Chapter 11
Prodrug Conjugate Strategies in Targeted Anticancer Drug Delivery Systems

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Abbreviations

ADCC	Antibody-dependent cellular cytotoxicity
ADEPT	Antibody-directed enzyme prodrug therapy
AuNP	Gold nanoparticles
BCR	Breakpoint cluster region protein
BTK	Bruton's tyrosine kinase
CDK inhibitor	Cyclin-dependent kinase inhibitors
CLL	Chronic lymphocytic leukemia
CPT	Camptothecin
CTC	Circulating tumor cells
Dox	Doxorubicin
DTC	Disseminated tumor cells
EGFR	Epidermal growth factor receptor
EPR	Enhanced permeability and retention
ERK	Extracellular signal-regulated kinases
FISH	Fluorescence in-situ hybridization
FOL	Folic acid
FR	Folate Receptor
GDEPT	Gene-directed enzyme prodrug therapy
GnRH	Gonadotropin-releasing hormone
GRP78	Glucose-regulated protein 78
HCC	Hepatocellular carcinoma

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HER2	human epidermal growth factor 2
HPLC	High-performance liquid chromatography
IGFR	Insulin-like growth factor receptor
KRAS	Kirsten rat sarcoma
LHRH	Luteinizing hormone releasing hormone
mAb	Monoclonal antibodies
MAPK	Mitogen-activated protein kinases
MCL	Mantle cell lymphoma
mCRC	Metastatic colorectal cancer
MDNS	Magneto-Dendritic Nano System
MDR	Multiple-drug resistance
mRNA	Messenger ribonucleic acid
mTOR	Mammalian target of rapamycin
NSCLC	Non-small-cell lung cancer
PCR	Polymerase chain reaction
PDGFRβ	Platelet-derived growth factor receptor
PI3K	Phosphatidylinositol 3-kinase
PIGF	Placental growth factor
RCC	Renal cell carcinoma
RET	Rearranged during transfection
RGD	Arginine-glycine-aspartic acid
SCCHN	Squamous cell carcinoma of the head and neck
scFv	Single chain variable fragment
siRNA	Small interfering RNA
Tf	Transferrin
Tf-PEG-AD	Transferrin-conjugated poly (ethylene glycol)-adamantane
TfR	Transferrin receptor
TPGS	D-α-Tocopheryl polyethylene glycol succinate
US FDA	United States Food and Drug administration
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor

11.1 Introduction

The primary dearth of treatment in chemotherapy is lack of molecular selectivity and severe toxicity associated from an anticancer drug. In general, chemotherapeutic drugs responds through anti-proliferative mechanisms; or by preventing cell cycle at a specific phases rather than producing a toxic effect to particular site or types of cancer cells [1]. Hence, for the effective anticancer therapy, polymeric prodrug conjugation methodology represents one of the most promising approaches in achieving selective chemotherapy. Many efforts in reducing the systemic toxicity of chemotherapeutic moieties have been clinically explored (Fig. 11.1). In particular, polymeric prodrug conjugate is a chemical modification of a biologically inert

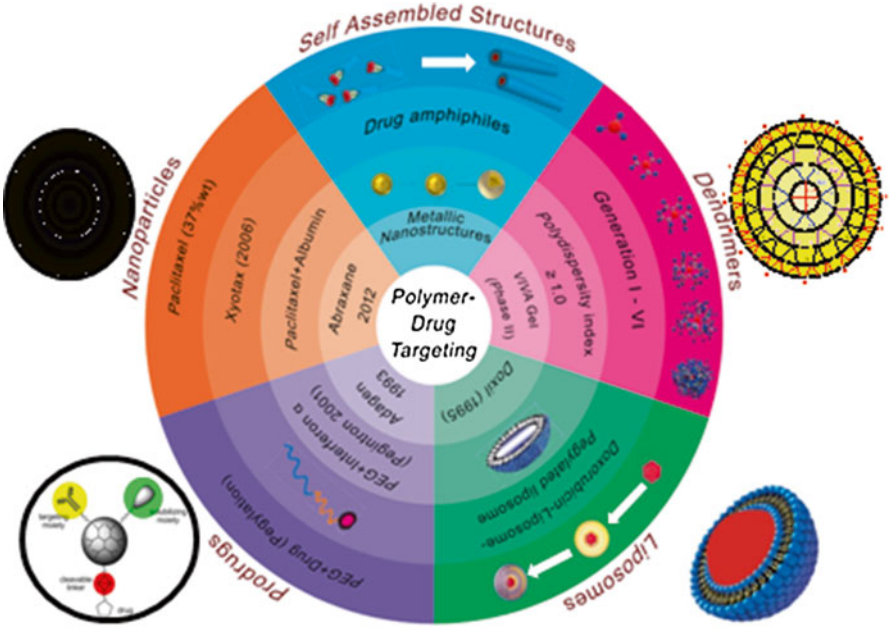

Fig. 11.1 Advances in drug delivery systems representing varied architectures, physicochemical traits such as size, shape, and surface charge (modified from [6])

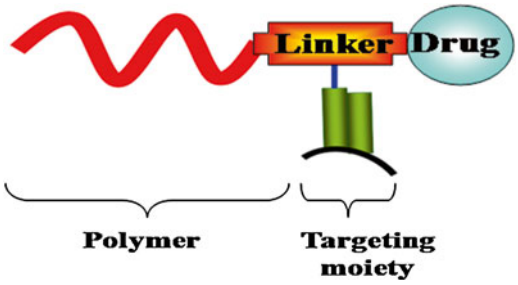

Fig. 11.2 Schematic representation of a polymeric prodrug conjugate with targeting moiety

component which is transformed to its active form, in vivo [2, 3]. Polymeric forms (e.g., poly(ethylene glycol) (PEG)) commonly referred in literature as "PEGylation" is a polymeric prodrug approach, offering an important tool to enhance the pharmacodynamics (PD) of the active pharmacologic component via a simple chemical alteration. Traditional prodrug design aims to offer: (1) enhanced aqueous solubility, chemical stability, brain permeability, and oral or local absorption of a drug; and (2) reduced undesired pre-systemic metabolism, and toxicity [4, 5].

An ideal polymeric prodrug conjugate system typically consists of multiple components as represented in Fig. 11.2.

1. A polymer as a drug delivery vehicle
2. Drug, protein or peptide as a biological active component
3. A spacer molecule and targeting moiety

Following approaches are generally used to target anticancer prodrugs to the tumor or cancer cells [7, 8]: (1) Passive Drug targeting and (2) Active Drug targeting.

Passive Drug Targeting: In passive targeting, drug is delivered to the targeted site by conjugating it with polymer which releases the drug outside the targeted site due to altered environmental conditions as represented in Fig. 11.3a. The general features of tumors and many inflamed areas of body include leaky blood vessels and poor lymphatic drainage which passively provides increased retention of macromolecules into tumor [9–12]. This phenomenon is commonly referred to as Enhanced Permeability and Retention (EPR) effect [9]. EPR effect is primarily a passive targeting due to the accumulation of prodrug, into the tumor. The phenomenon mainly occurs owing to hampered lymphatic drainage which allows them to release the drug into the tumor milieu. However, passive targeting approach has several limitations. This is because targeting of the cancer cells is not always achieved as the diffusion of some drugs is insufficient and the random chemical approach makes it difficult to control the process. The lack of control is expected to lead into multiple-drug resistance (MDR). This situation results in resistance of cancer cells towards one or more drugs, thereby leading to failure of chemotherapy treatments. Moreover, it is known that certain tumors do not show the EPR effect, and the permeability of vessels is unpredictable throughout tumor which further limits the passive targeting approach [13]. On the other hand, the more efficient way to obtain targeting is by "active targeting" process.

Active Drug Targeting: Active targeting approach involve interactions between specific biological systems, e.g., ligand–receptor, antigen–antibody, enzyme–substrate (Fig. 11.3a [14]). Active targeting is achieved by targeting ligand molecules that may interact with specific receptors on the cell surfaces—along with the bioactive prodrug system (Fig. 11.3b), designed by variety of synthetic conjugation methods. Most commonly used targeting components are small organic molecules, antibodies (mAbs), peptide ligands, sugar residues, aptamers (specific to particular receptors), selectins, antigens, and mRNAs overexpressed in targeted cells or organs. It is imperative that the targeting moiety binds with high selectivity to molecular receptors that are uniquely overexpressed on the cell surface.

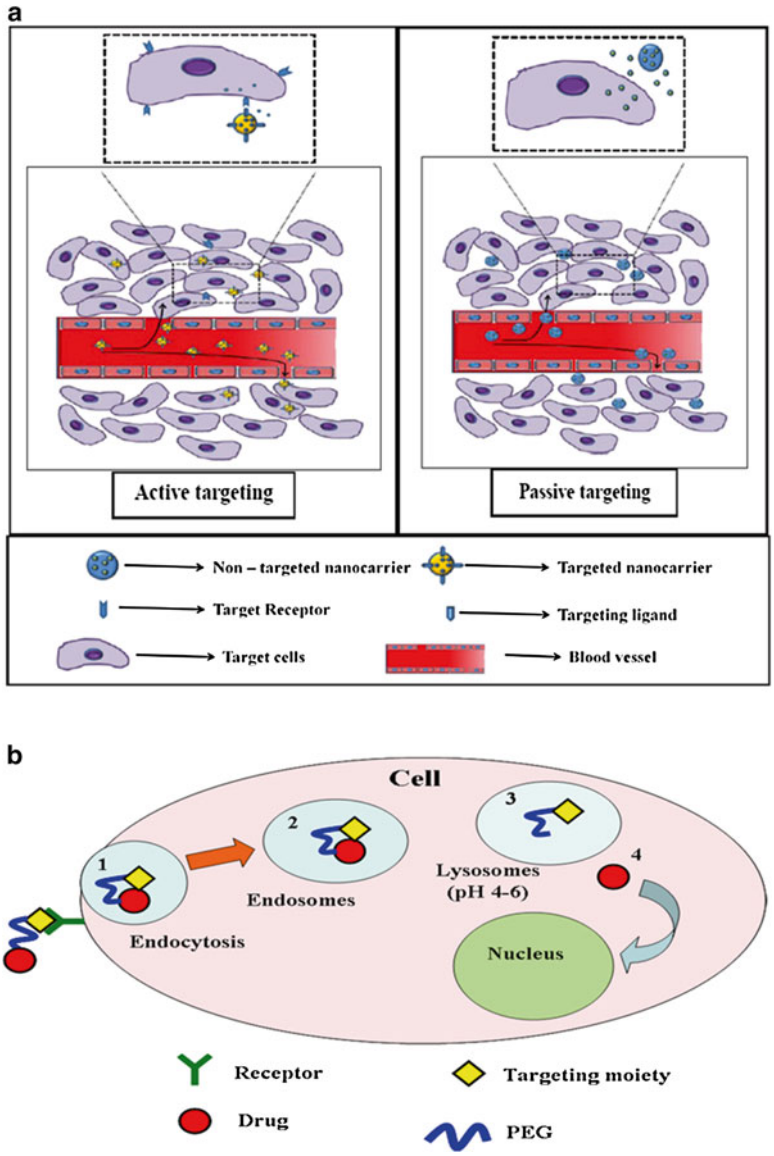

Fig. 11.3 (**a**) Active and passive targeting approaches though prodrug system, (**b**) (1) prodrug is docked at cell surface by ligand–receptor interaction and then internalized by tumor cells through receptor-mediated endocytosis, (2) transport of prodrug in membrane limited organelles, (3) fusion with lysosomes, (4) finally, drug is released intracellularly on exposure to lysosomal enzymes or lower pH (pH 6.5 to <4.0) [15, 16]

11.2 Prodrug Systems for Targeted Drug Delivery

The critical requirements for achieving selective targeting of prodrug to tumors are as follows: (1) it should be highly stable in blood circulation, (2) higher biodistribution to the targeted site, (3) adequate contact time with the target, (4) sufficient retention by the target, (5) retention of drug potency, and (6) adequate clearance fate of non-targeted compound [17]. Polymer therapeutics with various polymeric architectures have been reported to achieve cellular targetability and EPR effect (Fig. 11.4). Similarly, Table 11.1 shows different types of targeting through ligands, and their specific targets through various drug delivery systems. Furthermore, to target specific biological molecules (e.g., enzymes, peptide transporters, antigens) that are overexpressed in tumor cells in comparison to normal cells, new promising anticancer prodrugs can be designed which includes:

1. Enzyme-activated prodrugs—antibody-directed enzyme prodrug therapy (ADEPT) and gene-directed enzyme prodrug therapy (GDEPT) [17].
2. Targeting-ligand conjugated prodrugs—antibody–drug conjugates, peptide–drug conjugates, aptamer–drug conjugates, and folic acid–drug conjugates [18].
3. Enzyme-cleavable prodrugs.
4. Membrane transporter-associated prodrugs.
5. Polymeric prodrug conjugates.

The section below describes the classification and mechanisms of targeting.

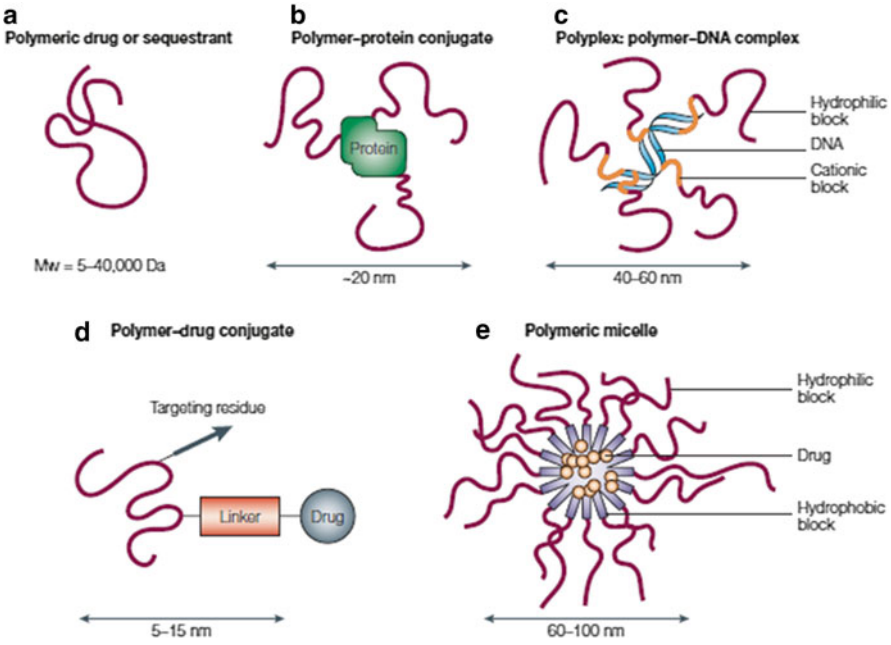

Fig. 11.4 (**a**-**e**) Different polymer therapeutics with various architectures for delivering biological actives (reproduced from [3])

Table 11.1 Examples of targeted molecular therapeutics approved by US FDA 1988–2014 (US FDA website [20], modified from [21])

Targeted molecular therapeutics	Brand name	Drug type	Type of cancer	Primary molecular targets	Year
Ibrutinib	Imbruvica	Small molecule	CLL, MCL	BTK, BCR	2014
Bevacizumab	Avastin	Monoclonal antibody	mCRC	VEGF Ligand	2013
Axitinib	Inlyte	Small molecule	Renal cell carcinoma	KITm PDGFRβ, VEGFR 1/2/3	2013
Sorafenib	Nexavar	Small molecule	HCC; RCC, DTC	RAF/MEK/ERK, VEGFR/ PDGFR	2013
Crizotinib	Xalkori	Small molecule	NSCLC	HGFR, c-MET, c-ros, RON	2013
Ziv-aflibercept	Zaltrap	Recombinant fusion protein	Colorectal cancer	PIGF, VEGFA/B	2012
Everolimus	Afinitor	Small molecule	RCC, breast cancer	mTOR	2012
Vandetanib	Caprelsa	Small molecule	Medullary thyroid Cancer	EGFR (HER1/ERBB1), RET, VEGFR2	2011
Sipuleucel-T	Provenge	Vaccine	Castrate-resistant prostate cancer	GnRH, LHRH	2010
Ofatumumab	Arzerra	Monoclonal antibody	CLL	ADCC, CDC	2009
Vorinostat	Zolinza	Small molecule	Cutaneous T-cell lymphoma	HDAC	2006
Cetuximab	Erbitux	Monoclonal antibody	SCCHN	EGFR, ADCC, KRAS	2004
Bortezomib	Velcade	Small molecule	Multiple myeloma, MCL	Proteasome	2003
Trastuzumab	Herceptin	Monoclonal antibody	Breast cancer, colon cancer	HER-2	1998
Methotrexate	Trexall	Small molecule	Breast, head and neck, leukemia	Folic acid	1988

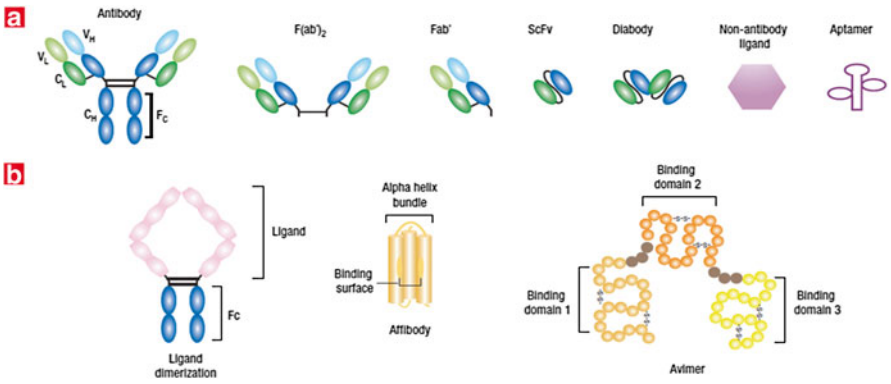

Fig. 11.5 (**a**) Various targeting molecules such as a monoclonal antibody or antibodies' fragments, non-antibody ligands, and aptamers. (**b**) Affinity and selectivity can be increased by dimerization of ligand or by screening for conformation-sensitive targeting agents such as intact antibodies and their fragments as well as antibodies, avimers and nanobodies (reproduced from [19])

11.3 Type of Targeting Moieties

Targeting agents can be classified broadly as proteins (mainly antibodies and their fragments), nucleic acids (aptamers), or other receptor ligands (peptides, vitamins, and carbohydrates) as shown in Fig. 11.5.

11.4 Implications of Molecular Targeting in Anticancer Therapy (e.g., CDK Inhibitors, mTOR, IGFR, VEGF)

The implementation of targeted cancer therapy for individual patient has revolutionized the existing ways for cancer therapy. There is an increasing importance of targeted therapy in the treatment of several cancer entities (e.g., colon, NSCLC, breast, lymphoma, and malignant melanoma) and its molecular targets such as human epidermal growth factor 2 (HER2) [22], epidermal growth factor receptor (EGFR) [23], cyclin-dependent kinase inhibitors (CDK inhibitor) [24], vascular endothelial growth factor (VEGF), etc. [25, 26]. However, the key issue in implementing targeted therapy may only be effective when the tumor carry certain molecular features; otherwise they can be ineffective or can create unwanted side effects. For example, panitumumab is active against colon cancer only when the tumor is Kirsten rat sarcoma viral oncogene (KRAS) wild type [27].

In order to avoid such limitations and designing diagnostic analysis of solid and hematological tumors, identifying target genes is an essential step. In order to have an identification process, the laboratories explore high-end techniques, such as

FISH assay, PCR, HPLC, protein arrays, DNA/RNA-array technology which are used to precisely detect the genetic alterations in the diseased state. For example, breast cancer is characterized by overexpression of HER2 which has been known to be more aggressive disease progression and a poorer prognosis [28, 29]. Hence, many researchers have focused on HER2 inhibitors as potential anticancer target which is achieved through gene therapy or by using drugs as Trastuzumab [30]. However, patients with HER2 positive breast cancer developed resistance towards the first FDA-approved therapeutic antibody for metastatic breast cancer "Trastuzumab" through hyperactivation of the phosphatidylinositol 3-kinase (PI3K)/Akt/mTOR signaling pathway [31]. The PI3K/Akt/mTOR signaling cascade has important regulatory functions in normal and oncogenic cellular growth, survival, proliferation, migrations and metabolism [32]. Several studies have shown that second mutations in this signaling pathway confers resistance mechanism to HER2-targeted therapies and direct inhibition of PI3K/Akt/mTOR signaling cascade may overcome trastuzumab resistance [33, 34]. Hence, combination of Trastuzumab and Anastrozole is targeted towards MAPK pathways and Akt pathway [35]. Likewise, inhibition of mTOR with drug Everolimus is efficacious when combined with Trastuzumab [36]. Recently, US FDA has approved Trastuzumab with Emtansine as a first antibody-drug conjugate for treating HER2-positive metastatic breast cancer [20]. In addition to many other molecular targets, sialic acid, a derivative of neuraminic acid, is one of the major targets in developing therapeutic treatment in cancer patients as hypersialylation has been shown to contribute cancer cell progression and metastasis [37]. However, till date, there is no therapeutic drug developed to interfere with sialic acid synthesis which might offer better treatment approach for cancer patients. Sialic acid as a targeting moiety with PEGylated doxorubicin (Dox) targeted prodrug conjugate demonstrated significant antitumor activity compared to free Dox and non-targeted conjugate counterpart. This significant effect is achieved by enhancing the permeation and prodrug uptake by cancer cells and cytotoxicity of the prodrug [38].

11.5 Role of Antibodies in Targeted Therapy

In 1975, Köhler and Milstein developed methods to recover antibodies in large amount which can be directed against specific antigens. Since then monoclonal have emerged as most important ligands for delivering contrast agents and chemotherapeutics for several different malignancies [39]. Monoclonal antibody are stable in blood and typically have nanomolar affinities for their target. The binding and non-binding domains of mAb are separated physically; hence, they could impart substitution with other chemical agents like, contrast agents and chemotherapeutics. Three targeting moieties, whole mAb, Fab′, and single chain variable fragment (scFv) were evaluated for targeting the same B-cell antigen CD19 [18]. The Fab′ immunoliposomes even though exhibited the most prolonged circulation times, exhibited statistically insignificant numbers of long-term survivors [18]. While, in

B-cell model, the anti-CD19 Fab′ immunoliposomes demonstrated increased circulation time and higher survival rates for Namalwa-bearing SCID mice as compared to the anti-CD19 mAb immunoliposome treatment [40].

More recently, the fragments of antibody containing only the variable region of the antibody are used for active targeting of therapeutics because they retain the specificity for their target [36]. In addition, they prevent complement activation due to the lack of constant Fc effector region or undesirable interaction with other cells. Furthermore, the smaller sizes of antibody fragments are important factor in the development of an actively targeting nanoparticle. Moreover, using antibody fragments can also help in efficient cell permeability [41]. Therapeutic agents must cross various biological barriers as well as the high interstitial pressure to reach their target cells. Towards this antibody fragments such as scFv and Fab are known to represent higher efficiency in penetrating tumor cells compared to intact antibody.

Immunogenicity caused by these antibodies is yet another important factor in using them for therapeutic targeting. Animal originated antibodies are obviously identified as foreign agents resulting in strong immune responses. However, genetic engineering tools can now design chimeric mouse-human mAbs. For example the anti-CD20 mAb rituximab (Rituxan) has revolutionized lymphoma treatment. On the other hand, humanized antibodies, containing only the binding regions of the mouse antibodies combined with a human antibody, exhibit reduction in immunogenicity [42]. However, they have shown reduced affinity in some cases. Further, antibodies could lose activity when translated into a conjugated form. Therefore, novel chemical strategies are essential to retain their potency even after the conjugation and at the same time are able to release the cytotoxic agent, either after binding to the cancer cell surface or after endocytosis into the cell. An example of such a conjugation is that of calicheamicin, a cytotoxic drug, conjugated to a tumor-targeting mAb through an amide linkage. The conjugate accumulates in the tumor but shows no appreciable cytotoxicity [43]. However, when calicheamicin is conjugated using a pH-sensitive bifunctional linker that permits its release intracellularly (Fig. 11.6), the conjugate shows potent antitumor activity [44].

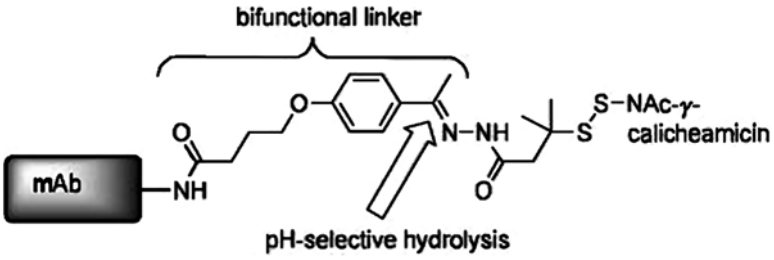

Fig. 11.6 Cleavable bifunctional linker for the conjugation of calicheamicin to monoclonal antibodies (reproduced from [17])

The prodrug was designed by coupling calicheamicin to various hydrazide bearing spacers through a disulfide bond, and the resulting moiety was conjugated to humanized anti-CD33 monoclonal antibody (clone P67.6) hinge region. Monoclonal antibody conjugated calicheamicin was then coupled to aldehyde-bearing oxidized carbohydrates, through hydrazone bonds [44]. In vivo study showed that active prodrugs rapidly cleaved at pH 4.5 whereas, they were stable at pH 7.4. On the other hand the efficacy of the prodrug system was found to be far less when calicheamicin was conjugated with a linker encompassing a non-pH-labile amide bond to mAb. This conjugate although had the same affinity for CD33 as Mylotarg, the system was found to be hundreds of times less cytotoxic in vitro and considerably less active in vivo and ex vivo [44]. In this system, the release of calicheamicin was believed to be due to intracellular oxidation of a hindered disulfide bond. This clearly indicated that the importances of linker chemistry for effective intracellular activation of calicheamicin–mAb conjugate.

11.6 Protein and Peptide Based Carrier Systems

Peptide ligands are being explored against a tumor-specific antigen or a peptide transporter that is overexpressed in cancer cells [40]. Peptide ligands can be directly conjugated to chemotherapy drugs to achieve a targeted delivery to cancer cells. Compared to antibody, peptides are more suitable targeting moieties because of (1) low molecular weight, (2) exceptional cell permeability, (3) ease in chemical conjugation, and (4) simple to produce [45].

The main approach in identifying appropriate peptide ligands is to screen peptide libraries produced by either phage display [46] or by chemical synthesis process [47, 48]. Phage display assists in identifying peptides that target a specific receptor, or certain cell types even if the receptors are unidentified [49]. Till date, various types of receptors or cells, have been discovered such as integrin receptors [50, 51], thrombin receptors [52], tumor cells [53–55], cardiomyocytes [56], and pancreatic β cells [57]. Tumor-targeting peptides have been effectively used in delivery vehicles for targeting small molecule drugs, oligonucleotides, liposomes, imaging agents, and inorganic nanoparticles to tumors. Furthermore, peptidomimetic self-assembled nanoparticles and peptide aptamers, which are peptide-related nanoparticles, also have shown great promise in targeted drug delivery. The former have wider applications in tumor imaging, tumor targeting delivery and vaccination [58], whereas the latter are directly used as drugs interfering with the function of receptors [59]. The use of these peptides has assisted in enhancing the specificity and efficacy of drug delivery with reduced side effects [60, 61]. The current discoveries of tumor lymphatic vessel targeting peptides present another route for targeted drug delivery for tumors [62–64].

Transferrin (Tf), a serum non-heme iron binding glycoprotein is a very pertinent targeting agent for cancer therapeutics due to overexpressed Tf receptors (TfR^+) on malignant cells compared to normal cells because of the higher demand for faster

cell growth and division. We and many others have demonstrated use of Tf for targeting TfR^+ in cancer therapeutics and diagnostics [65]. Tf is also extensively reported in human clinical trials with adriamycin [66], cisplatin [67], and diphtheria toxin [68].

An important study to demonstrate Tf mediated targeting was explored using 0.05 % PEG-AD containing Tf formulation [69]. The formulation was compared against AD-PEG-particles (with 0.0 % Tf-PEG-AD) using cells K562 for gene delivery. Tf-PEG-AD demonstrated a fourfold increase in transfection mainly due to Tf mediated uptake. However, presence of excess Tf added to 0.05 % Tf-PEG-AD particles eliminated the transfection enhancement [69].

An important characteristic of Tf ligand for targeting TfR^+ is the iron-binding efficiency. Transferrin ligand with low iron-binding efficiency resulted in a lower efficiency in binding to the TfR [68]. This was revealed from the flow cytometry study involving fluorescein-labeled transferrin (Tf-fluor) and holo-transferrin (holo-Tf, native transferrin), Tf-PEG-AD, or Tf-$(PEGAD)_2$. The Tf-PEG-AD and Tf-$(PEG\text{-}AD)_2$ showed lower binding affinities, while holo-Tf treatment showed the highest binding affinity mainly due to their oxidized state. As a result, a different synthesis route for Tf-PEG-AD was designed to improve the binding affinity to the Tf receptor. The synthesized Tf-PEG-AD nanoparticles (250 nM Tf-fluor/75 nM Tf-PEG-AD nanoparticles) showed a 15 % reduction in fluorescence compared to Tf-PEG-AD conjugates (250 nM Tf-fluor/75 nM Tf-PEG-AD in conjugate form) revealing high binding. The difference in receptor binding was mainly due to multiple interactions between each ligand-modified particle and cell surface receptors.

Davis et al. have also performed a novel study of small interfering RNA (siRNA) delivery in nonhuman primates using Tf-conjugated liposomes [70]. The efficacy of these Tf-conjugated liposomes had been proven effective in metastatic mouse models of Ewing's sarcoma, and consequently, the safety of the administration of these particles in nonhuman primates was the focus of this study [71, 72].

Interestingly, Tf-conjugated liposomes co-encapsulating Dox and verapamil (Tf-L-DOX/VER) have been shown to effectively overcome multi-drug resistance [73]. Cellular uptake of Tf-L-DOX/VER was 5.2 and 2.8 times greater with cytotoxicity (IC_{50} = 4.18 μM) than non-targeted liposomes having Dox and verapamil (IC_{50} = 21.7 μM) and Tf-conjugated liposomes loaded with Dox alone (IC_{50} = 11.5 μM) in a chronicmyelogenous leukemia cell line (K562 cells). In addition, the difference in cytotoxicity between the targeted and non-targeted liposomes was diminished within the presence of 2 mg/mL free Tf. This exhibits the effectiveness of the Tf moiety for cellular uptake and cytotoxicity [72].

We have recently shown that Tf conjugated multicomponent magneto-dendritic nanosystem (MDNS) can be efficiently used for rapid tumor cell targeting, isolation, and high-resolution imaging by a facile bioconjugation approach [65]. The bio-functionalized MDNS designed by combining multiple components such as Tf, iron oxide (Fe_3O_4) nanoparticles, fourth generation (G4) dendrimers, cyanine 5 NHS (Cy5) fluorescent NIR dye and glutathione (GSH) was able to capture TfR overexpressed cancer cells from an artificial circulating tumor cell (CTC)-like suspension (Fig. 11.7). The MDNS platform exhibited rapid capture (~5 min) of

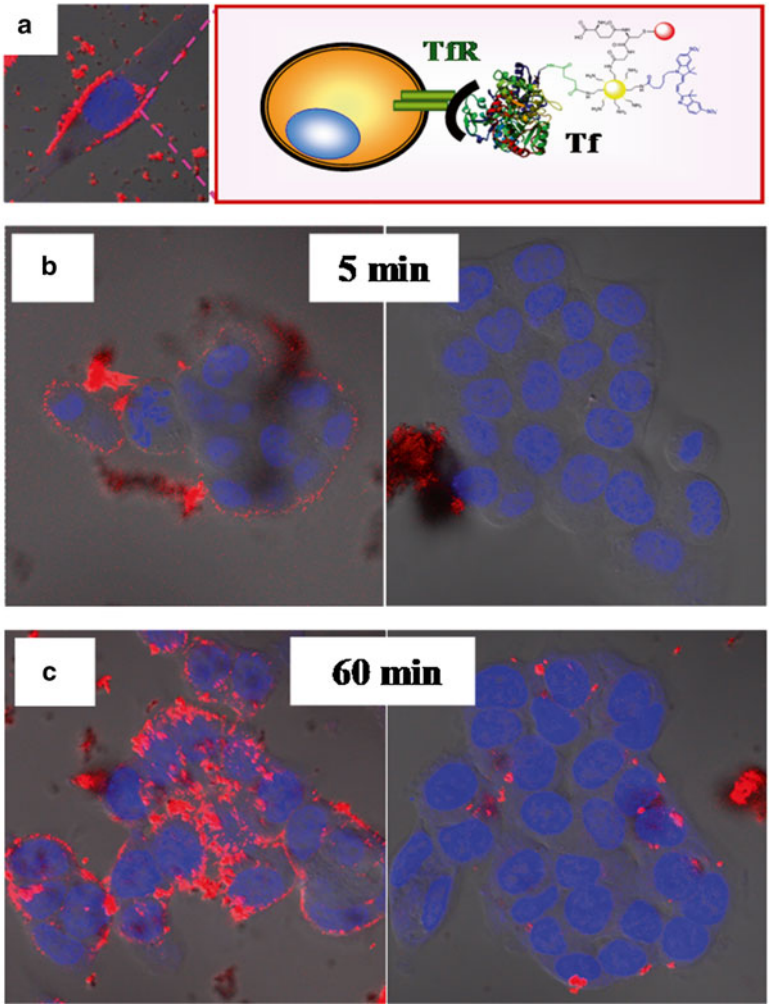

Fig. 11.7 Cellular targeting of a bio-functionalized Magneto-Dendritic Nano System (MDNS). (**a**) Magnified image of a cell showing localization of MDNS particles on the cell membrane after 5 min of incubation. (**b**) *Left image* shows Tf^{+} MDNS particles attached to the HCT116 cells, whereas, hardly any Tf^{-} MDNS particles present on the cell surface as shown in right image. (**c**) After 60 min of exposure only the Tf^{+} MDNS were present in large numbers on the cells revealing target specificity of MDNS interaction remains intact even after long exposure (reproduced from [65])

TfR-overexpressing (TfR^{+}) cancer cells at clinically relevant concentrations (approximately 1 CTC per 10^5 blood cells) [65].

Choi and his coworkers showed that Tf decorated PEGylated gold nanoparticles accumulations in the tumors and other organs are independent of Tf (Fig. 11.8) [74]. However, the nanoparticle localizations within a particular organ are influenced by

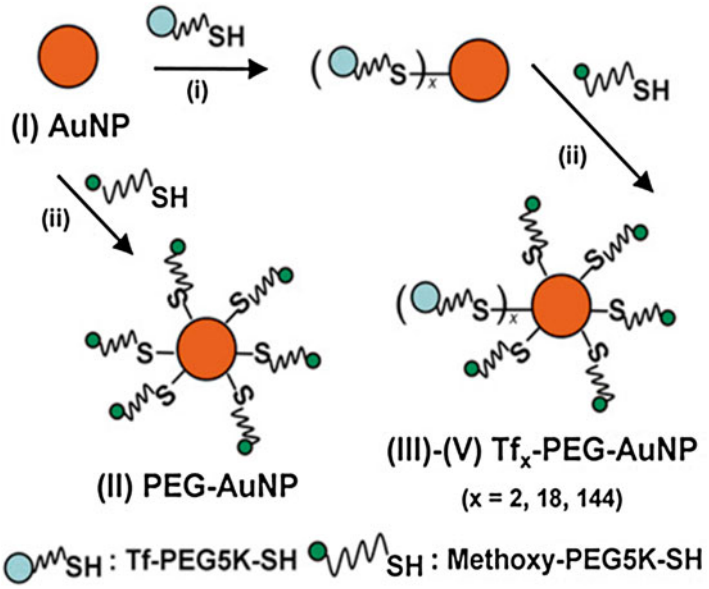

Fig. 11.8 Schematic of Tf-PEG-AuNPs. Unmodified 50-nm AuNPs (I) were reacted with excess mPEG-SH to form PEG-AuNPs (II) as untargeted particles or first were reacted with various amounts of Tf-PEG-SH and later excess mPEG-SH to form Tf-PEG-AuNPs (III: 2 Tf per particle; IV: 18 Tf per particle; V: 144 Tf per particle) (reproduced from [74])

the Tf content. They also demonstrated that in tumor tissue, the content of targeting ligands significantly influenced the number of nanoparticles localized within the cancer cells. Most nanoparticles remain in nonparenchymal cells, however, small amount of nanoparticles resided in hepatocytes due to higher Tf content [74].

Similarly, Lutenizing Hormone Releasing Hormone (LHRH) peptide-conjugated prodrug has shown promising results for cancer therapy [75, 8]. Khandare et al. conjugated LHRH peptide using PEG as the spacer to camptothecin (CPT), a cytotoxic drug [75, 8] (Fig. 11.9). The LHRH peptide-conjugated prodrug demonstrated higher efficacy with minimized side effects on healthy organs. In addition, the prodrug system showed targeting potential for both solid tumor tissue as well as a single tumor cell. Hence, this prodrug can effectively target tumor cells with a low toxicity to normal tissues [75]. Neamati and coworkers conjugated paclitaxel, an antimicrotubule agent commonly used in the treatment of metastatic breast cancer to a cyclic peptide E[c(RGDyK)]$_2$ (RGD). In vivo studies showed a specific tumor uptake of the RGD-paclitaxel system at 4 h post administration [76].

Similarly, Yoneda et al. have explored targeted delivery of paclitaxel and doxorubicin by using glucose-regulated protein 78 (GRP78) as a tumor-specific antigen [77]. GRP78 is overexpressed on many tumor cells, including skin, prostate, colon,

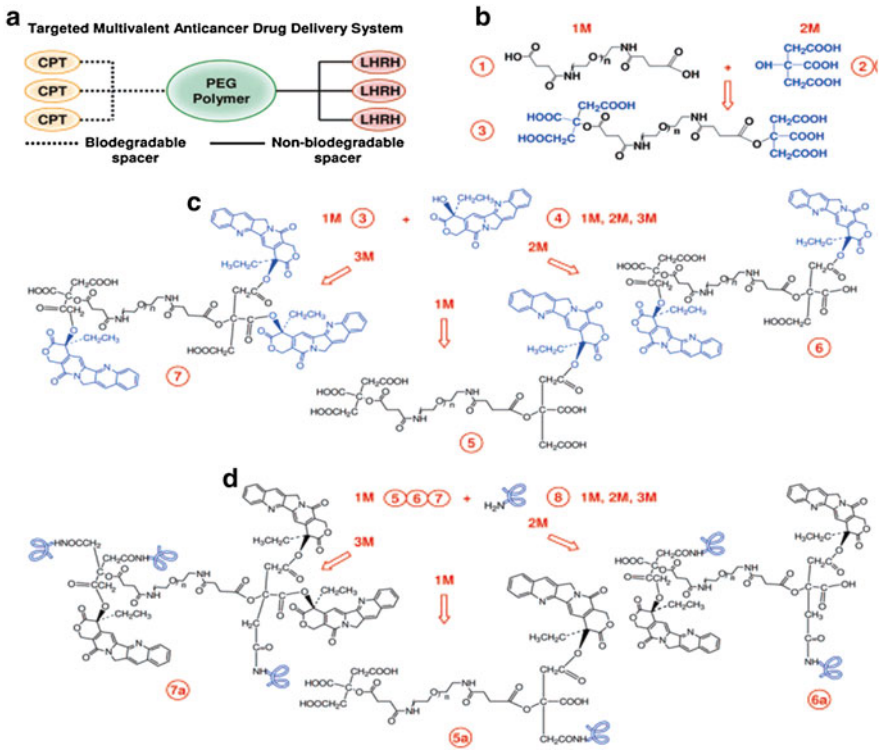

Fig. 11.9 Schematic of targeted multivalent anticancer prodrug. The prodrug conjugate system was designed with (**a**) bis-PEG polymer as a carrier- one, two, or three copies of CPT as an anticancer drug; and one, two, or three copies of LHRH peptide as a targeting agent. (**b**) α,ω-bis-PEG3000-CA conjugate (3) was designed by conjugation of bis(2-carboxyethyl) PEG (1) with CA (2). (**c**) The bis-PEG-CA conjugate (3) was conjugated with CPT (4) to obtain α,ω-bis(2-carboxyethyl) PEG-CA-CPT conjugates (5, 6, and 7). (**d**) α,ω-bis(2-carboxyethyl) PEG-CA-CPT-LHRH conjugates (d, 5a, 6a, and 7a) having one, two, and three copies of CPT (4) and LHRH (8) were synthesized by conjugating LHRH (8) with 5, 6, and 7 (reproduced from [75])

and breast cancers. On the other hand, its expression on normal tissues is very small [78]. Pep42, a cyclic 13-meroligopeptide (CTVALPGGYVRVC), internalizes through the GRP78 receptor-mediated endocytosis after specifically binding to GRP78 and then trafficked to the lysosome that contains protease cathepsin B. Therefore, Val-Cit motif, a cleavable linker, was used to link Pep42 to anticancer drugs. The Val-Cit linker is reasonably stable in the plasma, but cathepsin B in the cancer cells can cleave [79]. Both Pep42-paclitaxel and Pep42-Dox showed an enhanced toxicity in comparison to the free drug when cytotoxicity of the Pep42-prodrug was estimated in osteosarcoma cells, SJSA-1 [77].

11.7 Folic Acid-Drug Conjugate

Folic acid (FA) is a member of the vitamin B family and is one of the most commonly used targeting moiety for specific delivery of various imaging agents, therapeutic agents, and nano-scaled systems to tumor cells. It is known to bind with a very high affinity (K_d 0.1–1 nM) to folate receptor (FR). Folate receptor is overexpressed on the surface of many malignant cells including breast, lung, kidney, ovarian, and endometrial cancers [80]. On the other hand, the expression of FR on other normal tissues is low and restricted to some epithelial cells. Folic acid conjugated prodrugs enter cells via receptor mediated endocytosis after binding to folate receptors. In addition, FA has a low immunogenicity and relatively simple chemistry compared to other targeting moieties such as antibody, peptide, and aptamer [80–82]. For targeting tumor cells, a range of anticancer drugs have been conjugated with FA.

To enhance the specificity to tumor cells Dox was conjugated to FA [83]. Dox is an anthracyclinic drug used for a wide variety of cancers. However, poor solubility, extremely high toxicity and short half-life limit its therapeutic efficacy. D-α-Tocopheryl polyethylene glycol succinate (TPGS) was conjugated, to the FA modification, to Dox to enhance the solubility and drug permeability across cell membrane. The TPGS-Dox-FA prodrug exhibited enhanced half-life, high antitumor efficacy (45-fold more effective than the unmodified Dox), and less accumulation in the heart, which is the major organ affected by Dox's side effects [83].

Philip et al. demonstrated that FA on conjugation to campothecin, a poor water-soluble and highly toxic chemotherapy agent, via a hydrophilic peptide containing a disulfide bond [81]. They showed increase in specificity of the prodrug, while the cleavable spacer increased the solubility of campothecin and also provides an efficient release of camptothecin within tumor cells via the disulfide reduction [81].

11.8 Conclusions

Polymeric prodrug conjugate systems offer a potent and versatile tool for improving the therapeutic potential of low-molecular-weight drugs and proteins. Although considerable progress has been made in the field of prodrugs in clinic, prodrug systems consisting a targeting component still remains only as a perspective. There are numerous scientific challenges to overcome this goal, such as advanced biomaterials, molecular tunability, physicochemical strategies, immunogenicity, cell permeability, and cell specificity in clinically relevant targeted drugs. However, several prodrug systems having anticancer agents are currently under clinical trials. Towards this direction, in next few years many other anticancer conjugates could get regulatory approvals. In the future, requirement for advance drug delivery strategies such as targeted prodrugs will become more important since the discovery of new anticancer drugs will become increasingly challenging and expensive [1].

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