment (pH 5). In this section, we discuss the recent advances in smart nano delivery systems based on these acid-labile chemical bonds.

Polyacetals formed by condensation of polyols and divinyl ethers undergo rapid hydrolysis at acidic pH.[55] Acetals can be formed with wide range of alcohol functionalities and it is possible to tune their hydrolysis rate by choosing proper chemical structure. Hydrolysis of acetal linkages is generally first order relative to the hydronium ion, making

the probable rate of hydrolysis 10 times quicker with each unit of pH decrease.[56,57] Monodisperse prodrug micellar nanoparticles with average sizes in the range of 158.3-180.3 nm prepared by conjugating PTX onto water-soluble PEG-b-PAA copolymers *via* an acid-labile acetal bond exhibited high antitumor effect to human epidermoid carcinoma (KB) and HeLa cells (IC_{50} = 0.18 and 0.9 μg PTX equiv/mL, respectively) as well as PTX-resistant A549 cells. Folate modified micelle exhibited 12 fold reduction in IC_{50} value in KB cells. DOX was incorporated into this system for combination chemotherapy.[58] Zhao *et al.* developed comb-like amphiphilic copolymers bearing acetal-functionalized backbone based on poly[(2,4,6-trimethoxybenzylidene-1,1,1-tris(hydroxymethyl) ethane methacrylate-co-poly(ethylene glycol) methyl ether methacrylate] as effective nanocarriers for intracellular curcumin (CUR) release and evaluated efficacy on Hep G2 and EC-109 cells.[59] Same group have also developed dual trigger responsive (pH and reduction)-induced disassemblable nanoparticles based on an acid degradable cyclic benzylidenacetal groups-functionalized poly(2,4,6-trimethoxybenzylidene-1,1,1-tris(hydroxymethyl)ethane methacrylate)-g-SS-PEG graft copolymer for high loading efficiency and improved intracellular release of CUR. The nanoparticles were stable at physiological pH, and quickly disassembled in mildly acidic and reductive environments. While less than 15% of CUR was released in normal physiological conditions, 94.3% release was observed in the presence of reductive agent and mildly acidic conditions. Nanoparticles exhibited efficient internalization and growth inhibition toward EC-109 and Hep G2 cells.[60] Cyclodextrins have also rendered pH responsiveness by introduction of acetal linkages.[61]

Hydrazone[62] and cis-acotinyl linkages also undergo hydrolysis under acidic conditions and have been explored for making pH sensitive nano-drug delivery systems. Advantage of hydrazone linkage is that it will result in the release of drug as such rather than its modified form, which is the major limitation of using cis-acotinyl linkage to make pH sensitive nanocarriers. However, pH sensitivity of cis-acotinyl linkage in drug release at pH 5 over that at pH 7.4 is about 10 fold more, whereas same for hydrazone linkage is only four fold.[63] Stable polyionic micelles with charge conversion properties have been prepared by reversible conjugation with cis-acotinyl linkers which degrade at endosomal pH of 5.5 thereby exposing the primary amine groups of the proteins being delivered, and felicitating its release in the cytoplasm.[64] Kakinoki *et al*. synthesized PVA–DOX conjugates containing cis-aconityl acid-cleavable bonds which can release DOX inside lyosomal/endosomal compartments due to participation of the free carboxylic acid group in hydrolysis of the aconityl linker.[65] Cis-acotinyl linkage is more widely used for the preparation of pH

responsive prodrugs.[66] Micelles based on PEG-PAA block copolymer after modification with a hydrazone linkage demonstrated selective release of drugs at acidic pH (4-6).[67] Bae *et al.* prepared amphiphilic block copolymeric micelles based on PEG-poly(aspartate) conjugated to ADR *via* acid-sensitive hydrazone linkers that showed intracellular pH-triggered drug release capability, tumor-infiltrating permeability, and effective antitumor activity with extremely low toxicity.[68] DOX alone or in combination with a phosphatidylinositol-3 kinase inhibitor wortmannin was also covalently attached *via* hydrazone bond to this copolymer to achieve a synergistic drug action on MCF-7 human breast cancer cells.[69] Gold nanoparticles stabilized with a monolayer of folate-conjugated poly(L-aspartate-DOX)-b-PEG copolymer showed release of DOX more rapidly at pH 5.3 and 6.6 than at pH 7.4.[70] Core functionalized Pluronic micelles conjugated with DOX *via* hydrazone linkage showed major distribution in the cytoplasm, endosomal/lysosomal vesicles, and nucleus and greatly enhanced cytotoxicity for MCF-7 human breast cancer cells.[71] Albumin-derived core-shell DOX delivery system based on the protein denaturing-backfolding strategy where DOX molecules were covalently conjugated to the albumin polypeptide backbone *via* an acid sensitive hydrazone linker demonstrated high drug loading capacity, a two-step drug release mechanism based on pH and the presence of proteases, as well as fast cellular uptake. The low nanomolar range IC_{50} of these micelles for HeLa cells as well as leukemia cell lines indicate its attractive potential for anti-leukemia treatment.[72] Polymeric micelles prepared by conjugating DOX to adipic dihydrazide derivative of poly(styrene-co-maleic anhydride) and then encapsulating a P-gp inhibitor as well as an apoptosis inducer demonstrated enhanced cytotoxicity by increasing the intracellular accumulation of DOX and promoting the apoptotic response on drug-resistant breast cancer xenografts as compared to other combinations of both drugs.[73] Multifunctional unimolecular micelles based on hyperbranched amphiphilic block copolymer, Boltorn(®) H40-poly(L-glutamate-hydrazone-doxorubicin)-b-PEG conjugated with cRGD and NOTA have been found to synergistically integrate passive and active tumor-targeting abilities with pH-controlled drug release and PET imaging capabilities.[74] Dual endosomal pH-sensitive micelles based on poly(2-ethyl-2-oxazoline) (PEOz) conjugated to DOX (PEOz-hyd-DOX) *via* acid cleavable hydrazone linker were found to self-assemble into nanosized micelles. These micelles demonstrated enhanced antitumor efficacy and reduced side effects compared to free DOX.[75] Recently, Yu *et al.* reported the preparation of pH-responsive drug carrier, PEG-hyperbranched polyacylhydrazone (HPAH), which can form nanoscale micelles. The drug-loaded micelles (size 180 nm) showed the desired pH-dependent drug release properties and enhanced intracellular uptake with subcellular

localization mainly in the cytoplasm. PEG-HPAH-DTX (Docetaxel) micelles in combination with glucose exhibited a superior anti-tumor efficacy and a lower systemic toxicity *in vivo*.[76]

Orthoester is another type of acid labile bond introduced to polymeric back bone and side chain in order to make pH sensitive nanocarriers. Heller and coworkers have extensively investigated synthesis, characterization, properties and uses of poly(orthoesters) (POE). It is reported that ortho ester bond on the polymer back bone or side chain will be readily cleaved at accelerating rate under acidic pH by hydrolysis. Acid-sensitive micelle based on PEG–POE block copolymers showed degradation of core-forming POE block under acidic conditions and resulting micelles were stable for 3 days in PBS, at pH 7.4 and 37 $^{\circ}$C, but only for 2 h in citrate buffer, pH 5.5 at 37 $^{\circ}$C.[77,78,79] Micelles formed by self-assembly of amphiphilic diblock copolymer consisting of a hydrophilic PEG block and a hydrophobic poly(methacrylate) block (PEYM) bearing acid-labile ortho ester side-chains showed less than 10% degradation at pH 7.4 in 2 days. An accelerated degradation was observed at the lysosomal pH (~5) where hydrolysis was complete in no more than 5 h, suggesting that these micelles are potential candidates for delivering drugs into the lysosome. Micelles containing DOX were internalized efficiently by human glioma cells resulting in much higher intracellular drug concentration than cells treated with free drug leading to enhanced cytotoxicity.[80] Nanogels responsive to temperature, acidic and reductive environment have been prepared by the miniemulsion copolymerization of monomethyloligo(ethylene glycol) acrylate and an ortho ester-containing acrylic monomer, 2-(5,5-dimethyl-1,3-dioxan-2-yloxy) ethyl acrylate, with bis(2-acryloyloxyethyl) disulfide as a crosslinker. PTX-loaded nanogels exhibited concentration-dependent toxicity to MCF-7 cells envisaging potential of these nanosystems as carriers for hydrophobic anticancer drugs.[81] Deshielding of PEG also has been achieved by inserting orthoester linkage between PEG and copolymer of interest. Yuan *et al.* prepared micelles using 2-(ω-methoxy)PEGyl-1,3-dioxan-5-ylamine –g-poly(N-(acryloyloxy)succinimide-co-butyl methacrylate) polymer cross-linked by an acid-labile diamine cross-linker bearing two symmetrical cyclic orthoesters. By varying PEG content, cross-linked micelles of different morphologies were obtained; those with one mPEG segment exhibited 'echini' morphology whereas with three mPEG segments, nanowires were obtained. Crosslinked micelles exhibited pH-dependent hydrolysis with more rapid at mild acidic pH than physiological conditions. Hydrolyzates of the micelles also formed vesicles as new amphiphilic copolymers were formed. PTX loaded micelles revealed a controlled and pH-dependent release behavior.[82]

Linear pH-sensitive imine conjugate of mPEG with 4β-aminopodophyllotoxin (NPOD) demonstrated micelle formation in aqueous solution with significantly faster NPOD release at a mildly acidic pH of 5.0 and 4.0 than at physiological pH of 7.4 leading to enhanced cytotoxic effects in A549, HeLa, and Hep G2 cancer cells than the parent NPOD. IC_{50} of mPEG-NPOD was about one order magnitude lower than that of the free NPOD and reduced the size of the tumors significantly. These micelles with encapsulated hydrophobic PTX also showed pH-triggered fast release behavior and synergistic cytotoxic effects.[83] Micelle-forming amphiphilic polymer having benzoic imine linker has been found to be stable at physiological pH, while partially hydrolyzing at the extracellular pH of the solid tumor, and completely hydrolyzing at the endosomal pH. Due to the generation of amino groups from the cleavage of the imine bond at acidic tumor pH, the surface of the micelle changes from neutral to positively charged which facilitates the cellular uptake of the micelles. After endocytosis, complete cleavage of the micellar structure occurs at endosomal pH and the system becomes membrane-disruptive.[84] PEGylation of self-assembled noncovalently connected alpha-beta cyclodextrin dimer micelles *via* benzoic-imine bonds showed deshielding of the PEG segment at the tumor site and release of loaded drugs.[85] Polyurethane nanomicelle having a detachable mPEG linked *via* pH sensitive benzoic-imine linkage showed attractive self-assembly characteristics and stimuli-responsiveness, good cytocompatibility, and high loading capacity for DOX.[86]

In all these systems covalent modification of the polymer with drug molecules is performed which in one way helps in its exceptionally high loading given that the drug itself is a part of the micelle rather than merely an encapsulated agent. However the main disadvantage of this strategy is the prerequisite of covalently modifiable functional groups on the drug molecule and as not all drugs are capable of conjugation with polymers, the scope of this method is limited.

3.2. Redox responsive polymeric nanocarriers

Tumor cells and microenvironment are heterogeneous in nature.[87] A difference in redox states of tumor tissues as compared to normal healthy tissue is one such characteristic example of its heterogeneous state. There are many reports suggesting reductive environment of tumor cells with higher intracellular glutathione (GSH) levels compared to normal cells.[88] Also there are reports to exhibit oxidative stress in cancer cells, leading to an increased amount of intracellular ROS.[89] These different environments can exist in different type of cancers or at different regions within the same tumor or a single tumor cell depending on its

state.[88,89,90,91] Researchers have explored these enhanced levels of GSH and ROS as therapeutic targets for many nanoparticles designed for cytosolic drug delivery which is discussed in detail in the following subsections (Fig. 4).

3.2.1. Glutathione responsive nanocarriers

Glutathione (GSH) is a tripeptide, which is the most abundant reducing agent in mammalian cells having intracellular concentration in the range of 2 to 10 mM, about 100 to 1000 fold higher than its concentration in extracellular environment (2 -20 μM).[92] Being major endogenous anti-oxidant, GSH undergoes oxidation to form glutathione disulphide (GSSG). Both reduced (GSH) and oxidized (GSSG) forms of glutathione are present in normal cell, but an increase in GSSG/GSH indicates oxidative stress.[92] Besides this, tumor cells also have at least 4 fold higher concentration of GSH as compared to normal cells[12] making reducible nanocarriers of importance in tumor specific intracellular drug and gene delivery. Other than reducing environment in the cytosol due to GSH, the redox potential of the endosomes is modulated by an enzyme called Gamma interferon-inducible lysosomal thiol reductase in presence of cysteine.[93]

3.2.1.1. Reducible linkages within the polymer

Redox responsive nanocarriers are mostly prepared by using reducible polymers having disulfide linkages either in the polymer backbone or side chains. Polymers can be made redox responsive either by adding a disulfide cross linker, or oxidation of thiol groups, or thiol-disulfide exchange reaction. A large number of reduction responsive amphiphilic block copolymers have been used for exploiting the redox potential differences between tumor extracellular and intracellular spaces and most of these di and tri block polymer have a disulfide linkage between hydrophilic and hydrophobic units with the hydrophilic block being PEG.[17,47,94,95,96,97,98,99] Reduction sensitive polymeric nanocarriers have the advantage of fast intracellular release in response to reductive environment, high stability at physiological conditions, and enhanced anti-tumor activity as compared to reduction insensitive systems.

For carrying out *in vitro* studies for reduction sensitive systems, many researchers have used MCF-7 and Hep G2 cells as they have higher intracellular GSH levels. Also, it is possible to manipulate intracellular GSH levels of the cells by pretreating the cells with varying concentrations of glutathione ethyl ester.[100] Commonly used disulfide linker in synthesizing reduction sensitive polymeric systems include cystamine dihydrochloride,[17,101,102,103,104] bis(2-hydroxyethyl)disulfide,[84,97,99] 3,3′-dithiodipropionic acid (DTDP),[95,96,105,106] 2-iminothiolane,[107,108,109] N,N'-bis(acryloyl)cystamine,[110,111,112,113,114,115,116]

2-(2-pyridylthio)-ethanol as mentioned in Table 1.[117,118] Mezgharni *et al.* had prepared redox responsive polymeric nanocarriers using hyaluronic acid-glycyrrhetinic acid conjugate (HA-Cys-GA) which readily self-assembled in aqueous environment to form nanoparticles and were used for intracellular DOX delivery to Hep G2 cells. DOX release was significantly increased in presence of intracellular GSH concentration (10 mM) as compared to slow and sustained release in extracellular GSH (10 μM) or no GSH. The particle was claimed to be dually targeted to hepatocellular cells as they overexpress both HA and GA receptors, proved by higher affinity of these particles towards Hep G2 cells as compared to MDA-MB-231 breast cancer cell line. Nuclear localization of DOX and low IC_{50} was observed in Hep G2 cell line as compared to unresponsive particles. *In vivo* studies have shown higher tumor localization after systemic administration as well as high anti-tumor efficacy in Hep G2 xenograft mice model.[104]

Reduction responsive micelles of block copolymers have been used for GSH responsive intracellular drug release,[17,98,117] but these nanoparticles may show premature burst release of the drug. Also, these nanoassemblies tend to disassemble when the polymer concentration drops below CMC, which is one of the main reasons for drug loss while in circulation.[119] Crosslinking strategy has been employed to overcome this limitation but drug release becomes difficult from these cross linked micelles, so stimuli responsive cross linking has been employed to prevent drug leakage in circulation.[101,102,119,120] Amphiphilic triblock copolymer of methoxypoly(ethyleneglycol)-b-poly(ε-caprolactone)-b-poly(2-(2-oxo-1,3,2-dioxaphospholoyloxy)ethyl methacrylate) was cross-linked with cystamine to form a redox responsive copolymer.[101] These polymers were then self-assembled to form micelles for intracellular DOX delivery. When compared to uncross-linked micelles, core cross-linked micelles have shown lower CMC, higher drug loading and sustained release of DOX dependent on GSH concentration with highest release at 10 mM GSH. Core cross-linked micelles have shown higher intracellular localization in HeLa cells as compared to free DOX as well as DOX loaded in uncross-linked micelles.[101] Covalent cross linking of a core or shell of micelles is carried out to ensure stability in circulation and to prevent de-micellization however, it increases the complexity of manufacturing.

Another strategy used to obtain micelles which are robust while in circulation is preparing star shaped micelles which avoid drug release in circulation, and reduces the CMC without affecting the drug loading and delivering capability. Along with stability in circulation, accumulation at tumor site is also an important factor which can be enhanced by active targeting using tumor specific ligand, peptide or antibodies. One such example is that

of folic acid functionalized star shaped micelles of four armed PCL-PEG copolymer, which were synthesized using redox responsive DTDP linker.[95] It was found that the micelles having disulfide bonds showed higher release at intracellular GSH concentration of 10 mM, which was further enhanced at pH 5 as compared to pH 7.4. *In vitro* studies in HeLa cells have shown higher intracellular colocalization and lower IC_{50} of folate conjugated disulfide containing star shaped micelles. These micelles have also shown increased uptake by solid tumors and improved anti-tumor efficacy in murine breast cancer model.[95]

Polymeric nanocapsules have also been explored as reduction sensitive nanocarriers. Kim *et al.* had proposed a strategy for preparing template free, reduction responsive polymeric nanocapsules by self-assembly of amphiphilic cucurbit[6]uril, such that shell of these nanocapsules was crosslinked using a disulfide linker. These nanocapsules collapsed and aggregated after 30 minutes of DTT treatment and have shown triggered release of carboxyfluorescein in the presence of 100 mM DTT. Also, these nanocapsules were efficiently taken up by HepG2 cells with high intracellular accumulation of carboxyfluorescein.[106]

Apart from using disulfide linkers, reduction responsive monomers can also be used for preparing reduction sensitive polymeric nanocarriers. Among different monomers explored to design redox responsive polymers, 2-(pyridyldithio)ethylamine,[121] pyridyl disulfide,[117,122,123] and 2-(pyridin-2-yldisulfanyl)ethyl methyl acrylate[124] were found to be promising candidates as they can be modified with thiol capped molecules (drug/ligands) through covalent bond either before or after polymerization. Compared to the previously described systems which have to be functionalized before polymerization, these nanocarriers provide the option of functionalization after polymerization which can be carried out under milder conditions and gives more flexibility to the attached ligands. Similar advantage is also possible by attaching ligands through thiol-disulfide exchange reactions.[121,122,125]

Another interesting strategy recently employed to prepare bioreducible polymeric nanocarriers is the use of lipoic acid as one of the blocks of the copolymer.[126,127,128,129] The advantage of using disulfide containing lipoic acid (or thioctic acid) is it is naturally occurring and safe. Dextran-LA conjugates were synthesized and used to prepared nanocarriers for intracellular delivery of DOX. Efficient nuclear localization of DOX was seen in HeLa cells when these nanocarriers were used as compared to free DOX.[126] Also, a novel copolymer containing LA with PEG and Vitamin E was prepared such that these copolymers self-assembled to form micelles, which were used as carriers for tumor targeted PTX delivery. These nanocarriers have shown higher anti-tumor efficacy and 5-fold increase

in plasma concentration as compared to Taxol® in human ovarian cancer (SKOV-3) xenograft mice model.[129]

Reduction sensitive polymeric nanocarriers have also been explored for gene delivery. One such system which consists of PEG modified thiolated gelatin nanoparticles was proven to have high DNA transfection efficiency.[107] Modification of these particles was proposed later by preparing redox responsive EGFR targeted thiolated gelatin based nanoparticles for combination of gemcitabine and wild type p53 gene to target pancreatic adenocarcina. Gemacitabine was reacted with succinimidyl 3-[2-pyridyldithio]-propionate and the resulting product was then reacted with thiolated gelatin to form gelatin-gemcitabine disulfide conjugates. p53 plasmid DNA was added during particle preparation. *In vivo* efficacy was carried out in subcutaneous pancreatic adenocarcinoma model and higher tumor growth inhibition was seen in drug and gene combination group as compared to single agent therapy and superior activity was observed in case of targeted nanoparticles.[108] In another example of reduction responsive gene delivery, Kataoka and coworkers prepared a block catiomer (PEG-SS-P[Asp(DET)]) having bioreducible disulfide linkage between PEG and the polycation segment based on polyaspartamide with a flanking N-(2-aminoethyl)-2-aminoethyl group (DET) to condense DNA and facilitate endosomal escape. PEG-SS-P[Asp(DET)] formed stable micelles with pDNA, but these micelles aggregated when exposed to 10 mM DTT, indicating detachment of PEG as the disulfide linkages are cleaved. These micelles showed 1–3 fold higher luciferase gene transfection efficiency and gene expression in HeLa cells than micelles without disulfide linkages. Intracellular localization of these micelles carrying pDNA indicated that higher transfection by reduction sensitive micelles could be explained by endosomal escape due to the PEG detachment in the endosome.[130] Polymeric nanogels have also been explored for reduction responsive drug or gene delivery.[105,115,122,123,131,132]

3.2.1.2. Reducible linkages between polymer and drug molecules

Another class of redox responsive delivery vehicles apart from micelles based on di or tri block copolymer having disulfide linkages, is reduction sensitive polymeric drug conjugates which self assemble to form micelles.[97,113,125,133] As the conjugated drug in these systems becomes a part of the vehicle itself, another (or same) drug can also be physically encapsulated in these systems to make them dual drug delivery vehicles. PTX loaded PEG-disulfide-PTX (PEG-SS-PTX) conjugate nanoparticles were prepared; PEG-SS-PTX conjugates were self-assembled to form micelles and during this process free PTX was also added which remains in the hydrophobic PTX core of the micelles.[133] This results in rapid

burst release of physically entrapped PTX at once, and then sustained release of disulfide conjugated PTX in reductive cytosolic environment. PEG-SS-PTX had shown significantly enhanced release in the presence of 10 mM DTT (DTT used to simulate intracellular GSH). *In vivo* anti-tumor efficacy of PEG-SS-PTX evaluated in breast cancer xenograft mice model exhibited higher efficacy and lower toxicity as compared to Taxol®.[133] Polymer drug conjugates provides the advantage of high loading efficiency and prevention of unwanted leaching of the conjugated drug, which is released only in the intracellular reductive environment. However, it has a disadvantage that the drug has to be covalently conjugated to the polymer, so the drug must have suitable functional groups for the same and also the activity of the drug must not be altered while preparation of the conjugates or drug release at the tumor site.

3.2.2. ROS responsive nanocarriers

Among the most common intracellular ROS species such as hydrogen peroxide (H_2O_2), superoxide ions and hydroxyl ions, H_2O_2 has been more exploited for targeted release of anti-tumor agents due its high stability and its high cell concentration (0.5 nmol/10^4 cells/h).[13] The oxidative destabilization of polymeric vesicles was shown by Napoli *et al.* by preparing polymersomes using amphiphilic tri-block ABA copolymers such that A is hydrophilic PEG and B is hydrophobic poly(propylene sulfide) (PPS).[134] In an oxidative environment PPS is first converted from hydrophobic to hydrophilic poly(sulfoxide) and ultimately to poly(sulfone) on further oxidation, leading to destabilization of micelles as the hydrophobic core gets dissolved. Oxidation responsive nanocarriers for cancer therapy have been developed by using amphiphilic hyperbranched polymers consisting of alternating hydrophobic selenide groups and hydrophilic phosphate groups in a dendritic backbone. In an oxidative intracellular environment hydrophobic selenide is converted to hydrophilic selenone and selenium compounds can produce anticancer metabolites which cause apoptosis of cancer cells. So these nanocarriers have intrinsic anti-tumor activity which can act synergistically with the loaded drug. Intracellular nuclear localization of DOX loaded in such particles was seen in HeLa cells.[135] Similarly, pH and H_2O_2 responsive prodrug micelles were synthesized using β-cyclodextrin conjugated to DOX *via* hydrazone (for pH sensitivity) and ferrocine conjugated PEG (to impart H_2O_2 responsiveness). In presence of H_2O_2, ferrocine was converted to its ionic form and detached from the cyclodextrin cavity causing destabilization of the micelles.[136] Prodrug was cleaved to its active form at lysosomal pH at which hydrazone linkage is cleaved.[136]

ROS responsive polymeric nanocarriers were also explored for targeted gene delivery. Shim *et al.* prepared poly(amino thioketal)/DNA complexes which diassembled upon ROS exposure *via* cleavage of thioketal linkages. Intracellular disassembly of these reduction sensitive polyplexes, resulted in increased free labelled DNA content in PC3 cells (having high intracellular ROS levels).[137]

3.2.3. GSH and ROS responsive systems

Most of the nanocarriers described above are responsive to single redox trigger, either reduction sensitive or ROS sensitive. Wang *et al.* had prepared nanocarriers that could respond to both ROS and GSH.[138] A prodrug of SN-38, which is the active metabolite of irinotecan was prepared in this study, as its usage otherwise is limited by its very low solubility. For the preparation of the particle, (oligo(ethylene glycol) was conjugated to the phenol ester of SN-38 *via* thioether linkage such that the thioether chain could be easily oxidized by ROS and at the same time the phenol ester could undergo GSH triggered thiolysis and release SN-38. This amphiphilic prodrug was then self-assembled into nanocarriers that release drug in response to either ROS or GSH trigger. The prodrug nanoparticles have an additional advantage of high and fixed drug loading content along with almost 100% encapsulation efficiency. GSH is consumed during thiolysis and hence GSH mediated drug resistance can be reduced. These nanoparticles have shown lysosomal colocalization with low IC_{50} in Bcap37 cells as compared to the parent drug, irinotecan. *In vivo* studies in Bcap37 breast tumor xenograft model have shown higher tumor accumulation of the prodrug nanoparticles and better anti-tumor efficacy as compared to the free drug.[138]

Overall, redox responsive carriers provide benefits of higher difference in redox potential between intracellular and extracellular environment (approximately 100 times), which is much more than difference in pH and temperature differences for tumor environment making it an attractive trigger for intracellular cargo delivery to tumor cells. However, the exact location, mechanism and rate of reduction are not explicitly known and in depth analysis is needed to obtain these details. Further, most of the studies exploit higher value of 10 mM GSH to evaluate the *in vitro* release behavior whereas the actual concentration inside the cell may vary from 2 – 20 mM. So there is a need to develop sensitive systems which can be tuned to small changes in redox potential.

3.3. Enzyme responsive polymeric nanocarriers for cancer therapy

Cancer cells overexpresses certain enzymes differing from normal healthy cells, owing to different metabolism and requirements of cancer cells in terms of growth,

progression and invasion to new metastatic sites. These enzymes mainly include extracellular enzymes such as urokinase plasminogen activator (uPA), matrix metalloproteinases (MMP), hyaluronidase, β-glucouronidase and intracellular cathepsins (Table 2). Enzyme responsiveness is a comparatively less explored area among different responsive nanocarriers, but it exhibits vast potential and certain characteristic advantages as compared to other triggers like light, temperature, magnetic, ultrasound etc. Advantages of using cancer specific enzymes as trigger include their specificity and high expression in tumors such that their expression correlates with their prognosis and invasiveness, and hence this strategy can be used to target highly invasive tumor cells.[139] Secondly enzyme responsive carriers are inherently smart as they do not require any additional moiety; also prior knowledge of tumor localization is not needed and they can even act on small metastatic lesions which are not detected microscopically. This is not possible for external triggers like ultrasound, light or magnetic field as the application of their respective sources is dependent on the knowledge of area affected by tumor. Enzyme responsive drug delivery vehicles employ a based peptide substrate specific for the targeted enzyme activity such that this peptide acts as a linker between the polymer and functional units or is directly tagged to the therapeutic agent (like in prodrugs) (Fig. 5).

3.3.1. Response to extracellular enzymes

MMPs are an important class of extracellular enzymes which have gained importance in tumor targeted therapy as they play an important role in growth and progression of tumors.[140] Among MMP family, MMP2/9 (also known as Gelatinases A/B or Collagenase IV) are widely overexpressed in most tumor cells.[141] So, unlike redox responsive carriers which are explored for intracellular delivery, most of the enzyme responsive systems have tumor extracellular environment as their active site.

The use of MMPs for targeted drug release had started in late 1990's, which initiated with peptide prodrugs, where the activity of the drug is restored after the peptide is cleaved by MMPs. These are however associated with drawbacks such as fast clearance from circulation and non-specific accumulation at non-target tissues. To overcome these limitations, carrier protein conjugates were investigated. Kratz *et al.* prepared DOX albumin conjugates such that the maleimide DOX peptide sequence, specific for MMP2 cleavage was bound to cysteine 34 residue of serum albumin.[142] This albumin DOX conjugate was efficiently cleaved by MMP2 and MMP9 releasing DOX tetrapeptide, which was further cleaved to get free DOX. Further evaluation of this conjugate for its *in vivo* activity exhibited

higher maximum tolerated dose and efficacy than free DOX in A375 human melanoma xenograft which is known to have high MMP2 expression.[143]

The MMP responsive peptide linked polymer drug conjugate was reported by the Langer group in 2004 to obtain dual advantage of polymer drug conjugates and peptide prodrugs.[144] They have prepared and characterized PEG linked to methotrexate through (Pro-Val-Gly-Leu-Ile-Gly) PVGLIG peptide which is a target for both MMP2 and MMP9 which are highly expressed by tumors as identified through a combinatorial library. *In vivo* efficacy of these conjugates in xenograft tumor models keeping controls as free drug and polymer conjugated to drug through insensitive linker, demonstrated enhanced efficacy without significant side effects at lower therapeutic doses as compared to free drug.[145]

Several researchers have also worked on liposomes containing PEGylated lipids through MMP cleavable linker so that the PEG gets cleaved in the tumor extracellular environment and liposomes can be efficiently endocytosed by the tumor cells.[146,147,148,149,150] PEG-lipid functionalized conjugates has also been used to prepare self-assembled PTX prodrug nanomicelles in such a way that one of the conjugates contain PEG attached to PTX through MMP2 sensitive linkage and another TAT peptide conjugate which is used for cell penetration. This system has shown enhanced cellular uptake and higher tumor targeting in non-small cell lung cancer xenografts as compared to unresponsive system and free Taxol®, resulting in higher *in vitro* and *in vivo* efficacy.[151]

PEG-PCL micelles have also been explored for MMP responsive DTX delivery.[139,152] mPEG-PCL nanoparticles having gelatinase sensitive peptide linkages exhibited aggregation after gelatinase action (Fig. 5).[139] Their cellular uptake and *in vitro* cytotoxicity in cancer cell lines was dependent on gelatinase levels and was higher than that of Taxotere® and unresponsive nanoparticles. Effectiveness of these nanoparticles was also shown in primary cell line isolated from pericardial fluid of lung cancer patients. Dong *et al.* had prepared pH and enzyme dual responsive nanoparticles for DOX delivery.[153] Initially polyGC(double strand DNA fragments)-DOX was prepared such that DOX is intercalated in this nucleic acid sequence and then it was combined with gelatin to give enzyme responsiveness by MMPs. To impart pH responsiveness, this was then conjugated with PEG-Histidine tagged alginate. *In vivo* studies in murine sarcoma tumor model have shown higher accumulation of DOX in tumor and reduced accumulation in heart ultimately leading to increased anti-tumor efficacy and decreased cardiotoxicity.[153]

Another cancer associated protease is urokinase plasminogen activator (uPA) which has been exploited by Basel *et al.* by preparing responsive polymer caged liposomes.[154] To

develop protease responsive polymeric shell, a cholesterol anchored copolymer of PAA and short peptide substrate of uPA were used. This shell was then cross-linked using diamine to completely cover the liposomal surface. The design of the particles was optimized in the study and desired release of carboxyfluorescein was obtained in presence of uPA. Aside from these proteases, hyaluronidase in tumor ECM has also been explored as a target for drug delivery systems.[155,156] Chen *et al.* had proposed an easy strategy for enzyme responsive targeted drug delivery by coating mesoporous silica nanoparticles with hyaluronic acid. This hyaluronic acid layer plays dual role of targeting as well as capping agent.[155] This offers advantages like simplicity, biocompatibility, colloidal stability, TME responsiveness and increased cellular uptake through CD 44 receptors. Increased release rate of rhodamine B in presence of hyaluronidase 1 enzyme along with higher cellular uptake in targeted MDA-MB-231 cell line had been shown in this study. β-Glucouronidase, which is expressed in tumor necrotic extracellular regions was exploited by Talelli *et al.* for preparing biodegradable and thermosensitive polymeric micelles using PEG-b-poly[N-(2-hydroxypropyl)methacrylamide-lactate] such that DOX prodrug doxorubicin-glucuronide is covalently conjugated to the core of these micelles and free drug is released in the presence of enzyme.[157]

3.3.2 Response to intracellular enzymes

Among intracellular enzymes, cathepsins have been mainly considered for tumor targeted delivery.[158,159,160,161,162] Biodegradable polymersomes were prepared using block copolymers of mPEG-PLA such that peptide substrate for Cathepsin B was introduced between the two blocks.[158] These polymersomes were also modified with EGFR antibody for active targeting to EGFR overexpressing tumors. The disintegration of polymersomes was found to be dependent on both the presence of cathepsin B and lysosomal pH. *In vitro* experiments had confirmed that peptide linker was cleaved in the lysosomal compartment.[158] PEGylated peptide dendron nanocarriers conjugated to DOX through cathepsin B substrate peptide (GFLG) were prepared and their *in vivo* efficacy in 4T1 murine breast cancer model have shown higher tumor accumulation and anti-tumor efficacy of this delivery system.[161] PEG-PTX prodrug was prepared by conjugating PEG and PTX using p-aminobenzylcarbonyl spacer and valine–citrulline (VC) substrate of cathepsin B (CB), as linker. The synthesized PTX conjugate had shown increased water solubility, high therapeutic index and environment sensitive release as compared to Taxol® *in vivo* in human breast cancer xenograft model.[160]

The challenge for exploitation of enzymes as trigger could be the heterogeneous expression of different enzymes in various tumors, which depend on the tumor physiology and metastatic potential. This might vary from patient to patient. Another crucial factor is

specificity and stability of the peptide substrates used, while in circulation as they should be designed to cleave specifically at the tumor site by a tumor specific enzyme.

4. External triggers

Unlike internal triggers, external triggers have an inherent advantage of providing remote control over the release of drug at target site in a spatiotemporal manner. The polymers which are sensitive to external triggers like temperature, light, magnetic field and ultrasound, are potential agents for external stimuli responsive nanocarriers mediated on-demand drug delivery in cancer.[163] In this section we will be discussing about various external stimuli responsive polymeric nanoparticles in detail.

4.1. Temperature responsive polymeric nanoparticles

Temperature is an important factor to sustain the metabolic activity of cells. The body temperature of 37 ºC is an optimum condition for functioning of intracellular enzymes. If cellular temperature increases above tolerable limit 43-45 °C (hyperthermia) then it becomes difficult for the cells to maintain their activity, mainly because of denaturation of enzymes. Temperature may be an important physical agent to kill the cells by altering it in spatiotemporal manner.[164,165,166] Temperature mediated killing of cells is one of the fast growing field for anticancer therapy because of its cost affective nature and easy availability. Recently, hyperthermia has been used along with temperature responsive drug loaded nanoparticles to induce sufficient level of cytotoxicity to cancer cells.[167,168] There are many factors that need to be considered while treating cells by combinational therapy of drug and temperature. For instance, the drug should release at the target site in adequate amount while hyperthermia is being applied. So, researchers are looking for some temperature sensitive polymer which can be decanted on exposure of heat at tumor site and increase the drug availability. Apart from the pH, temperature is also a factor which has been found to be distributed oddly in tumor as compared to normal tissue. For example, *in vivo* brain tumor temperature was higher than body temperature, in some cases 38 ºC and in others it is lower than 34 ºC. There is discrepancy in temperature of different type of tumors of brain.[169] In breast tumor, temperature of core area is significantly higher than the neighboring tissue.[170] Because of the architectural difference and bit higher temperature the blood flow in tumor region is found to be very high which results into higher extravasations and can be exploited for drug delivery.[171] However, this temperature difference has not been exploited as internal trigger for the design of temperature responsive polymeric drug delivery systems mainly

because of lack of even distribution of temperature on tumors and unavailability of polymers sensitive to this range of temperature.

For drug delivery systems, mainly polymers having lower critical solution temperature (LCST) near body temperature or where the LCST can be fine-tuned by copolymerization with hydrophobic or hydrophilic residues have been preferred. N-isopropylacrylamide (NIPAM) based polymers are one of the most studied thermosensitive polymers of LCST category.[172,173] Some other LCST based thermosensitive polymers which have been used so far include, poly(N-vinylalkylamide), polysaccharide derivatives, pluronics, tetronics, PLGA–PEG–PLGA triblock copolymers, poly (N-isopropyl acrylamide) (PNIPAM), poly(N-vinylcaprolactam), poly(N,N-diethylacrylamide), phosphazene derivatives, chitosan derivatives, poly(ethylene oxide) (PEO) and poly(propylene oxide) (PPO).[172,174] UCST class polymers will be in soluble phase above their critical temperature but become insoluble below their UCST. These are less commonly used for drug delivery because they require high temperature for phase separation which is unfavorable for heat sensitive drug or other labile biomolecular agents that are to be delivered.[175]

Rao *et al.* developed pluronic based thermosensitive co-polymers in order to make CUR encapsulated pluronic F127-chitosan co-polymeric nanoparticle (nCCM). Authors have reported temperature dependent change in size of nanoparticles, 22 and 300 nm at 37 and 22 °C respectively and because of the smaller size of nanoparticles at body temperature, a high uptake by prostate cancer PC-3 cell lines was seen. When mild hyperthermia was applied intracellular concentration of drug was increased significantly due to thermal responsive nature of nCCM. Hyperthermia also increased the nuclear localization of the nanoparticles, which might be because of electrostatic interactions between negatively charged nuclear membrane and positively charged nanoparticles at 43 °C. Hyperthermia along with nCCM decreased the IC_{50} value by 7 fold on PC-3 cell line. Authors have employed dry bath for applying hyperthermia to cells, which do not simulate the *in vivo* conditions for translation.[168] Poly (N-isopropylacrylamide-co-acrylamide-co-allylamine) (PNIPAM-AAm-AA) conjugated to the surface of adriamycin (ADR) encapsulated BSA nanoparticles have been designed for thermal targeting.[176] Authors claimed that PNIPAM-AAm-AA tethered BSA nanoparticles circulate through blood and precipitate around cancer cells by phase transition upon increasing local temperature around 42 °C. PNIPAM-AAm-AA prevented unnecessary release of drug under normal physiological conditions. The amount of nanoparticles adhered to the cell surface at 43 °C was higher as compared to that at 37 °C.[176] Xu *et al.* developed thermoresponsive PNIPAM-co-AM-b-PLA polymers which self-assemble to form micelles in

aqueous medium. Micelles were tested by encapsulating three drugs, PTX, 10-hydroxycamptothecine (HCPT) and prednisone in order to obtain the efficient micelles-drug formulation. The cloud point of co-polymeric micelle was reported as 43-54 °C. PTX loaded micelle was quite efficient as compared to two other drug micelles regarding higher drug loading capacity, entrapment efficiency and thermoresponsive behaviour towards release of drug.[177] Another acrylamide based polymer, poly(acrylamide-co-acetonitrile)-g-polyethyleneglycol (Poly (AAm-co-AN)-g-PEG) developed by Li *et al.* had a UCST of 43 °C and was capable of undergoing self-assembly to form micelles in aqueous solutions at ambient temperature. Almost 80% of the encapsulated DOX was released from micelles at 43 °C but at body temperature, release was slow with minimal release. The cytotoxicity was evaluated on BEL-7402 human hepatic carcinoma cell line; IC_{50} of drug was reduced significantly from 4.91 μg/ml of DOX loaded micelles without hyperthermia to 1.56 μg/ml of DOX loaded micelles accompanied by hyperthermia. The IC_{50} of free DOX without hyperthermia had not shown any significant difference when hyperthermia treatment was given.[178]

One of the advantages of PNIPAM based block polymer is that they do not require toxic organic solvents for nanoparticle formation and can self-assemble in aqueous phase. However, major disadvantage is its non-biodegradability.[179] Several attempts have been made to reduce its toxicity and increase biodegradability, for instance, by conjugating it with a biodegradable polymer like chitosan. Rejinold *et al*. reported a thermoresponsive CUR loaded nanoparticle from co-polymer chitosan-g-PNIPAM by simple ionic gelation method.[180] Different weight ratios of chitosan and PNIPAM-COOH were chosen to synthesize a co-polymer with the desired LCST for hyperthermia applications. Same group also prepared 5-flurouracil encapsulated biodegradable thermoresponsive polymer chitosan-g-poly(N-vinylcaprolactam) nanoparticles by ionic cross-linking method having LCST of 38 °C.[181] Like other PNIPAM based polymeric particles, this nanoparticle has also shown higher amount of drug release above LCST. Authors reported that the prepared nanoparticles without drug were nontoxic to MCF-7, L929, KB and PC3 cell lines. Apoptosis of MCF-7 breast cancer cells were increased when treated with 5-FU nanoparticles, as compared to normal cells (Table 3). Some other NIPAM based polymers have been developed in order to make temperature sensitive carrier for heat trigged drug delivery in spatiotemporal manner.[182,183,184]

Mi *et al.* have synthesized a multimodality treatment nanoparticle (MMNP) system of a co-polymer PLA-TPGS and TPGS –COOH by blending these components along with

iron oxide nanoparticle and docetaxel.[167] This particle was further surface modified by coupling with ethylenediamine followed by reaction with Herceptin using EDC/NHS coupling method. Authors have investigated the efficacy of this MMNPs formulation on HER2 positive cell line SK-BR-3 *in vitro*. There were four anti-therapeutic agents in this formulation of MMNPs; Herceptin, docetaxel, IONPs and hyperthermia induced by IONPs along with magnetic field. Authors have demonstrated that this multimodality treatment was satisfactory and IC_{50} of the MMNPs was reduced drastically about 2130 fold more efficient than the just mixture of equivalent single modality treatments.[167]

Although these findings are indispensable but still long way to go in order to make it more efficient for anticancer therapy. One of the limiting factors is sensitivity of polymer towards the small temperature difference between normal cells and cancer cells, if we are considering only internal hypothermia as a sole stimulus for drug release. So the polymer should be highly efficient as far as the sensitivity towards change in temperature is concerned. Within that small window the polymer has to change their phase to release the drug and moreover the amount of dispensed drug must reach to the optimum level for proper treatment. Because of this limitation the hyperthermia has often been generated by some external physical stimuli. This should not only increase the local temperature but also maintain it for a while to induce release of drug in adequate amount. Some of such external agents which are being used for hyperthermia include magnetic field, ultrasound/HIFU and light. These stimuli are discussed in the following subsections.

4.2. Magnetic field responsive polymeric nanoparticles

Magnetic nanoparticles (MNPs) have shown promise in cancer therapy because of its specialized property of undergoing continuous magnetization and demagnetization under applied alternating magnetic field (AMF) leading to generation of heat due to Neel relaxation or Brownian movement and hysteresis loss.[185] This can induce cytotoxicity in cancer cells either directly by temperature shock[186] or stimulus based release of therapeutically active substance from nanoparticle. MNPs are commonly made up of either magnetite (Fe_3O_4) or maghetite (γFe_2O_3), both of them in oxide form.[187,188] Superparamagnetic nanoparticles (SPIONs) used for magnetic based hyperthermia can be categorized in three groups according to their hydrodynamic diameter; oral SPION (300 nm-3.5μm), standard SPION (SSPIO, 50-150 nm) and ultra-small SPION (USPIO, <50 nm). SPION with size range 10-100 nm are found to be most favorable for intravenous injection. Magnetic nanoparticle with sizes more than 200 nm are more susceptible to sequestration by spleen whereas particles with size of

less than 10nm are more open to fast renal clearance.[189,190,191,192] Further, MNPs has the ability to form aggregates when subjected to strong magnetic field because of dipole–dipole interaction amongst particles. In order to minimize their aggregation and increase their stability, magnetic nanoparticles have been coated with polymers such as dextran and chitosan.[193,194,195] As these polymers can be either synthetic or natural biodegradable polymers, it enables the functional group modification which can be used to covalently attach the therapeutic drug through heat labile bond to facilitate the temperature sensitive release of drug from the nanoparticles. For magnetic field responsive release, one can tailor the nanoparticles in such a way that the drug can be released in a spatiotemporal manner by encapsulating both magnetic nanoparticles and the drug in the same compartment. For example, polymerosomes composed of a di-block polymer Poly(trimethylene carbonate)-block-poly(l-glutamic acid) (PTMC-b-PGA) showed encapsulation of both hydrophobic as well as hydrophilic species, 6% w/w encapsulation of DOX and 30% w/w encapsulation of SPION (USPIO; γ-Fe2O3) simultaneously and was able to induce 18% increase in toxicity on HeLa cell line under high frequency AMF (14 mT at 750 kHz).[196] Authors reported that the increase in cell toxicity is because of release of DOX from polymerosome disintegration, not because of magnetic hyperthermia alone. PNIPAM based amphiphilic block polymer may also be used to coat the magnetic nanoparticles which permits the encapsulation of both hydrophobic as well as hydrophilic drug. As this co-polymeric material would have temperature sensitivity because of this particular component and be capable of change their conformation on increase in temperature owing to AMF application. When temperature increases above 32 °C it undergoes phase transition from swollen hydrated state to shrunken desiccated state and releases the encapsulated drug (Fig. 6).[173] Similarly, a different group Koppolu *et al.* have developed PNIPAM coated iron oxide nanoparticles (IONPs). The IONPS were functionalized by vinyltrimethoxysilane (VTMS) and then PNIPAM was coated over it by free radical polymerization of NIPAM monomer.[197] Further, these NIPAM coated nanoparticles were encapsulated in PLGA by double emulsion solvent evaporation technique along with PVA in a role of surfactant. BSA and CUR were used as model drugs for encapsulation. The BSA was first encapsulated in the covering shell of PNIPAM nanoparticles whereas CUR was encapsulated in outer covering shell made up of PLGA which was surrounding bunch of PNIPAM nanoparticles. The group had studied the release kinetics to show the temperature sensitive release of BSA.[197] PNIPAM based polymers coating IONPs have been widely exploited section as far as thermosensitive trigger based magnetic nanosystems are concerned.[198,199,200,201,202]

Magnetic nanoparticles were developed by co-precipitation and coated with mesoporous silica to form MNP-MSNs for controlled as well as targeted delivery to glioma cells.[203] DOX was encapsulated in this mesoporous silica coating and another drug PTX was encapsulated in PLGA covering around MNP-MSNs by process of double emulsion solid-in-oil-in-water evaporation. In order to provide tumor selectivity, these particles were conjugated with transferrin, as latter is over expressed in glioma (U-87) cell lines.[204] The final nanoparticles were designated as MNP-MSN-PLGA-Tf NPs. The efficacy of system was evaluated both *in vivo* and *in vitro*. For *in vitro* efficacy U-87 cell line was treated with final formulation followed by application of magnetic field. The authors reported that the cytotoxicity of the nanoparticles increased when magnetic field was applied after nanoparticle injection, as compared to free drug DOX, PTX and DOX–PTX–NP–Tf alone. Higher efficacy of formulation was also obtained *in vivo* in U-87 tumor bearing BALB/c nude mice (Table 4).[203]

Size of the magnetic nanoparticles plays an important role in attaining superparamagnetism.[205] However, very small size of nanoparticle does not provide enough specific loss power necessary for temperature rise at target site, especially when MNPs are not targeted.[206,207] Very small nanoscale particles with size in order of 10 nm are vulnerable to glomerular filtration by kidney and will be having very less circulation time *in vivo*. For larger nanoparticles, there is an increased tendency for conversion of magnetic property from superparamagnetism to ferromagnetism which may lead to instability because of agglomeration at < 32 °C. Deka *et al.* devised a strategy to overcome this by encapsulating a cluster of IONPs inside a shell of temperature sensitive polymer PNIPAM.[194] Cluster of nanocrystals (nanobeads) were used as an alternative to individual IONPs to provide enough magnetic moment and moreover now each and every single domain would be able to sustain their superparamagentism and collectively they would provide enough magnetic moment to generate necessary heat for therapeutic purpose. However, size of the IONPs encapsulating PNIPAM nanoparticle need to be fine-tuned for maintaining adequate circulation time.[198,208]

There are few examples where magnetic nanoparticles are used in combination with other stimuli like pH in order to increase their efficacy. Yadavalli *et al.* developed a dual responsive system which can respond to changes in both, pH as well as hyperthermia, for targeted cancer treatment. Chitosan is well known for its pH sensitivity whereas PNIPAM is known for its temperature labile phase transition from swollen to shrunken state that facilitates the release of encapsulated content. A complex polymer was generated by 2% glutaraldehyde treatment to induce cross linking between NIPAM and chitosan.

Nanoparticles prepared from this complex polymer had plenty of amine functional groups on the surface that makes it possible to covalently attach the folic acid and fluorescein by EDC-NHS coupling to provide an active targeting moiety on the final particle. Nickel ferrite magnetic nanoparticles doped with gadolinium were encapsulated to provide magnetic property and CUR was the encapsulated drug.[209]

4.3. Ultrasound responsive polymeric nanoparticles

Ultrasound mediated drug delivery systems have advantages of being noninvasive and established safety due to its widespread use in diagnosis.[210] Therefore, if the therapeutic efficacy of ultrasound is evaluated then it would have dual feature of imaging and as well as therapy, term is also known as theranostics. There are two main components in ultrasound mediated drug delivery; ultrasound contrast agent, which is a microbubble in most cases and drug, which is necessary to induce therapeutic ability to this contrast agent. A microbubble is a small vesicular structure having core shell feature and filled with a gas. The shell may be of either lipid, protein or any other polymer in order to increase the shelf life and circulation time in blood after injection. The interior cavity is made up of some heavy gases like sulphur hexafluoride, perfluoropropane, perfluorohexane and nitrogen or some volatile liquid which can change its phase from liquid to gas as an after effect of increase in temperature. This core has the ability to reflect ultrasound strongly.[211] Due to the difference in the density of gas present in bubble core and the surrounding fluid the bubbles start oscillating when subjected to high frequency ultrasound (1-10MHz).[212,213] In an ultrasound field with low acoustical pressure microbubbles are stably cavitating and start oscillating around a given diameter, but when the same microbubbles are subjected to an ultrasound field with high acoustic pressure then inertial cavitation occurs, and oscillation of the microbubbles become more violent eventually disintegrating the bubble (Fig. 7).[214] When microbubbles collapse they cause the plasma membranes in close proximity to become permeable temporarily through formation of transient pores, induced by shockwaves produced due to collapsing.[215,216,217] This phenomenon is known as sonoporation[218] and such transient pores have shown to be beneficial for enhanced uptake of drugs from extracellular environment.[216]

The principal aspect that need to be optimised is the design of a polymer which is either having direct sensitivity towards ultrasound so that it can undergo disintegration induced by ultrasound, or can undergo conformational changes because of secondary manifestations of ultrasound. For instance, increase in local temperature and ROS generated due to ultrasound can lead to heat and free radical mediated depolymerisation of some

polymers respectively.[219,174] Meanwhile some other liposome based ultrasound mediated drug delivery had been developed and tested *in vitro* and *in vivo.* In order to provide contrast ability to such drug carrying liposomes or temperature sensitive liposomes, microbubbles were conjugated to them.[210,220] Gourevich *et al*. reported that cyclodextrin based polymeric nanoparticles have the potential to show ultrasound mediated thermal degradation and increase in drug uptake by monolayer of cancer cells (MCF-7 and A375M). It had been found that the drug uptake was increased 5.5 fold on treatment of focused ultrasound (FUS) when mechanical effect was taken into consideration without increasing temperature. In FUS based thermal induction, the cellular internalization of encapsulated DOX was enhanced by a factor of 9.6. However, when hyperthermia was induced without using ultrasound then internalization was enhanced by factor of just 5.7.[221] Besides this Ninomiya *et al.* studied N-isopropylmethacrylamide-co-NIPAM (poly (NIPMAM-co-NIPAM)) modified liposomes which were able to release DOX sufficiently by ultrasound stimulus.[222] This study demonstrated that the polymer modified liposomes released drug due to ultrasound trigger alone and not because of ultrasound mediated thermal effect. Cavitation derived shear forces enhanced the cellular uptake and cytotoxicity in Hep G2 cell line as well. In addition to this, Rapoport *et al*. described that PEG-PLLA copolymer can be used to make nanodroplets encapsulating both ultrasound contrast agent as well as antitumor agent PTX.[223] On ultrasound treatment these nanoemulsions were converted to bubbles because of temperature driven phase transition of encapsulated perfluoropentane from liquid to gas. This transition leads to conversion of nanoemulsion droplets to microbubbles, which act as ultrasound contrast agents. Higher *in vivo* efficacy of the ultrasound responsive microbubbles were observed in human breast cancer MDA-MB-231 and ovarian cancer A2780 tumor xenograft mice models (Table 5).[223] In a different approach, Eggen *et al*. had reported a PEGylated NPs of the polymer, poly-N-butylcyanoacrylate (PBCA) nanoparticle stabilized microbubble.[224] These PEGylated NPs with model drug demonstrated heterogeneous distribution inside the tumor but revealed significant tumor uptake because of ultrasound induced extravasation of nanoparticles. Nestor *et al*. had reported development of nanocapsules having property of ultrasound contrast agent and nanoscale size of 370 ± 96 nm with a shell thickness of 50 nm.[225] The covering shell of contrast agent was a biodegradable polymer PLGA and the core cavity was filled with air. This study was carried out with an intention to make nanoscale contrast agents which can access to the fenestrations of the vasculature feeding cancerous cells by EPR effect, as opposed to most of the reported or commercially available microbubbles which are of micron size and hence may have limited penetration. Apart from

the size, another important parameter is the stability of the bubbles. Once the ultrasound pressure is applied then the bubble's shell should be stable enough to sustain the pressure of regular pulsation or compression-rarefaction. In this regard the polymeric shell shall be more prominent as compared to single layered lipid shelled microbubbles. Although the polymeric shell may be less flexible and may compromise contrast ability to some extent but it will increase the shelf life or retention time *in vivo*.[225]

Considering the drawback of large sized conventional microbubble based ultrasound contrast agents, Yang *et al*. developed a new type triple responsive (ultrasound/pH/GSH) biodegradable PMAA-PFH (Poly(methyl acrylic acid)-Perfluorohaxane) nanocapsule.[226] The nanocapsule was filled with PFH and DOX loaded PMAA. The covering sheath of the nanocapsule was of PMAA and DOX was cross linked to this sheath by disulfide linkage. The size of the nanocapsule was around 300 nm which is less than the conventional microbubbles and can easily reach to the tumor site by passive EPR effect. The encapsulation efficiency of DOX was quite high 93.5%. The nanocapsules were able to enhance the imaging signal after acoustic vaporization of PFH liquid to gas. Authors stated that PMAA is biodegradable and safe for *in vivo* use. The group has tested the biocompatibility of UCA (ultrasound contrast agent) nanocapsules on HEK 293 cells and cytotoxicity of drug containing nanocapsules on HeLa cell line. Pancreatic tumor xenograft bearing nude mice and healthy rats were used for *in vivo* imaging. However, *in vivo* therapeutic efficacy was not reported.[226] Chen *et al*. designed a dual responsive (ultrasound and pH) self-assembled vesicles of block polymer PEO-b-P(DEA-stat-TMA) composed of PEO, 2-(diethylamino)ethyl methacrylate (DEA) and (2-tetrahydrofuranyloxy)ethyl methacrylate (TMA). The formulation was non-toxic below 250 mg/ml. From *in vitro* release study of encapsulated DOX, authors concluded that the formulation showed ultrasound as well as pH responsiveness.[227]

Ultrasound has also been used for the purpose of gene delivery. As chitosan is positively charged at physiological pH, it can be used for entrapping negatively charged DNA. Cavalli *et al*. developed chitosan nanobubbles for ultrasound triggered gene delivery. The core of nanobubbles consisted of perfluoropentane as a source of ultrasound contrast. The diameter of DNA coated nanobubbles was around 300 nm with positive zeta potential. *In vitro* transfection efficiency of DNA coated nanobubbles was evaluated in COS7 cells in presence of ultrasound. Nanobubbles have not shown any transfection in absence of ultrasound. *In vitro* release study of DNA was tested with and without ultrasound. Authors

reported that the chitosan nanobubbles were stable for 3 minutes even after insonation with a frequency of 2.5 MHz at 37 °C.[228]

Ultrasound can also be used to disrupt weak bonds by overcoming the energy barrier. Wang *et al.* reported one such co-polymer, poly (ethylene oxide)-block-poly (2-tetrahydropyranyl methacrylate) (PEO-b-PTHPMA) to make ultrasound responsive disruption of micelles. pH of the micelle solution was decreased upon high intensity focussed ultrasound (HIFU) exposure at room temperature. HIFU hydrolysed the THPMA at room temperature which resulted in cleavage of the THP group. This study had shown that ultrasound has potential to induce breakage of bonds but it depends on the power of ultrasound, concentration of micelle and volume of focal point of ultrasound.[229]

Overall, patient compliant nature of ultrasound is well established in terms of diagnosis. Therefore, clinical translation is expected to be easier. However, there has been limited development of ultrasound responsive polymeric nanoparticles for drug delivery. It needs to be studied thoroughly with respect to their stability, biocompatibility, ultrasound responsive nature, and ultrasound contrast ability (for theranostics).

4.4. Photoresponsive polymeric nanoparticles

Light is another attractive stimulus for the drug delivery in cancer cells like other external triggers as it can be remotely controlled and so many parameters like wavelength, intensity and duration of exposure can be modulated to increase the release profile of drugs. Light is being used in several ways for the treatment of cancer; photodynamic therapy, photochemical internalization[230] and photoresponsive polymeric nanoparticles. Photodynamic therapy is the process of treatment of various diseases by using photosensitizer along with the particular light source. This combination of light and photosensitizer leads to the generation of ROS from molecular oxygen which facilitates killing of cancer cells by reacting with various biomolecules like proteins, lipids, DNA etc.[231]

Apart from photodynamic therapy, there is one different aspect of photon based cancer therapy, photoresponsive polymeric nanoparticles for light triggered release of anticancer therapeutic agent. Therapeutic agent can be encapsulated in polymer having ability to release encapsulated agents after exposure of light. Attaching a photochromic group to polymers is a popular approach for making light responsive block polymers which shows responsiveness towards light either by physical (i.e. change in polarity or hydrophobicity) or chemical (i.e. cross linking and isomerisation) phenomena.[232] Out of all light responsive phenomena, the hydrophilic and hydrophobic switching of polymer side chains in response to

light has been studied most elaborately. Others approaches include light induced breaking of block junctions of polymer, backbone degradation and reversible cross linking.[232] The photochromes like spiropyrans (SPs), azobenzene, diazonaphthoquinone (DNQ), O-nitrobenzyl and coumarin have been used a lot in order to make light responsive polymers.[19,232,233,234,235,236,237]

4.4.1. Spiropyran based photoresponsive polymer

Spiropyran has the ability to show light driven change in fluorescent property form ring open to closed form reversibly. Ring closed form is colourless spiropyran (SP) and ring open form is coloured merocyanine (MC). The SP and MC forms are sensitive to different light sources UV light (365 nm) and visible light (550 nm) respectively. This SP and MC states have different physical and chemical properties. So once the SP form is incorporated inside the polymer, then after photo irradiation hydrophilic – hydrophobic imbalance occurs in the copolymer due to isomerization of SP to MC, which enables disruption of the nanoassembly. Nanocarriers made up of this polymer can be used as a probe for cellular imaging and tracking simultaneously.[232] Tong *et al.* reported development of spiropyran (SP) based PEGylated lipid nanoparticles which undergo light induced reduction in hydrodynamic diameter from 150 nm to 40 nm. It was proposed that, as SP forms induce destabilization of the monolayer, the packing of alkyl chains in the core would be decreased and lead to an increase in the volume of particles. UV induced conversion of hydrophobic SP to zwitterionic MC enables close packing of core alkyl chain due to shifting of MC from core region to more hydrophilic PEG peripheral layer, leading to a reduction in size of nanoparticles. Authors hypothesized that photoswitchable isomerization of hydrophobic SP-C9 to amphiphilic MC-C9 would lead to the increase in hydrophilicity which would change the physical assembly properties of the nanoparticles and trigger drug release.[238] The same group also evaluated the *in vivo* efficacy of nanoparticles on human fibrosarcoma HT-1080 tumor xenograft in nude mice. Docetaxel containing nanoparticles were found to be more effective in reducing tumor volume when irradiated with UV light (365 nm) as compared to free docetaxel and encapsulated docetaxel without irradiation.[239]

Yu *et al.* synthesized amphiphilic spiropyrans based di-block polymer PEG-b-1'-(2-methacryloxyethyl)-3'-3'-dimethyl-6-nitro-spiro (2H-1-benzopyran-2,2'-indoline) (PEG-b-PSPMA) by reversible addition–fragmentation chain transfer (RAFT) polymerization in order to make self-assembled micelles which can undergo disassembly by irradiation of light.[232] The copolymer showed the property of light switchable fluorescence response. Spiropyran based folic acid conjugated copolymer was synthesized by Xing *et al.*[240]

Lanthanide upconversion nanoparticles were fabricated with folate conjugated copolymer (PSMN-FA) in order to make core-shell nanoparticles. *In vitro* cytotoxicity assay revealed that DOX loaded nanocarriers were able to reduce cell viability of KB cells significantly after treatment with NIR light. DOX loaded nanoparticles along with NIR (980 nm) treatment showed more efficient regression of folate receptor positive human KB tumor xenograft in nude mice as compared to control groups.[240]

4.4.2. Diazonaphthoquinone based photoresponsive polymer

Diazonaphthoquinone (DNQ) based amphiphilic micelles or polymers have the ability to undergo wolf's rearrangements of bonds when exposed with light source of 795 nm. Amphiphilic polymer having DNQ as hydrophobic portion, upon NIR treatment, undergoes wolf rearrangement to form a more hydrophilic form with a carboxylic moiety, leading to the release of content from the micelle.[241,242] Goodwin *et al.* demonstrated dissociation of micelles having PEG as the hydrophilic component and 2-diazo-1,2-naphthoquinone as the hydrophobic component via two-photon photoreaction triggered by NIR, leading to the release of the encapsulated dye.[242] Liu *et al.* reported development of NIR responsive DNQ based polymeric micelles for drug delivery in cancer. The amphiphilic grafted polymer dextran-graft-(2-diazo-1,2-naphthoquinone) (Dex-DNQ) was synthesized by chemical modification of hydrophilic dextran with hydrophobic DNQ molecule.[243] DOX was encapsulated and was released when the micelles underwent destabilization upon exposure of NIR (808 nm) light. *In vitro* cyotoxicity assay showed very high intracellular release of DOX at this wavelength and reduced cell viability of Hep G2 cancer cells (Table 6).[243]

4.4.3. O-nitrobenzyl based photoresponsive polymer

O-nitrobenzyl alcohol derivatives were used primarily as photo-labile protecting groups of carboxylic acid and amine groups in polypeptide or organic synthesis.[234,244] Later it was shifted to photoresponsive polymer chemistry for the generation of amphiphilic di- or tri-block polymers in order to make micelles for light driven release of cargo in spatiotemporal manner. This hydrophobic photosensitive group acts as protector of hydrophilic carboxylic group, preventing its exposure to the outer aqueous environment and the whole polymer exhibits hydrophobicity. Upon irradiation of UV light, O-nitrobenzyl group is cleaved off leading to dissolution of the whole polymer in aqueous base. Most importantly this photo induced cleavage of O-nitrobenzyl does not lead to any acidic photoproducts.[234] Cheng's group recently reported synthesis of polymeric conjugates of camptothecin having pendent o-nitrobenzyloxyl-1-carbonyl or phenylboronic pinacol ester protecting groups to obtain UV and H_2O_2 responsiveness. When exposed separately to the triggers, there was more than 10

fold decrease in IC_{50} of the polymer drug conjugates in HeLa cells. *In vivo* efficacy evaluation on syngenic 4T1 tumor model revealed improved antitumor efficacy by inducing higher apoptosis index.[245]

Liu *et al.* developed lactose terminating NIR light sensitive block polymer lactose-PEO-b-poly(S-(o-nitrobenzyl)-L-cysteine) [Lac-PEO-b-PNBC].[246] Block polymers were used to fabricate the UCNP (upconversion nanoparticle) loaded polypeptide nanoparticles. UCNP absorbs NIR 980 nm and emits high energy photons of UV-Visible region.[247] UV photons were further absorbed by nitrobenzyl groups of copolymer to initiate the process of photocleavage. It results in disassembly of composite nanoparticles and release of encapsulated DOX (Fig. 8). IC_{50} of the composite nanoparticles decreased 1.7 and 3.5 fold after irradiation with NIR (980 nm) for 5 min and 10 min respectively, as compared to non–irradiated samples. IC_{50} had dropped down 4.7 fold as compared to non-targeted nanoparticle on Hep G2 cells.[247] Song *et al.* developed light responsive plasmonic vesicles for targeted delivery of anticancer drug by using external phototherapy as stimulus. Amphiphilic gold nanoparticles modified with photoresponsive hydrophobic poly (2-nitrobenzyl acrylate) (PNBA) were prepared to self-assemble to form plasmonic vesicles. On application of light, destabilization of the micelles followed by release of drug occurred due to transformation of light sensitive PNBA into hydrophilic PAA. The authors evaluated the *in vitro* efficacy of formulation on MDA-MB-435 cell line and it was reported that upon light (UV 365 nm) irradiation the cytotoxicity was enhanced.[248]

4.4.4. Coumarin based photosensitive polymer

Another method of making photoresponsive polymer is the polymerization of monomers having capability to show property of photo induced dimerization by cross linking (UV light $\lambda > 310$ nm) and de-cross linking (UV light $\lambda < 260$ nm) such that both polymerization as well as depolymerization is induced by light only. Coumarin is one of the well-known monomer having such property. Advantage of coumarin based polymer is that the light sensitive groups remain attached to the monomer separating unit and no other toxic byproducts are generated apart from monomer after light exposure.[249,250,251]

4.4.5. Other photoresponsive polymeric systems

In addition to photoresponsive polymers, there are some other polymers which had shown photo triggered release of drug without having above mentioned photoresponsive groups. Kwag *et al.* demonstrated that glycol chitosan (GC)-grafted fullerene (GC-g-C60) copolymers self-assembled to form nanoparticles because of their amphiphilic nature. When nanoparticles were irradiated with laser light of wavelength 670 nm, light responsive GC-g-

C60 nanoparticle (around 30 nm) generated singlet oxygen in tumor cells and induced tumor toxicity. Also these nanoparticles had shown preferential tumor accumulation in KB-tumor bearing nude mice and the amount of Ce6 dye tagged particles accumulated in the tumor region was significantly high as compared to other organs.[252]

Although, enormous progress has been made so far in the area of development of photoresponsive polymers, clinical translation remains a challenge. It needs to be characterized regarding its responsiveness towards particular wavelength of light, in order to make it more favorable for drug delivery in cancer. For instance, most of the photoresponsive polymer absorbs UV/visible light. UV light has limited penetrating power and is toxic for tissue which makes it questionable for *in vivo* application. As an alternative, NIR is being used along with upconversion nanoparticles but its feasibility of *in vivo* use has not been well studied. The selection of appropriate upconversion nanoparticles is also important for efficient photon conversion at target site. NIR has penetration ability till 10 cm depth in living tissue[253,254] and induces very minimal tissue damage at target site.

5. Combination of triggers

Trigger responsive nanocarriers have been designed to release the drug in response to different tumor specific internal and external triggers, as discussed above. The main aim while designing such nanocarriers is to get rapid release at tumor site and prevent the drug leakage in circulation. The specificity of these nanocarriers can be further enhanced and fine-tuned by making them responsive to more than one trigger. Dual and multi-responsive polymeric nanoparticles for site specific delivery have been reviewed in detail by Cheng *et al.*.[255] Polymeric nanocarriers responsive to combination of triggers including pH/redox,[17,47,60,81,86,111,114,119,124,128,136] pH/enzyme,[153] pH/ultrasound,[227] pH/magnetic field,[256,257] pH/NIR,[258] temperature/magnetic field[197,198,199,200,201,202,208] and temperature/ultrasound[221] have been studied to make dual responsive polymeric nanoparticles, with pH or temperature being one of the trigger in most of the combinations as these are the two most widely used triggers.

pH and redox are attractive triggers as they exist naturally in tumor tissues and their combination have been extensively exploited to achieve higher intracellular concentration of drugs in cancer cells (Fig. 9). Many such examples have been discussed in the previous sections of this review, like pH and redox dual responsive copolymeric micelles for intracellular delivery of DOX which were prepared using pH sensitive PbAE having disulfide linkages in their backbone.[47] These micelles have shown higher DOX release in acidic and

reducible environment, along with their nuclear localization in hepatocellular carcinoma (Hep G2 cell line).[47] The combined trigger response can either take place simultaneously or in a sequential manner depending on the design of the particle as well as conditions at the targeting site. Sequential release of DOX from the PEG-SS-poly(2,4,6-trimethoxybenzylidene-pentaerythritol carbonate) (PTMBPEC) micelles in different intracellular compartments was hypothesized as pH sensitive acetal linkages will be hydrolysed at endosomal pH causing release of DOX and once the micelles escape into the cytoplasm, the disulfide bonds will be reduced by GSH resulting in DOX release in cytosol (Fig. 9).[17] It was shown that the release of DOX was higher in presence of both triggers as compared to single trigger. Also higher anti-tumor efficacy of these dual responsive micelles was shown in HeLa cells *in vitro*.[17] To prevent drug loss during storage and in circulation, interlayer crosslinking via disulfide linkages have been proposed to prepare pH and redox responsive micelles such that the core is composed of pH responsive polymer encapsulating DOX.[119] These interlayer crosslinked micelles were prepared from triblock copolymer of mPEG, 2-mercaptoethylamine (MEA)-grafted poly(l-aspartic acid) (PAsp(MEA)), and 2-(diisopropylamino)ethylamine (DIP)-grafted poly(l-aspartic acid) (PAsp(DIP)). In these micelles, presence of both DTT and pH 5 results in much higher release as compared to these individual triggers. Also, it was found that *in vitro* nuclear localization of DOX was faster with dual responsive micelles as compared to unresponsive micelles. Higher tumor growth inhibition was also observed with dual sensitive micelles in hepatocellular carcinoma (Bel-7402) xenograft mice model.[119] Another interesting strategy for preparing smart dual sensitive micelles was proposed by Song *et al.*, where targeting ligands were added to disulfide containing alkynyl sites for active uptake and PEG shell was attached by pH sensitive benzoic imine linkages which were cleaved at extracellular tumor pH of 6.5. This system combines the advantage of increased circulation time by PEGylation and higher intracellular uptake through active receptor mediated endocytosis.[86] pH and reduction sensitive polymeric nanohydrogels have also been evaluated as dual responsive vehicles for cancer therapy.[81,115] Apart from these reduction and pH responsive nanocarriers, micelles sensitive to intracellular H_2O_2 and pH have also been developed and evaluated for DOX delivery.[136]

pH can also be used in combination with cancer specific enzymes as triggers to prepare dual responsive polymeric nanocarriers. PolyGC-DOX (DOX complexed with DNA) was complexed with cationic gelatin to obtain gelatinase responsive complex which was further coated by negatively charged pH responsive PEGylated histamine modified alginate

to provide stealth characteristics to the nanocarriers. The coating detaches at the tumor extracellular pH of 6.5 to 6.7 and the nanoparticles become positively charged (Fig. 9).[153] Stealth property of these nanocarriers helps in higher tumor accumulation of the drug which otherwise gets accumulated in the liver as gelatinase expression is comparatively higher in liver, causing hepatocellular toxicity. These DOX loaded dual responsive nanocarriers have shown higher anti-tumor efficacy along with reduced cardiotoxicity of DOX in heterotopic murine sarcoma (S180) mice model.[153]

Among external triggers, temperature is an important trigger which may be combined with ultrasound or magnetic triggers to obtain optimum drug release at the cancerous site. PNIPAM based polymer coated iron oxide nanoparticles have been exploited for preparing temperature and AMF dual responsive polymeric nanocarriers.[197,198,199,200,201,202,208] Poly(N-isopropylacrylamide-acrylamide-allylamine)-coated MNPs have been prepared and evaluated for dual responsive DOX delivery to prostate cancer cells. Temperature sensitive nanoparticles showed increased release at 41 ºC as compared to 37 ºC.[202] Further, these nanoparticles were targeted with prostate specific polyarginine peptide and have shown higher tumor accumulation in subcutaneous prostate cancer (PC3-KD) bearing xenograft mice.[201] Temperature can also be used as a trigger in combination with ultrasound as studied by Gaurevich *et al.*.[221] It was shown that cellular internalization of DOX encapsulated in cyclodextrin based polymeric nanoparticles was dependent on both heat and focused ultrasound (FUS) treatment. When compared to treatment without trigger, there was upto 5.5 and 5.7 times increase in DOX uptake in presence of FUS and heat alone respectively, whereas DOX uptake increased by a factor of 9.6 when hyperthermia treatment was given along with FUS.[221]

Nanocarriers responsive to both internal and external triggers like pH/magnetic field,[256,257] pH/ultrasound,[227] pH/NIR[258] have also been evaluated. Ultrasound and pH responsiveness of the self-assembled vesicles of block polymer PEO-b-P(DEA-stat-TMA) was shown by the effect of varying ultrasound radiation time and solution pH on DOX release *in vitro*.[227] To impart pH responsiveness to magnetic nanocubes, hydrazone bearing PMMA was functionalized on their surface through which DOX is loaded on these nanocarriers. External magnetic field was used for remote targeting of the nanoparticles to the tumor tissue where pH acts as a stimulus for drug release (Fig. 9).[255]

Multi-responsive nanocarriers which can respond to more than two triggers like pH/ temperature/redox,[81] pH/redox/enzyme,[102] pH/ultrasound/redox,[226] pH/temperature/magnetic field,[209] pH/temperature/NIR[259] have also been developed recently to further improve release

kinetics and prevent drug loss. Dual or multi-responsive nanocarriers have definite advantage of better control over drug release as compared to unresponsive or single trigger responsive nanoparticles and have a great potential to be used in cancer therapy. But these advantages come at a cost of increased complexity in their method of preparation, which adds to the cost of the system, and can pose a difficulty in their scale up and clinical translation in future. Also research in the field of polymeric nanocarriers responsive to combination of triggers is still in early stages with many non-biodegradable and toxic polymers being used to get proof of concept and very few reports of their *in vivo* evaluation. So along with adding multiple functionalities to a nanocarrier, the research should be aimed at developing a facile strategy for preparation of these nanoparticles using biodegradable and non-toxic polymers.

6. Challenges in clinical translation of trigger responsive polymeric nanocarriers

Trigger responsive polymeric nanoparticles represent a class of smart delivery systems, which have been researched extensively over the last few years to overcome the limitations of first generation nanoparticles. A large number of nanocarriers have been characterized and studied for their *in vitro* efficacy, with a considerable number of studies with *in vivo* evaluation resulting in promising outcomes. However, most of these are studied in subcutaneous xenograft mice models, which are not the accurate representation of tumor systems in the body and the results of these studies cannot be directly extrapolated to predict clinical outcomes. In humans, physiology of tumor tissue and its vasculature is highly heterogeneous; the amount of blood supply to tumor interstitium in patients with solid tumors is much less as compared to the subcutaneous tumors in mice.[260] So, one of the main prerequisite for these "smart" nanocarriers to translate from laboratory to clinics is their extensive preclinical evaluation in relevant orthotopic animal models. Secondly, adding multiple dimensions to these nanocarriers in terms of tumor targeting, longevity in circulation and trigger responsiveness makes their preparation multi-step, complex and tedious process which also add to the overall cost of these nanocarriers. So cost effectiveness, reproducibility and scale up remain another challenge for clinical translation of these trigger responsive nanocarriers. The use of inexpensive materials which are regarded as safe for human use and exploring novel facile methods for preparation can help in overcoming these challenges. The shelf life of these nanocarriers is another important aspect for clinical translation. The stability of these nanocarriers on storage should be studied and measures should to be taken towards enhancing the stability for further translation. Also, the treatment regimen should be simple and complex methods of administration should be avoided to make the therapy patient

compliant and easy to adopt for clinicians. If research in the field of trigger responsive nanocarriers is carried with the clinical translation as the main focus by addressing the above mentioned concerns, it holds a great potential in the field of cancer therapeutics as it exploits the same nature of tumor cells and microenvironment to target the tumor, which otherwise helps the tumor to grow and metastasize in biological systems.

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List of abbreviations

Abbreviations	**Full Form**
5-FU	5-fluorouracil
ADR	Adriamycin
AMF	Alternating magnetic field
BSA	Bovine serum albumin
CB	Cathepsin B
CER	Ceramide
Chol	Cholesterol
CMC	Critical micellear concentration
CPT	Campothecin
CUR	Curcumin
DEA	2-(diethylamino)ethyl methacrylate
DNQ	Diazonaphthoquinone
DNQ	Diazonaphthoquinone
DOX	Doxorubicin
DTDP	3,3´-dithiodipropionic acid
DTT	Dithiothreitol
DTX	Docetaxel
ECM	Extracellular matrix
EDC	1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide
EPR	Enhanced permeability and retention Effect

FUS	Focused ultrasound
GA	Glycyrrhetinic acid
GSH	Glutathione
GSSG	Glutathione disulfide
H_2O_2	Hydrogen peroxide
HA	Hyaluronic acid
HCPT	10-hydroxycamptothecine
HER2	Human epidermal growth factor *receptor* 2
HPAH	Hyperbranched polyacylhydrazone
IC_{50}	Half maximal inhibitory concentration
IONPs/IONPS	Iron oxide nanoparticles
LA	Lipoic acid
LCST	Lower critical solution temperature
MDR	Multidrug resistance
MMNPs	Multimodality treatment nanoparticles
MMP	Matrix metalloproteinase
MNP	Magnetic nanoparticle
mPEG	Methoxy poly(ethylene glycol)
MSNs	Mesoporous silica nanoparticle
NHS	N-Hydroxysuccinimide
NIR	Near infrared
NPOD	4β-aminopodophyllotoxin
NPs	Nanoparticles
P(NIPAM-co-AM)-b-PLA	poly(N-isopropylacrylamide-co-acrylamide)-block-poly(d,l-lactide)
PAA	Poly(acrylic acid)
PAMAM	Poly(amidoamine)
PBAA	Poly(butyl acrylic acid)
PbAE	Poly(β-amino ester)
PBCA	poly-N-butylcyanoacrylate
PCBS	Poly(l-cystinebisamide-g-sulfadiazine)
PCL	Polycaprolactone
PDEAEM	Poly(N,N′-dimethylaminoethyl methacrylate

PEAA	Poly(ethyl acrylic acid)
PEG	Poly(ethylene glycol)
PEG-b-PSPMA	(Poly(ethylene glycol)-b-1'-(2-methacryloxyethyl)-3'-3'-dimethyl-6-nitro-spiro (2H-1-benzopyran-2,2'-indoline))
PEI	Polyethylenimine
PEO	Poly(ethylene oxide)
PEOz	poly(2-ethyl-2-oxazoline)
PEYM	Copolymer of PEG and poly(methacrylate)
PFH	Perfluorohaxane
PG	poly(l-glutamic acid)
PGA	Poly(glycolic acid)
P-gp	P-glycoprotein
PHIS	Poly(L-histidine)
PL	Poly(D,L-lactide)
PLA	Poly(lactic acid)
PLGA	Poly (d,l-lactide-co-glycolide)
PMA	Poly(methacrylic acid)
PNBA	poly (2-nitrobenzyl acrylate)
PNBC	poly(S-(o-nitrobenzyl)-L-cysteine
PNIPAM	Poly (N-isopropylacrylamide-co-acrylamide)
PNIPAM-AAm-AA	Poly (N-isopropylacrylamide-co-acrylamide-co-allylamine)
POE	Poly(orthoesters)
PPAA	Poly(propyl acrylic acid)
PPO	poly(propylene oxide)
PPS	poly(propylene sulphide)
PSD	Poly(methacyloylsulfadimethoxine)
PSDM	Polysulfadimethoxin
PTHPMA	poly (2-tetrahydropyranyl methacrylate)
PTMC	Poly(trimethylene carbonate)
PTX	Paclitaxel
PVA	Polyvinyl alcohol
RES	Reticuloendothelial system
ROS	Reactive oxygen species
SDM	Sulfadimethoxine

SPION	Superparamagnetic iron oxide nanoparticle
Tf	Transferrin
TMA	(2-tetrahydrofuranyloxy)ethyl methacrylate
TME	Tumor microenvironment
TPGS	d-α-Tocopheryl polyethylene glycol 1000 succinate
TRITC	Tetramethylrhodamineisothiocyanate
UCST	Upper critical solution temperature
uPA	urokinase plasminogen activator
USPION	Ultra-small SPION
VC	Valine–citrulline
VTMS	Vinyltrimethoxysilane
WHO	World Health Organization

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Table 1: Redox responsive polymeric nanocarriers used in cancer therapy.

Chemical moiety used to introduce disulfide linkage	Key studies	
	Polymers used	***In* vitro/*In vivo* studies**
Glutathione (GSH) responsive polymeric nanomaterials for cancer therapy		
Cystamine dihydrochloride	PEG block copolymers[17,101,102]	*In vitro* cytotoxicity in human cervical carcinoma (HeLa) cell line[17,101] *In vitro* cytotoxicity in Human ovarian adenocarcinoma (OVCAR-3) and human epidermoid (KB) carcinoma cell line[102]
	PAA[103]	*In vitro* cytotoxicity in HeLa cells[103]
	HA[104]	Human liver hepatocellular carcinoma (Hep G2) xenograft mice model[104]
Bis(2-hydroxyethyl)disulfide	PEG block copolymers[94,99]	*In vitro* cytotoxicity in Lewis lung carcinoma cells[94] *In vitro* cytotoxicity in human lung adenocarcinoma (A549), human breast cancer (MCF-7), and HeLa cell lines[99]
3,3′-dithiodipropionic acid	PEG block copolymers[95,96,105]	Murine breast cancer (4T1) model[95,96] *In vitro* cytotoxicity in HeLa cells[105]
2-Iminothiolane	Thiolated gelatin[107,108]	*In vitro* transfection in murine fibroblast (NIH3T3) cells[107] Subcutaneous pancreatic tumor (Panc-1) xenograft model[108]
	PAMAM[109]	*In vitro* cytotoxicity in HeLa cells[109]
2,2′-Dithiodipyridine	PEG based prodrug[133]	Breast cancer (MCF-7) xenograft mouse model[133]
	Poly (2-(pyridin-2-yldisulfanyl)ethyl acrylate)[124]	*In vitro* cytotoxicity in human colon cancer (HCT-116) cell line[124]
	PbAE[121]	*In vitro* transfection in human hepatocellular carcinoma cell line[121]

N,N′-bis(acryloyl)cystamine	PEG based prodrug[113]	*In vitro* cytotoxicity in mouse fibroblast cell line (L929)[113]
	PAMAM[111,112,114]	*In vitro* cytotoxicity in MCF-7 and Hep G2 cell lines[111] *In vitro* transfection in monkey kidney cells (COS-7 cells)[112] *In vitro* transfection in HEK293, COS7, MCF-7 and Hep G2 cell lines[114]
	Poly(methacrylic acid)[115]	*In vitro* cytotoxicity in human glioma (U-251MG cells)[115]
	Poly acrylamide[116]	*In vitro* cytotoxicity in HeLa, MCF-7 and human glioblastoma (U-87 MG) cells[116]
Dithiobis(sulfosuccinimidylpropionate)	mPEG block copolymer[120]	Human breast cancer (MDA-MB-231) xenograft mice model[120]
2,2′-dithiodiethanol diacrylate	PEG based copolymer[47]	*In vitro* cytotoxicity in Hep G2 cell lines[47]
2-(2-pyridyldithio)-ethanol	PCL block copolymer[117,118]	*In vitro* cytotoxicity in P-gp overexpressing human breast adenocarcinoma (MCF-7/Adr) cell line[117] *In vitro* cytotoxicity in A549 cell line[118]
Lipoic acid	Dextran based polymer[126]	*In vitro* cytotoxicity in HeLa cell line[126]
	PEG block copolymer[127,128,129]	*In vitro* cytotoxicity in HeLa and Hep G2 cells[127,128] Human ovarian carcinoma (SKOV-3) xenograft mice model[129]
ROS responsive polymeric nanomaterials for cancer therapy		
Selenium	Polyphosphate[135]	*In vitro* cytotoxicity in HeLa cell line[135]
Ferrocine	PEGylated nanoparticles[136]	Cellular internalization in HeLa cells[136]

Table 2: Enzyme responsive polymeric nanocarriers used in cancer therapy.

Linker peptides substrate and corresponding enzyme	Key studies	
	Polymers used	***In vitro*/*In vivo* studies**
Gly-Pro-Leu-Gly–Ile-Ala-Gly-Gln **MMP2/9**	Albumin drug conjugate[142,143]	*In vitro* efficacy in murine renal cell carcinoma (RENCA) cell line[142] Human amelanotic melanoma (A-375) xenograft mice model[143]
	PEG based prodrug[151]	Non-small cell lung cancer (A549) xenograft mice model[151]
Pro-Val-Gly-Leu-Ile-Gly **MMP2/9**	Dextran based prodrug[144,145]	*In vitro* cytotoxicity in human fibrosarcoma HT-1080 and BT-20 cell lines[144] Human fibrosarcoma (HT-1080), human glioblastoma (U87) and human bladder carcinoma (RT-112) xenograft mice model[145]
	PEG block copolymers[139,152]	Primary cells from pericardial fluid of human lung cancer patients[139] Murine hepatic (H22) tumor mice model[152]
Gly- Gly- Gly-Val-Pro-Leu-Ser-Leu-Tyr-Ser- Gly- Gly-Gly- Gly **MMP2/9**	PEGylated liposomes[147,148]	Human fibrosarcoma (HT-1080) xenograft mice model[147,148]
Hyaluronic acid **Hyaluronidase**	Hyaluronic acid[155]	*In vitro* efficacy in human breast cancer (MDA-MB-231) cell line[155]
Glucuronide **β-Glucouronidase**	PEG block copolymer linked to glucuronide based prodrug[157]	*In vitro* efficacy in human head and neck squamous carcinoma cells[157]
Gly–Phe–Leu–Gly **Cathepsin B**	PEG block copolymer[158,161,162]	Intracellular uptake in human breast cancer (SKBR3) cells[158] Murine breast cancer (4T1) mice model[161] Human ovarian cancer (SKOV-3) xenograft mice model[162]
Valine–Citrulline **Cathepsin B**	PEG prodrug[159,160]	Murine fibrosacrcoma (Meth-A) mice model[159] Human breast cancer (MCF-7) xenograft mice model[160]

Table 3: Temperature responsive polymeric nanocarriers used in cancer therapy.

Trigger	**Response mechanism for drug release**	**Key studies**	
		Polymer	***In vivo/In vitro* Model**
Temperature (38 ºC)	poly(N-vinylcaprolactam) phase transition to destabilize the nanoparticle	chitosan-g poly(N-vinylcaprolactam)[181]	Apoptosis assay on MCF-7 cell line
Temperature (38 ºC)	PNIPAM phase transition to destabilise the nanoparticle	chitosan-g-poly (N-isopropylacrylamide)[180]	*In vitro* cytotoxicity on KB, MCF-7 and PC3 cell lines
Temperature (37 ºC)	Micelles phase transition above LCST of PNIPAM	$(PNIPAM)_2$-b-HTPB-b-$(PNIPAM)_2$ [182]	MDA MB 231 human breast cancer cell line
Temperature and magnetic field (42 kA/m alternating electric current field with 240 kHz frequency and 42 ºC)	Hyperthermia induced by magnetic nanoparticle	Iron oxide NPs coated with TPGS-PLS/TPGS-COOH[167]	SK-BR-3 (HER2-positive)cell line as *In vitro* model for cytotoxicity assay
Temperature (43 ºC)	Thermosensitive swelling and shrinking of nanoparticle	F127-chitosan copolymer[168]	*In vitro* Cytotoxicity on PC-3 prostate cancer cell line

Table 4: Magnetic field responsive polymeric nanocarriers used in cancer therapy.

Trigger	**Response mechanism for drug release**	**key studies**	
		polymer	***In vivo/ In vitro* Model**
AMF (14 mT at 750 kHz))	Release of DOX because of heating disintegration of polymerosome by magnetic field	PTMC-*b*-PGA[196]	*In vitro* cytotoxicity on HeLa cell line
AMF (35 mT at 250 kHz)	Temperature mediated phase transition of PNIPAM	PNIPAM and chitosan co-polymer[209]	*In vitro* Cytotoxicity assay on MDA MB 231, MCF-7, DU-145 and SK-OV-3 cell line.
AMF (0.1 T neodymiumei-ron-boron (NdFeB) permanent magnet)	Magnetic field enhances uptake of MNP-MSN-PLGA-Tf NPs	Magnetic NPs coated with mesoporous silica and PLGA. NPs encapsulated both DOX as well as PTX[203]	U-87 MG-luc2 xenograft in BALB/c mice

Table 5: Ultrasound responsive polymeric nanocarriers used in cancer therapy.

Trigger	**Response mechanism for release/role of trigger**	**Key studies**	
		Polymer	***In vivo/In vitro* study**
Ultrasound (1MHz frequency, 3.4 W/cm^2 power for 30 s)	Disruption of bubble	PEG-PLLA[223]	Ovarian cancer A2780 and MDA MB 231 tumor xenograft in nude mice
Ultrasound (1MHz with mechanical index(MI) 0.15 for 2 min)	Extravasations of nanoparticle/ enhanced penetration through extracellular matrix	PBCA[224]	PC-3 human adenocarcinoma tumor xenograft in nude mice
Ultrasound (MCF7 cells: frequency(f) 0.95 MHz, power (P)2 W, intensity (I)37.54 W/cm^2, MI 0.77, for 25 s. and For A375m cells: f 0.95 MHz, P1W,I 18.77 W/cm^2, MI 0.54 for 10 s.)	Mechanical and hyperthermal release of drug	Cyclodextrin based polymer [221]	Internalization of DOX was increased in A375m and MCF-7 cell line .
Ultrasound (1MHz, 0.5 W/cm^2 for 120 s)	Mechanical disruption by shear forces/ cavitation	poly (NIPMAM-co-NIPAM)[222]	*In vitro* cytotoxicity on HepG2 cell line

Table 6: Photo- responsive polymeric nanocarriers used in cancer therapy.

Trigger	**Response mechanism for release of drug/ role of trigger**	**Key studies**	
		Polymer	***In vtiro/In vivo***
Light (980 nm NIR radiation with power 60 mW/cm^2 for 30 min each day till 19 days)	Hydrophilic –hydrophobic switching	PSMN-FA[240]	Human KB tumor xenograft in nude mice
Light (NIR 808nm with power 300 mW for 5 min)	Wolf rearrangement of DNQ in polymer	Dex-DNQ[243]	HePG2 cells.
Light (980 nm NIR irradiation for 5 or 10 min)	Cleavage of O-nitrobenzyl in polymer	Lac-PEO-b-PNBC[246]	*In vitro* Cytotoxicity on HeLa and HePG2 cell line
Light (2.3 mW/ cm^2 for 15 min)	Hydrophobic to hydrophilic conversion of polymer	PNBA[243]	*In vitro* efficacy study on MDA MB 435 cell line
Light (100 mW/cm^2 670 nm light illumination for 10 min)	ROS generation by C60 portion of polymer	Glycol chitosan (GC)-grafted fullerene (GC-g-C60)[252]	Preferential accumulation of nanoparticle in KB tumor xenograft/*in vitro* cytotoxicity on same cell line
UV light (365 nm, 1 W/cm^2 for 20 sec)	Hydrophobic-hydrophilic imbalance in polymer physically destabilize the nanoparticles and trigger drug release	DSPE-PEG and SP-C9[238,239]	Higher *in vivo* efficacy of nanoparticles in HT-1080 tumor xenograft model when given along with UV irradiation

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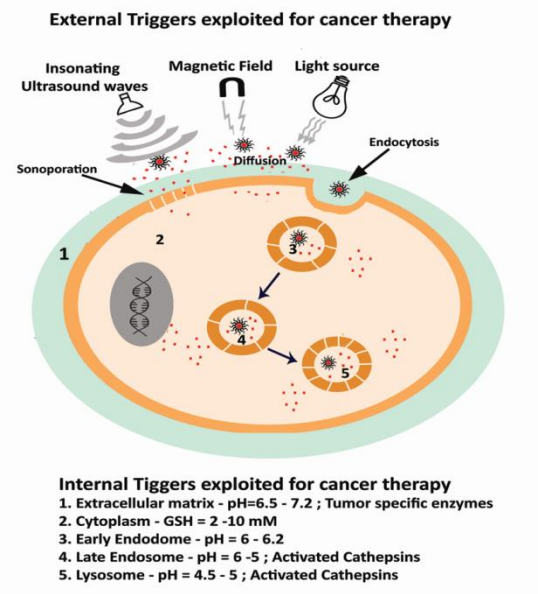
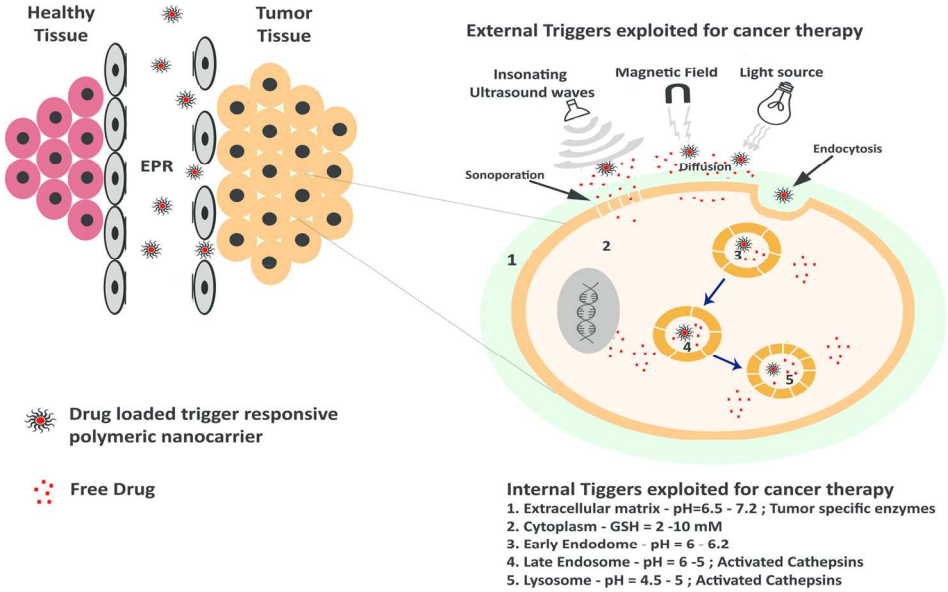
Trigger responsive polymeric nanocarriers for cancer therapy

Shahdeep Kaur, Chandrashekhar Prasad, Biji Balakrishnan, Rinti Banerjee*



Strategies for development of polymeric nanocarriers responsive to different internal and external triggers, modulating the drug release in tumor environment are reviewed.



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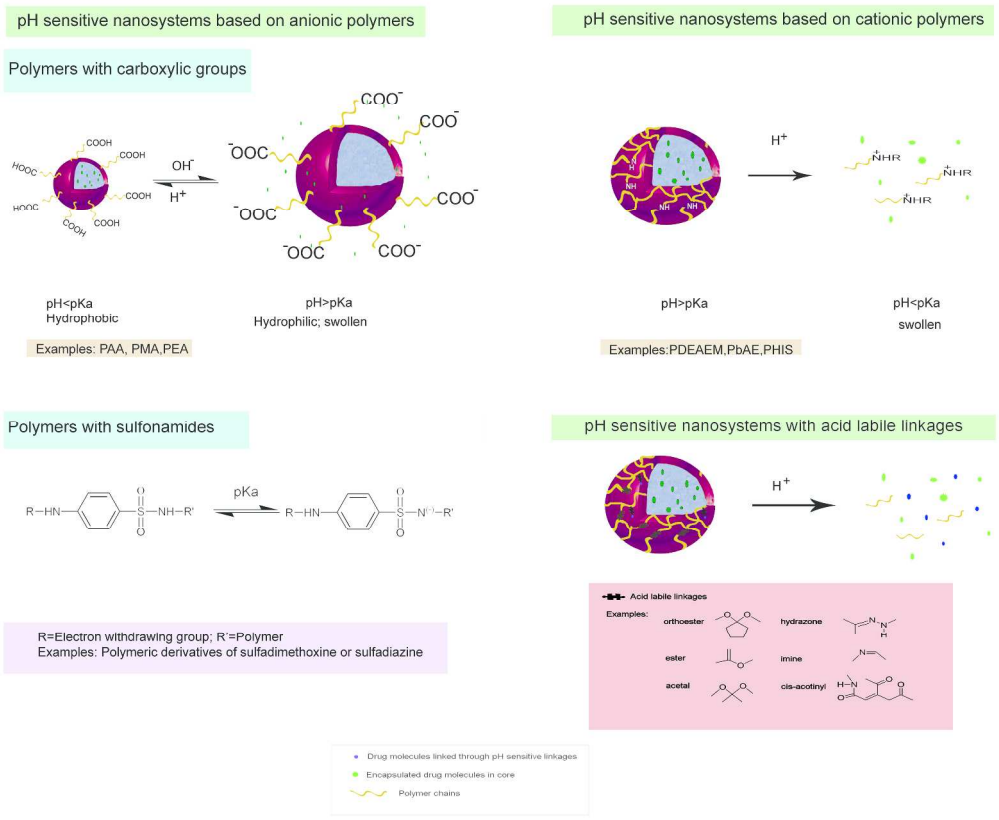

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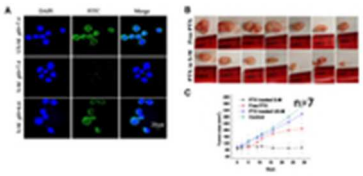

A) Internalization of US-M and S-M into MCF-7 cells observed by CLSM at pH 6.6 and 7.4; B&C) Tumor size for mice receiving long term treatment with PTX. Reprinted with permission from Elsevier 2013 [ref. 51].
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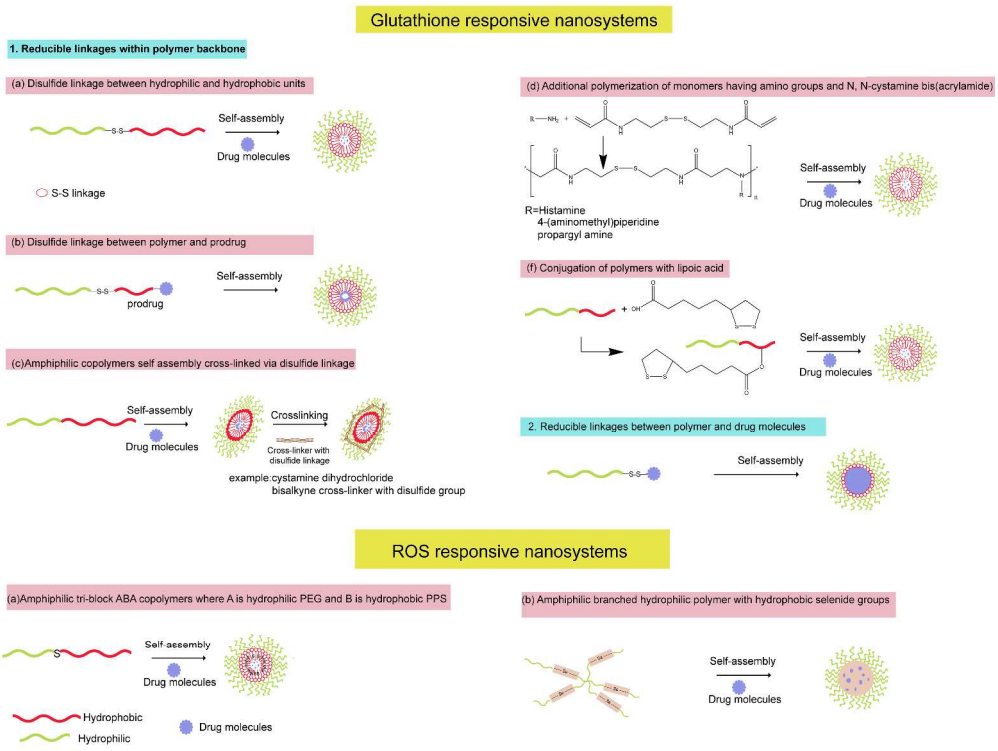

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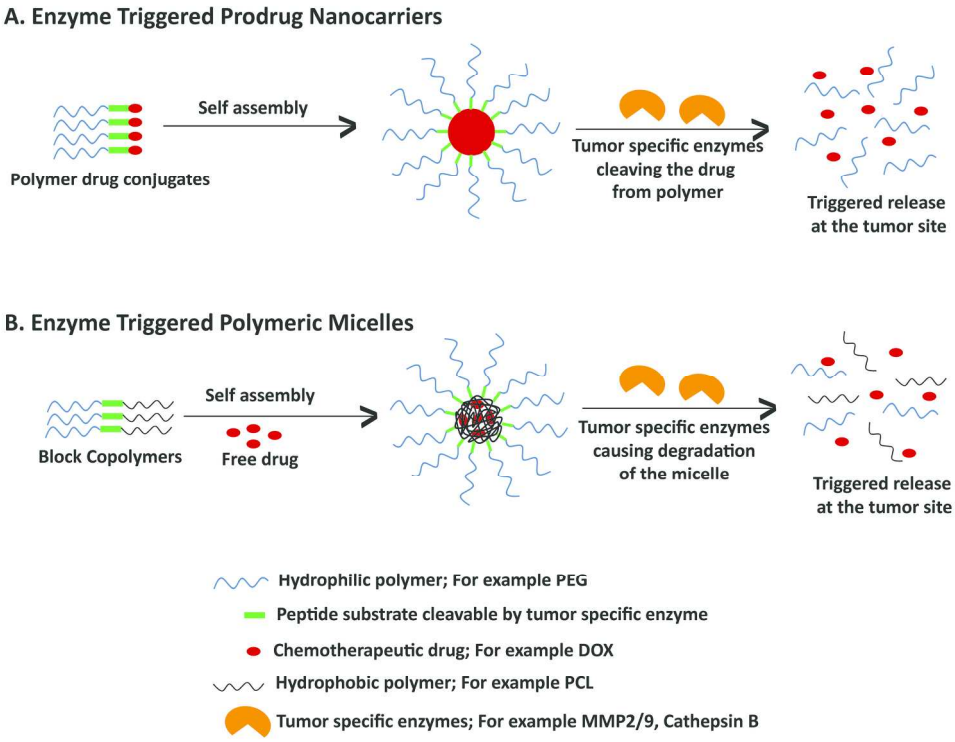

Schematic representation of enzyme triggered drug release from polymeric nanocarriers.
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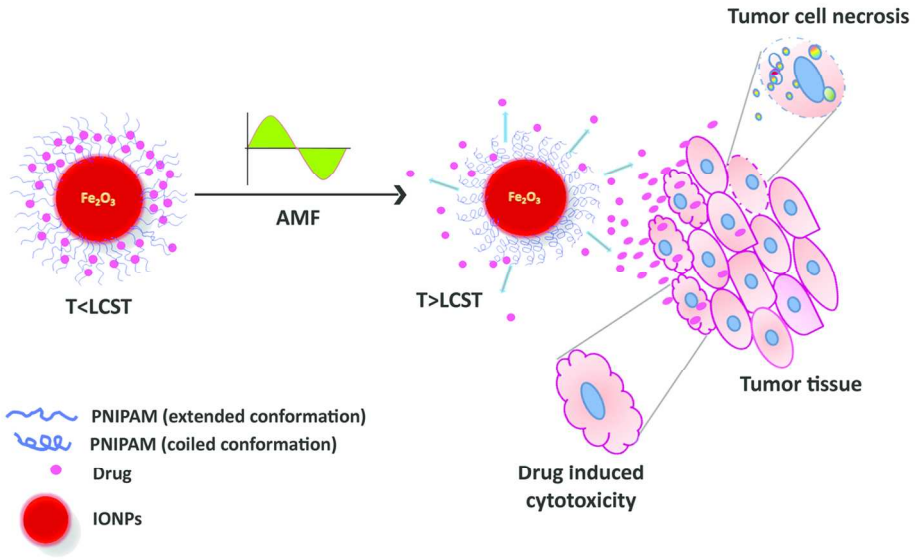

Schematic illustration of NIPAM based AMF responsive nanoparticle showing release of encapsulated drug due to conformational change in polymer from extended to coiled state.
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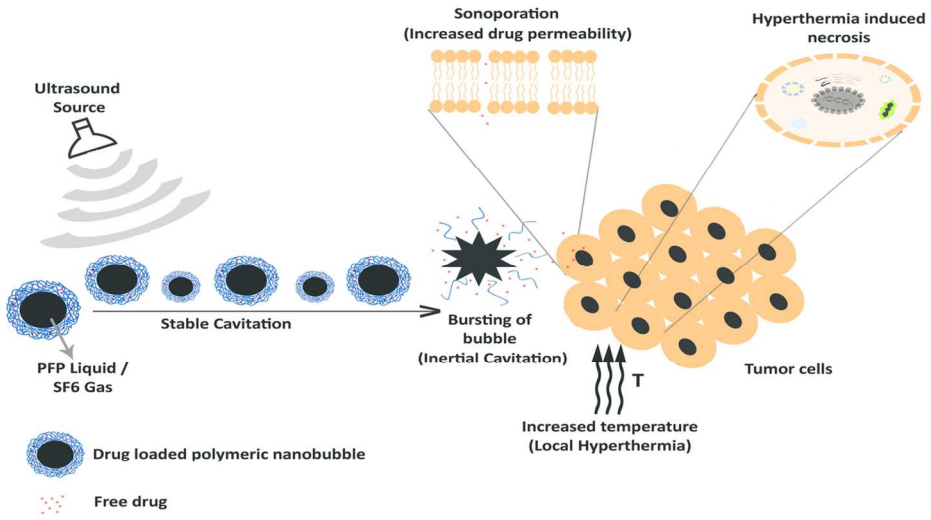

Schematic representation of ultrasound aided drug release and cancer cell death.
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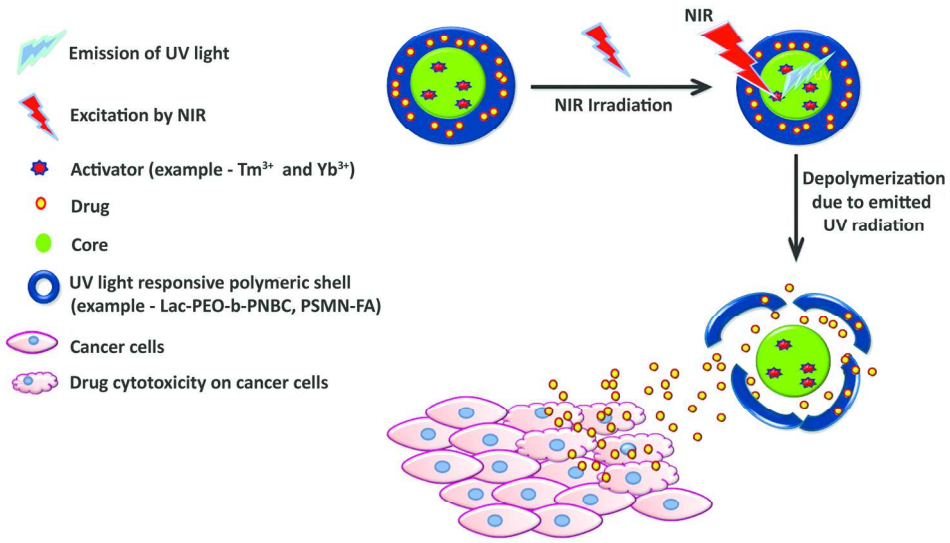

Schematic representation of photoresponsive upconversion nanoparticle showing absorption of NIR by activator followed by emission of UV light which facilitates depolymerization of polymeric sheath and release of drug.
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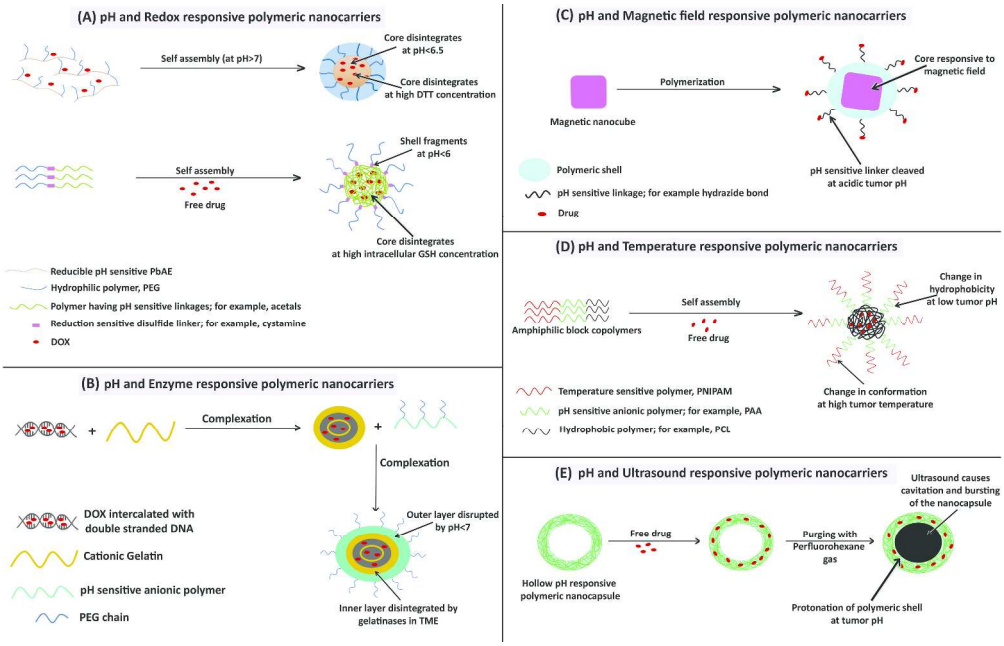

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