Targeting of nanoparticles in cancer: drug delivery and diagnostics

Meghna Talekar[a], Jackie Kendall[b], William Denny[b] and Sanjay Garg[a]

Anticancer agents continue to be a preferred therapeutic option for several malignancies. Despite their effectiveness, oncologists are continually looking for tumor-specific anticancer agents to prevent adverse effects in patients. Targeting of imaging agents to cancerous tissue is another area that is enthusiastically explored to circumvent some of the drawbacks that current imaging agents possess, including the inability to target small tumor cells, inadequate imaging period, and the risk of renal damage. Formulation scientists have explored nanotechnology-based delivery systems for targeting anticancer agents and tumor-imaging agents to cancer tissue. Targeting with nanotechnology-based delivery systems has been investigated by both passive and active mechanisms with significant clinical success. This review presents a discussion on targeting strategies used for the delivery of nanoparticles by passive and active mechanisms, focusing more specifically on active targeting of nanoparticles using albumin, folic acid, transferrin, and aptamers as targeting ligands. ***Anti-Cancer Drugs*** **22:949–962 © 2011 Wolters Kluwer Health | Lippincott Williams & Wilkins.**

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[a]School of Pharmacy and [b]Auckland Cancer Society Research Centre, Faculty of Medical and Health Sciences, The University of Auckland, Private Bag, Auckland, New Zealand

Correspondence to Dr Sanjay Garg, School of Pharmacy, Faculty of Medical and Health Sciences, The University of Auckland, Private Bag, Auckland 92019, New Zealand
Tel: +64 9 923 2836; fax: +64 9 367 7192;
e-mail: s.garg@auckland.ac.nz

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Introduction

Cancer is a condition in which cells divide without control and can invade nearby healthy tissue spreading through the bloodstream and the lymphatic tissues to other organs. In 2008, 7.6 million people died of cancer and it is predicted that by 2030, cancer will cause more than 11 million deaths worldwide [1,2]. Even with fascinating advances in the field of medical sciences, several types of cancers are still incurable or are associated with severe therapy-related adverse effects. To resolve this dilemma, pharmaceutical scientists around the world are continuing to advance research in anticancer therapy, more specifically toward targeted delivery of these anticancer agents to tumor sites.

The National Nanotechnology initiative, a US government program that was initiated in 2001, defines nanotechnology as 'the understanding and the control of matter at dimensions of approximately 1–100 nm, where unique phenomena enable novel applications' [3]. In pharmaceutical terms, nanoparticles are submicron colloidal systems where the drug is incorporated, adsorbed, or dispersed on their surface [4,5]. Nanotechnology-based delivery systems (NBDS) have been extensively explored for targeted delivery of cancer imaging and therapeutic agents.

NBDS are often targeted to tumor sites using passive or active mechanisms. In this review, we will briefly cover aspects of passive targeting, followed by in-depth review of active targeting of nanoparticles using albumin, folic acid (FA), transferrin, and aptamers as targeting ligands.

Passive targeting of nanoparticles

In passive targeting, nanoparticles accumulate at the tumor site due to their nanometer size range. Normal vasculature is impermeable to molecules larger than 2–4 nm, except for kidney, liver, and spleen (fenestrated endothelium of kidney glomerulus has 40–60 nm and sinusoidal endothelium of liver and spleen have up to 150 nm of intercellular gaps) [6]. In contrast, tumor tissues have an increased permeability to macromolecules and colloidal carriers of diameter up to 600 nm. This allows nanoparticles to easily extravasate into the interstitial matrix at the tumor site, reducing drug distribution and toxicity to normal tissues. Along with this, the lymphatic drainage system on the tumor tissue does not clear nanocarriers effectively, which results in decreased clearance of the nanocarriers from the tumor site. The cumulative effect of this is the enhanced permeation retention (EPR) effect (Fig. 1), which allows the drug to remain in contact with the tumor site for an extended period of time allowing sustained drug release [7–9]. The EPR effect was first described by Maeda *et al.* [10] and has been a vital mechanism for passive targeting of NBDSs to tumor tissues. However, these nanoparticles are quickly recognized as foreign particles and are opsonized by plasma proteins in the blood stream and then rapidly sequestered into reticuloendothelial system (RES) cells. This leads to their

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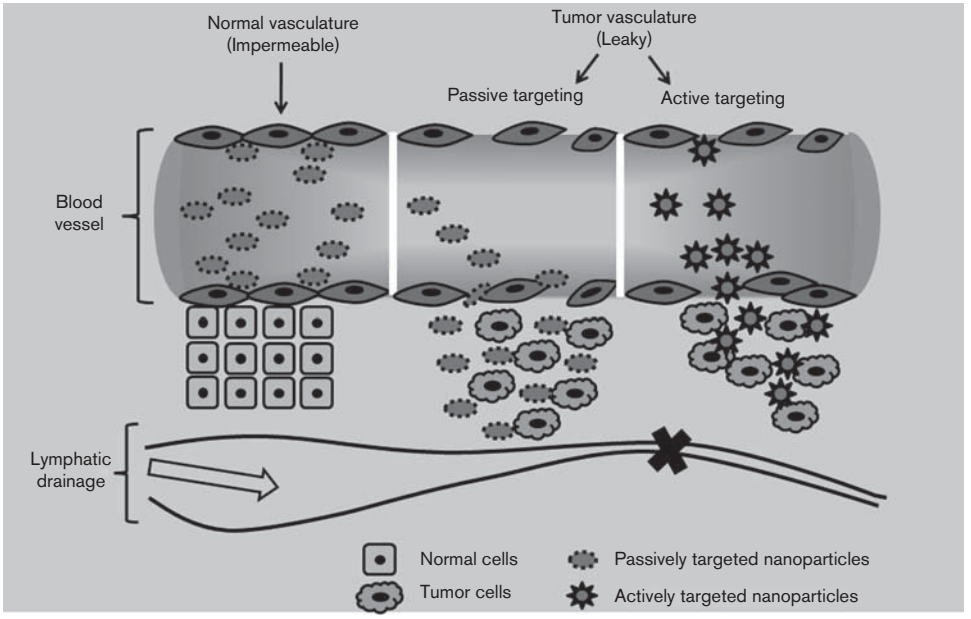
Fig. 1



Passive and active targeting of nanoparticles. Healthy vascular endothelial cells are impermeable to nanoparticles. In contrast in tumor tissues, passively targeted nanoparticles accumulate due to increased intercellular gaps between vascular endothelial cells and due to poor lymphatic drainage from the tumor interstitium, a phenomenon coined as the enhanced permeation retention effect (EPR). Actively targeted nanotechnology-based delivery systems, which are coated with targeting ligands, accumulate in the tumor interstitium by the EPR effect. These ligands allow selective uptake of the nanoparticle into the tumor cell, leading to a targeted cytotoxic effect.

rapid clearance from the systemic circulation. To enhance the circulating half-life of nanoparticles, hydrophilic polymers such as polyethylene glycol (PEG) or PEG-containing copolymers (poloxamers, poloxamines, and polysorbates) are added to their surface to increase the sustainability of the drug in the tumor tissue.

DaunoXome (liposomal daunorubicin; Gilead Sciences, Foster City, California, USA) and Doxil (PEG-coated liposomal doxorubicin; Centocor Ortho Biotech, Raritan, New Jersey, USA) are the first two NBDS against cancer that were approved by the US Food and Drug Administration (FDA), and have proved very successful in the clinic. In DaunoXome, daunorubicin is encapsulated in self-assembling lipid vesicles called liposomes; whereas in Doxil, the doxorubicin is incorporated in anionic liposome and the surface of the liposome is coated with PEG, which allows the drug to escape from the RES cells, resulting in an enhanced circulation time, an improved accumulation in tumor tissue, an increased antitumor activity, and avoidance of cardiotoxicity [11,12].

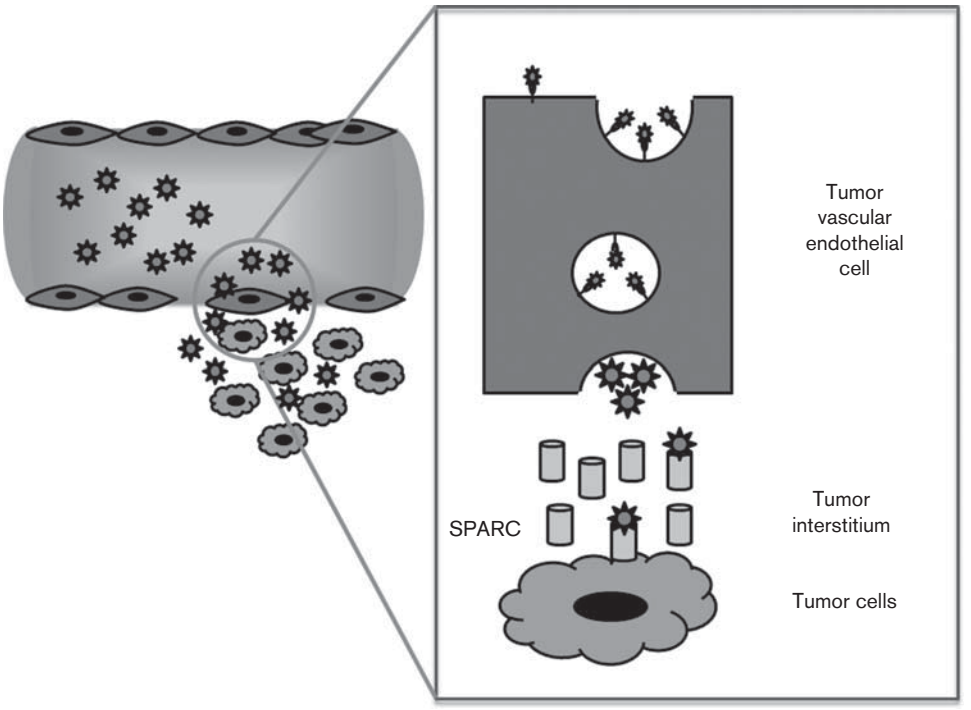
Passively targeted NBDS can target anticancer drugs to tumor tissues due to the EPR effect. However, the therapeutic agent needs to be internalized from the tumor interstitium into the tumor cell to exhibit any effectiveness. This uptake can be achieved by actively targeted NBDS (Fig. 1).

Active targeting of nanoparticles

In the 19th century, Paul Ehrlich first proposed the theory of active targeting by idealizing a delivery system that would target drugs to specific areas in the body, which he described as the 'magic bullet' [13]. Active targeting of nanoparticles is achieved by attaching a component to the nanocarrier structure that recognizes a target within the tumor-affected organ, tissue, cell, or intracellular organelle, leading to preferential accumulation of the nanoparticles. Regardless of the location of the target, it is more important that the target is tumor specific, is homogenously expressed on the tumor cell, and is not shed or downregulated.

During the preparation of NBDS, the different components of these systems are subjected to harsh chemicals (solvents) and processing conditions (homogenizing, milling); hence, the selected ligand should be able to withstand such conditions. As the surface of the nanoparticle interacts with the target, the ligand incorporated on the surface of the nanoparticle should be in sufficient quantity to allow multipoint binding at the tumor site. After administration of the actively targeted nanoparticle, the ligand should not be easily degraded by endogenous enzymes nor should it have any immunogenic effect. The ligand should be internalized easily into the tumor cell and then the drug should be released at the tumor site at a therapeutically effective

Fig. 2



Delivery of albumin-bound anticancer agents to tumor cells. Albumin nanoparticles are targeted to the tumor tissue by the enhanced permeation retention effect effect and also due to binding of albumin to glycoprotein 60 receptor, which stimulates formation of caveolae that transports the albumin-bound anticancer agent into the tumor interstitium. In the tumor interstitium, the nanoparticle binds to secreted protein, acidic and rich in cysteine (SPARC), which leads to uptake of the drug into the tumor cell.

level. After delivering the chemotherapeutic agent at its site of action, the ligand should be readily hydrolyzed and cleared from the body without causing any toxicity.

To prepare such an effective carrier system, several receptors or antigens have been identified and nanoparticles have been designed. Some of these targeting agents include medium-sized molecules such as albumin, FA [14,15], galactose [16], peptides (RGD [17,18], ATWLPPR [vascular endothelial growth factor (VEGF) peptide [19]), aptamers (pegaptanib) [20], proteins (transferrin [21–23], luteinizing hormone-releasing hormone [24]], and antibodies [herceptin (trastuzumab) [25], rituxan (rituximab) [26], CD19 antibody [27]]. On the basis of our interest and ongoing research in the remainder of this paper, we will focus on the latest developments that have been reported on targeted delivery of NBDS using albumin, folate, transferrin, and aptamers as the targeting ligand.

Albumin-based targeting

Background and mechanism of targeting

Albumin is a medium-sized compound (Mw = 67 kDa) found in abundant levels in the plasma (42–54 g/l). It is synthesized in the liver and is important for various physiological processes including delivery of nutrients to cells, solubilization of long chain fatty acids, balancing plasma pH, providing colloidal osmotic pressure in the blood, and for binding to bilirubin and therapeutic agents.

Tumor cells have a high demand for amino acids; hence, albumin can be used as a carrier for the delivery of anticancer agents [28]. When albumin-bound nanoparticles are administered, they accumulate at the tumor tissue by a combination of the EPR effect and also due to the binding of albumin to glycoprotein 60 (gp60) receptor; this subsequently binds to caveolin-1 (intracellular protein), resulting in the formation of transcytotic vesicles that are delivered intracellularly and transported to the tumor interstitium (Fig. 2) [29]. In the tumor interstitium, tumor cells secrete an albumin-binding protein (secreted protein, acidic and rich in cysteine, also called BM-40) that binds to the albumin–nanoparticle complex. This allows the drug to be in close contact with the tumor cell, which leads to a preferential uptake of the drug into the tumor cells, stimulating cell death [30].

Benefits of albumin as a targeting ligand

Albumin can be successfully used as a targeting ligand because, unlike other endogenous proteins, it is stable over a wide pH range (4–9), it is unaltered by denaturing agents and solvents at moderate concentrations, and it is

stable for upto 10 h at 60°C [28,31]. Owing to these properties, albumin can remain stable under the typical processing conditions encountered during nanoparticle preparation. Albumin is also known to have a cryoprotectant effect due to which albumin-based nanoparticles can be lyophilized immediately after preparation [29,32].

In the past, bovine serum albumin and human serum albumin (HSA) were the only sources of albumin for nanoparticle preparation. Lately, alternative blood-derived recombinant HSA [Recombumin (Novozymes, Cambridge, Massachusetts, USA); Cellastim (Invitria, Fort Collins, Colorado, USA)] has been synthesized in yeast cells (Recombumin) or rice seeds (Cellastim), which have shown comparable safety and efficacy profile to HSA [33,34]. Albumin is biodegradable in nature due to which it is nontoxic and nonimmunogenic, and hence it is incorporated in several actively targeted NBDS [31].

Research and clinical success of albumin-based nanotechnology-based delivery systems

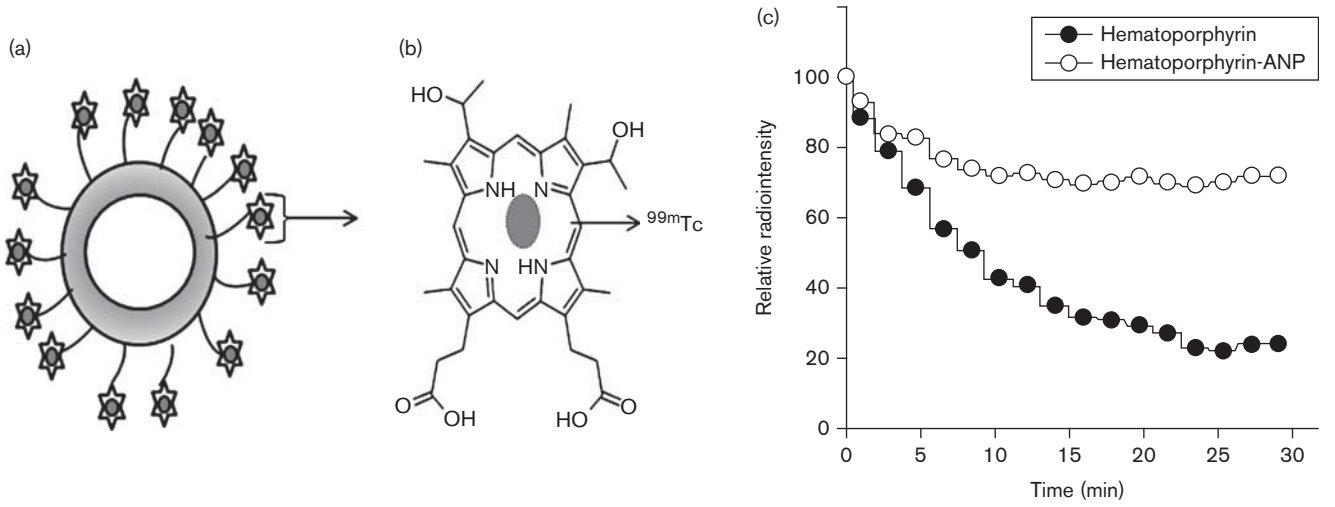
Traditionally, albumin nanoparticles (ANP) have been successfully prepared by desolvation or emulsification followed by denaturation, heating, or chemical cross-linking [35–38]. Although albumin is a robust protein, overheating can induce its denaturation, which would cause an irreversible change in its structure. Similarly during chemical cross-linking, the amines and hydroxyls on albumin can cross-link nonspecifically, resulting in a reduced activity [29]. American Bioscience developed 'nab-technology' to overcome some of these processing issues. In this process, the drug is mixed with HSA (3–4%) in an aqueous solvent and passed under high pressure through a jet to form ANP within the size range of 100–200 nm. Nanoparticle albumin-bound paclitaxel [Nab-paclitaxel (Celgene Corporation, Summit, New Jersey, USA), abraxane] is one such nanoparticulate system that was approved by the FDA in 2005 for the treatment of metastatic breast cancer. Development of this technology avoided processing problems commonly encountered in albumin nanoparticle preparation. Furthermore, this formulation did not require polyethoxylated castor oil (Cremophor EL; BASF Corp, Ludwigshafen, Germany) and ethanol, which were commonly used to solubilize paclitaxel. Phase I and II clinical studies showed that nab-paclitaxel could exhibit antitumor activity and patients did not require premedication with corticosteroids and antihistamines due to the absence of Cremophor EL. However, Gradishar *et al.* [39] conducted a crucial study in 2005 that compared the administration of nab-paclitaxel against solvent-based paclitaxel in patients suffering from metastatic breast cancer and observed a greater tumor response and a significant increase in survival with nab-paclitaxel compared with solvent-based paclitaxel [40]. Thus, this new technology provided a useful alternative toward the preparation of albumin-based nanoparticles.

The blood–brain barrier (BBB) is a complex layer of endothelial cells that separates the blood compartment and cerebral parenchyma. Its primary role is to prevent the entry of high-molecular weight molecules into the brain, due to which delivery of anticancer agents to tumor tissues in the brain continues to be a challenge in oncology [41]. Albumin NBDS have also been designed for targeting nanoparticles to the brain using adsorptive-mediated transcytosis. This is a mechanism by which cationic molecules are transcytosed into the brain. For targeted anticancer delivery to the brain, bovine serum albumin can be cationized with ethylenediamine in the presence of 1-ethyl-3-(dimethylaminopropyl) carbodiimide hydrochloride to prepare cationic ANP [42–44]. This positively charged albumin binds to the negatively charged sites on the brain endothelial cells (e.g., glycoprotein) undergoing transcytosis across the BBB [45]. Such cationized nanoparticles have also been explored for gene delivery into brain tumors, especially for the treatment of malignant gliomas. In these situations, the NBDS are localized in the glycoproteins within the brain and tumor microvasculature, delaying tumor growth and stimulating apoptosis of tumor cells [46].

A number of proteins with anticancer potential have recently been discovered. Apo2 ligand or tumor necrosis factor-related apoptosis-inducing ligand, is one such protein that is known to bind to tripartite death receptors, which are overexpressed on tumor cells. It has been investigated with tremendous interest due to its specificity toward tumor cells compared with normal cells. However, the use of this protein has been limited due to its poor solubility and pharmacokinetic properties. To overcome this problem, an albumin-based Apo2L-nanoparticle system has been designed by desolvation technique. This system maintains the cytotoxic capacity of the Apo2L and it also helps to improve its pharmacokinetic properties [47]. Hence, albumin-based nanoparticles can be explored as a promising delivery system for improving pharmacokinetic properties of proteins used for anticancer therapy.

As ANP are easily reproducible, are biodegradable, and allow functionalization of other groups on their surface; they have been combined with other targeting agents to allow effective delivery of therapeutic and diagnostic agents [48,49]. Yang *et al.* [49] recently reported the development of a core-shell multifunctional albumin nanoparticulate system, where albumin was used to target the nanoparticles to the tumor site, hematoporphyrin was added for it photodynamic and cancer-targeting properties, and ^{99m}Tc-γ-emitting nuclide was used for scintigraphic imaging. Pharmacokinetic studies conducted in rabbits with ^{99m}Tc-hematoporphyrin showed an instantaneous distribution through the body with a rapid clearance through the kidney in comparison with ^{99m}Tc-hematoporphyrin-ANP,

Fig. 3



(a) Schematic illustration of ^{99m}Tc-hematoporphyrin–albumin nanoparticles [hematoporphyrin–albumin nanoparticles (ANP)]. (b) ^{99m}Tc chelated to hematoporphyrin. (c) Radiointensity of ^{99m}Tc-hematoporphyrin-ANP and ^{99m}Tc-hematoporphyrin indicating extension of terminal half-life by grafting ^{99m}Tc-hematoporphyrin on albumin nanoparticles. Adopted from Yang *et al.* 49 – Reproduced by permission of The Royal Society of Chemistry. *http://dx.doi.org/10.1039/C0JM01544J.*

which showed limited organ distribution and also an extended terminal half life (Fig. 3).

Folate-based targeting

Background and mechanism of targeting

FA is a small-molecular weight vitamin (441 Da) that is required by eukaryotic cells in the biosynthesis of nucleotide bases (purines and pyrimidines) [50,51]. Folate can be internalized in cells by a low-affinity (K_D of approximately 1–5 μmol/l) membrane-spanning protein, which transports reduced folates directly into the cytosol or it can be endocytosed by a high-affinity glycoprotein (K_D of approximately 100 pmol/l).

The glycoprotein-based FA transport system is expressed at high levels on the surface of many cancer cells (especially tumor cells of the ovaries, mammary gland, colon, lung, prostate, nose, throat, and brain), which makes it a rational target for drug delivery to tumor tissues [50,52,53]. When FA-conjugated NBDS (FA-NBDS) are administered, they accumulate at the tumor site and bind to the folate receptor (FR), which leads to the formation of an endosome. When the pH within the endosome decreases, lysozymes are activated, which allows the folate–drug conjugate to be detached from the FR. The folate–drug conjugate then enters the cell cytoplasm by translocation, anion exchange, or simple leakage. The FR then returns to the cell membrane to start a second round of transport (Fig. 4) [52].

Benefits of folate as a targeting ligand

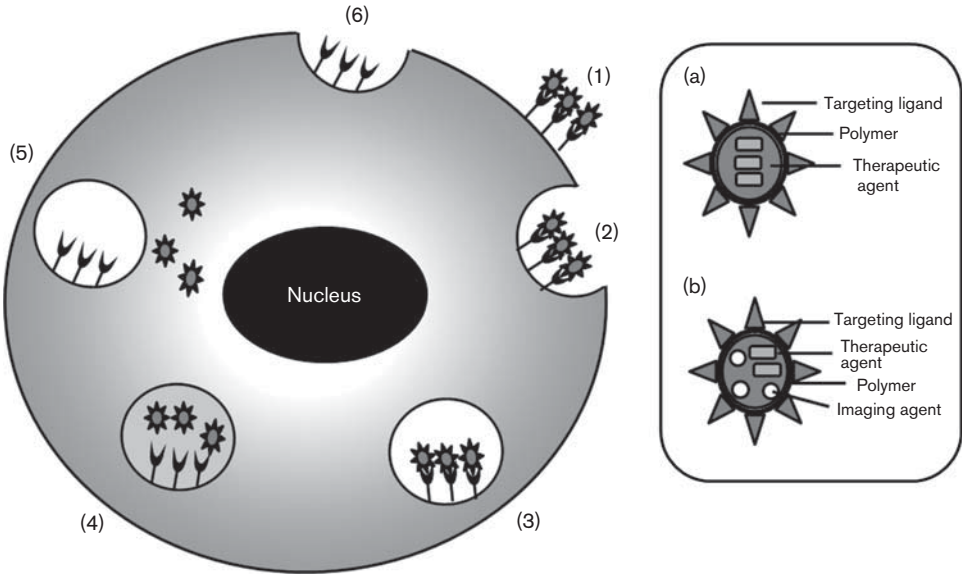
A number of properties of FA makes it a suitable ligand for targeted drug delivery. It is a stable and relatively inexpensive vitamin, which eliminates any processing problems. At the tumor site, it has a very high affinity for tumor cell surface FR and the complex is rapidly internalized into tumor cells (3×10^5 FA molecules/h) [54,55].

Research and clinical success of folate-based nanotechnology-based delivery systems

A range of polymers with an improved biocompatibility have been used for the development of FR-targeted NBDS [56–60]. In a typical FR-targeted nanoparticle, the anticancer agent is encapsulated in a stabilizing polymer and the folate is conjugated on the surface of the polymer. PEG is often used as a polymer in a FR-targeted nanoparticulate system to enhance its circulation time and also to improve the association of the targeted nanoparticle with the tumor cells [61,62]. The surface density and length of PEG chains should be optimal to maintain the system targeting and stealth properties. The surface density of PEG on the FA-NBDS can be improved by increasing the amount of PEG incorporated in the nanoparticle. An increase in the amount of PEG leads to a decrease in the distance between two adjacent PEG–lipids on the surface of the nanoparticle. This increases the lateral pressure between adjacent PEG chains, resulting in extension of the polymer from the surface providing a linear PEG confirmation. The linear conformation allows the PEG to be exposed appropriately on the surface of the nanoparticles, allowing it to fulfill its targeting and stealth functions [63].

The length of the PEG chain is also an important parameter for the development of an effective nanoparticulate

Fig. 4



Receptor-mediated endocytosis of actively targeted nanotechnology-based delivery systems (NBDS). (1) Actively targeted NBDS bind to cell surface receptor. (2) The receptor and the NBDS complex are endocytosed. (3) The NBDS are encapsulated in the early endosome. (4) The pH of endosome decreases, which leads to release of the drug from the receptor. (5) The NBDS are released intracellularly by translocation, anion exchange, or simple leakage. (6) The receptor is recycled to the cell surface. Insert: (a) NBDS, which consists of the anticancer agent, the polymer, and the targeting ligand; and (b) NBDS, which consists of the anticancer agent, the polymer, the targeting ligand, and the imaging agent.

system. The use of higher chain length PEG (PEG5000 compared with PEG3400) provides an increased association between FR-positive cells. However, if varying PEG chain lengths are incorporated in the nanoparticle, then the folate ligand can be hidden within the brush layer of PEG, resulting in a reduced interaction with the tumor cell [63,64].

The mole fraction of folate added to a nanoparticle system is also thought to affect the cytotoxic capability of the system. It is presumed that higher ligand content would give an enhanced targeting ability. However, these increased levels of FA would lead to a reduction in the stealth properties of PEG resulting in an increased uptake by the RES cells [63]. Furthermore, when excessive folate molecules are present on the surface of the nanoparticles, they can self-assemble to form dimers, trimers or tubular quartets, which cannot interact with FR (only one molecule of FA can bind to FR) [65]. Hence, it is important to determine an appropriate mole fraction of FA that is added to the targeted NBDS.

Similar to PEG, poly lactic-*co*-glycolic acid (PLGA) is a copolymer that has been incorporated in a number of nanoparticle systems due to several benefits, including biocompatibility and biodegradability. From a drug-delivery perspective, when a cytotoxic solution is administered in the tumor interstitium it is transported by passive diffusion into the tumor cell. During passive diffusion, the cytotoxic agent may interact with P-glycoprotein, which effluxes the drug back into the tumor interstitium. PLGA NPs are taken up into the cells by endocytosis, which results in higher cellular uptake of the entrapped cytotoxic agent as the drug is able to escape P-glycoprotein-mediated efflux. To further enhance accumulation of these nanoparticles into tumor cells, PLGA NPs can be coated with FA to target the FR [58,66].

Chitosan (2-amino-2-deoxy-β-D-glucan) is another natural cationic polymer that has been used in FR-targeted NBDS as it is easily available, relatively inexpensive, easy to manipulate, nonallergic, and nonimmunogenic [67]. Chitosan NBDS have also been conjugated to FA to target contrast dye to tumor tissues. The mucoadhesive property of chitosan provides sustained interaction with the target cells and the FR-mediated uptake leads to an enhanced imaging effect [68]. The cytotoxic activity of chitosan NP conjugated to FA has also been explored to show a higher cellular cytotoxicity due to enhanced uptake by receptor-mediated endocytosis complemented with a depot effect, which leads to sustained drug release providing greater apoptosis and enhanced cell cycle arrest [69]. Thus, chitosan-based FA-conjugated NBDS can be used for targeted drug delivery.

To prepare biocompatible delivery systems, researchers are now investigating delivery of NBDS using endogenous lipoprotein. Low-density lipoprotein (LDL) is one such endogenous lipoprotein, which allows lipids such as

cholesterol and triglycerides to be transported within the bloodstream. LDL has been explored as a NBDS due to its high loading capacity, biocompatibility, biodegradability, and nonimmunogenicity [70]. However, this system offers very little selectivity between tumor cells and normal cells (especially of the liver, adrenal glands, and reproductive organs) due to a high expression of LDL receptors on the surface of these cells. LDL-based FA-NBDS are specifically targeted to tumor tissue due to greater expression of FR on the surface of tumor cells. It has been observed that *in vitro*, fluorescently labeled FA showed accumulation of FA-conjugated LDL-NPs in FR-positive cells with minimal uptake in FR nonexpressing cells [71]. This concept of LDL NBDS has been further explored by functionalizing the LDL-NPs with a near-infrared dye, 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindotricarbocyanine iodide and targeting the system to FR-rich tumor tissues. In this case, in-vitro and in-vivo data showed that the targeted system was successfully directed to FR-positive tumor tissue and the incorporation of 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindotricarbocyanine iodide within the system allowed optimal cancer cell imaging in live animals [72]. Thus, conjugation of LDL-NPs with FA has enabled this system to be used for diagnosis and targeted drug delivery.

Earlier single ligand-based systems were used for nanoparticulate targeting, but researchers have now focused toward the development of multifunctional NBDS. In these multifunctional systems, the anticancer agent is usually incorporated in the central core of the nanoparticle, and the surface is coated with functional groups to consolidate several properties together. These groups can be modified as desired with cell-penetration peptides, stimulus-sensitive components (pH, temperature, or photosensitivity) or imaging properties (magnetic nanoparticles or quantum dots; Fig. 4 insert). Gold nanoparticles have been identified as an excellent intracellular targeting vector due to its ability to be synthesized in a range of nanosizes (0.8–200 nm). These NBDS are biocompatible with various materials and can be tracked throughout the body due to their visible excitation behavior. A significant amount of research has been diverted toward the preparation of multifunctional gold nanoparticle conjugated with FA to combine the advantages of both of these systems [73–76]. Similarly, superparamagnetic iron oxide nanoparticles are crystalline magnetite structure coated with dextran and dextran derivatives. They are also emerging as promising candidates for a number of biological applications, including magnetic resonance imaging and as targeted drug delivery systems. A multifunctional superparamagnetic nanocarrier with FR targeting and pH-mediated drug release can also be developed to achieve a decrease in tumor volume, improved MRI sensitivity, and decrease in incidence of adverse effects [77–79].

A novel 'dual-drug' FR-targeted delivery system was recently developed by Leamon *et al* [80]. Their team constructed a molecule called EC0225, which consisted of FA, a hydrophilic peptide spacer (-Asp-Asp-Asp-βDpr-Cys), vinca alkaloid, and mitomycin as the anticancer agents. This targeted system showed a high affinity for FR-positive cells, potent dose–response activity *in vitro*, and significant tumor activity *in vivo* [80]. At the end of 2010, the phase I clinical study on EC0225 for the treatment of metastatic tumors was completed and a safe and effective dose for humans was identified [81].

Transferrin-based targeting

Background and mechanism of targeting

Transferrin (Mw = 80 kDa) is the fourth most abundant serum nonheme iron-binding glycoprotein that is mainly produced in the liver. It helps to transport iron to rapidly growing cells, which is required as a cofactor for DNA synthesis [22]. Normally at a cell, transferrin offloads the iron onto a transferrin receptor (TfR, CD71, K_D = 1–100 nmol/l), and the complex is internalized through clathrin-coated pits.

Cancer cells rapidly proliferate, and hence there is a greater demand for iron in tumor cells; this leads to a greater expression of TfRs on the surface of cancer cells [82]. This enhanced TfR expression can be exploited for actively delivering anticancer agents specifically to tumor tissues.

Benefits of transferrin as a targeting ligand

Transferrin has a number of properties that allow it to be successfully incorporated as a targeting ligand in nanoparticle systems. Transferrin is stable over a wide pH range (3.5–11) and has shown to be unaffected by repeated freeze–thaw cycles; hence, it can be subjected to processing conditions commonly encountered during nanoparticle preparation [83,84]. Previously, transferrin used to be isolated from human and bovine sources, which increased the risk of infection and contamination. There was also a shortage of commercially available human sources of transferrin. However, this issue of transferrin availability has been overcome by the development of recombinant transferrin (Optiferrin) [85]. TfRs are overexpressed on the surface of cerebral endothelium and brain tumor cells; hence, transferrin has been explored as a ligand for actively targeting NBDS to the brain (discussed later) [23].

Research and clinical success of transferrin-based nanotechnology-based delivery systems

Transferrin-conjugated NBDS have been explored in a number of studies for the delivery of anticancer agents. These studies have reported that transferrin-conjugated NBDS have a longer retention time in the circulation, higher tumor accumulation, and an enhanced cytotoxic activity in the tumor site [86–89]. Hence, transferrin-NBDS can be further explored to improve pharmacokinetic properties and to enhance cytotoxic activity of anticancer drugs.

Normally, transferrin can be conjugated to nanoparticles less than 100 nm in size to obtain an enhanced cytotoxic activity. If the nanoparticles are greater than 100 nm, it may lead to poor accumulation of these nanoparticles in the tumor cells, which results in moderate anticancer activity. To overcome this issue, the actively targeted system can be directly administered into the tumor tissue by intratumoral injection [90]. Hence if a tumor is localized and accessible, intratumoral injection of nanoparticles would lead to a better therapeutic outcome compared with systemic administration.

Traditional strategies for drug delivery to the brain have involved neurosurgical options (e.g., intracerebral drug injection, intraventricular drug infusion, or disruption of BBB), pharmacological strategies (e.g., lipidation or chemical modification of the drug), and physiological strategies (e.g., carrier or receptor transport systems) [91]. TfR is one such physiological strategy that has been extensively investigated for the delivery of anticancer agents, gene vectors, and imaging agents to the brain [92–95]. This is mainly due to the overexpression of TfR on the surface of these endothelial cells [93]. Jain *et al*. [96] reported the preparation of surface-engineered long-circulating PLGA–PEG–transferrin NP for targeting of temozolomide to the brain and showed an increased uptake of transferrin-appended NPs, localization of the NPs in the brain tissue, and an enhanced cytotoxicity. An improvement in cytotoxicity was attributed to receptor-mediated uptake of the transferrin-conjugated nanoparticle compared with simple diffusion of the drug from the free drug solution. Incorporation of the drug in the nanoparticles also masked the drug from multidrug-resistance proteins (PgP), resulting in a greater uptake of the anticancer agent into the cell. Hence, a combination of receptor-mediated uptake, masking of the therapeutic agent from efflux proteins, and greater intracellular retention leads to greater therapeutic efficacy from these targeted systems. These findings indicate that TfR can be used as a target for effective delivery of anticancer agents to the brain.

Transferrin has been used as a targeting ligand as part of several NBDS. However, the concentration of transferrin in blood (25 μmol/l) is greater compared with its binding affinity to the TfR. Thus, when transferrin-conjugated nanoparticles are administered in the cerebral tissue, they may compete with endogenous transferrin molecules leading to lack of endocytosis of these targeted nanoparticles. To overcome this problem, a unique targeting agent HAIYRPH (T7) can be used, which is known to bind to a different site than transferrin. Hence, T7-bound NBDS can be used to enhance antitumor activity, even in the presence of endogenous transferrin [97–99].

Several transferrin-NBDS have been successfully entered into clinical trials. CALLA-01, designed by Bartlett and Davis [100], is one of the first clinically successful transferrin-conjugated nanoparticulate system. This system consists of a duplex of synthetic nonchemically modified siRNA, which self-assembles to a cationic copolymer containing cyclodextrin, adamantane-PEG (AD-PEG) as a stabilizing agent, and AD-PEG-transferrin as the targeting moiety. After administration, the nanocomplex provides siRNA protection from nucleases in the serum, minimizes erythrocyte aggregation, and reduces complement fixation. At the tumor site, the transferrin binds to the tumor cell TfR, which leads to preferential uptake of the complex within the tumor cell. In the cell, the polymer unpacks from the small interfering RNA allowing it to interfere with RNA resulting in reduced tumor growth [101]. This system is currently being tested in phase I clinical trials for the treatment of solid tumors and has shown promising results [102,103]. Liposomal oxaliplatin (MBP-426) is another TfR-targeted system that has successfully completed phase I clinical trial for patients with advanced solid tumors [104]. Currently, MBP-426 is being tested in combination with leucovorin and 5-fluorouracil in Phase Ib/II study for esophageal adenocarcinoma [105].

Aptamers

Background and mechanism of targeting

Aptamers are short nucleic-acid-based ligands (DNA, RNA, oligonucleotide) that can be incorporated in NBDS to target therapeutic agents to tumor tissues [106,107]. The word aptamer is derived from the Greek word 'Aptos' (to fit). Owing to the intramolecular forces in the aptamer strand, it has the ability to fold into a 3-dimensional structure that binds to specific proteins with high affinity.

Traditionally, a number of compounds were assayed to isolate a ligand for the production of aptamers. However, development of a technique called systematic evolution of ligands by exponential enrichment has allowed the rapid and selective production of aptamers. In the preparation of aptamers by the systematic evolution of ligands by exponential enrichment process, the following steps are undertaken [107]:

A random library of nucleotides with fixed 5′ and 3′ ends are first selected and incubated with the target protein.

The bound sequences are then partitioned by affinity chromatography and the 5′ and 3′ ends are used as primers; the sequences are amplified by polymerase chain reaction. The selected sequences are cycled through the above steps (usually for 6–10 cycles) until the affinity of the sequences to the target reaches a plateau.

The tightest bound sequences are then cloned in plasmid, amplified, and sequenced to obtain highly specific aptamers.

Benefits of aptamers as a targeting ligand

Aptamers similar to other ligands that have been discussed above can tolerate a moderate change in

temperature, pH (4–9), and ionic strength and can be processed with organic solvents without a loss of activity [108]. These properties in aptamers enable them to withstand the common production conditions encountered during nanoparticle preparation. After production, aptamers can be transported at ambient temperatures and can remain stable even after long-term storage [109].

Aptamers (10–15 kDa) are smaller in size compared with antibodies (150 kDa) and single-chain variable fragment antibodies (25 kDa) due to which they accumulate quickly within the tumor tissue. However, due to their small size, aptamers can be cleared quickly by the kidneys. To delay their clearance, PEG or cholesterol can be added to aptamer NBDS [109,110]. Aptamers are chemically synthesized and bind with very high affinity (K_D = 10 pmol/l to 10 μmol/l) and specificity to the target on the tumor tissue. Owing to this specificity, there is a reduced risk of immunogenicity associated with the administration of aptamers [111].

Research and clinical success of aptamer-based nanotechnology-based delivery systems

A number of papers have reported the therapeutic and targeting capabilities of aptamers. Pegaptanib sodium aptamer (Macugen, Pfizer, and Eyetech) was the first pharmaceutical aptamer that sought approval by the FDA for the treatment of age-related macular degeneration. Pegaptanib is a pegylated anti-VEGF aptamer that binds specifically to VEGF 165, a protein critical for angiogenesis. In Macugen, the aptamer is used as a therapeutic agent. However, as aptamers are expensive to produce, it is more economical to use aptamers as targeting agents rather than as therapeutic agents. Hence, in this study, we have focused on the use of aptamers as a targeting ligand.

Farokhzad *et al.* [112] have reported the targeting capability of aptamers, especially the A10 RNA aptamer. The A10 RNA aptamer is a competitive inhibitor of prostate-specific membrane antigen (PSMA), a prostate cancer tumor marker that is overexpressed on prostate acinar epithelial cells [112]. In 2004, the development and efficient uptake of a controlled release nanoparticle bioconjugated to A10 aptamer was reported. The capability of this system to target anticancer drugs was explored by preparing bioconjugates of A10 aptamer with docetaxel nanoparticles. This system showed an enhanced cellular cytotoxicity and provided greater tumor reduction compared with nontargeted systems by a combination of enhanced intracellular delivery of docetaxel, increased retention time, and reduced circulation clearance at the tumor site [107]. This provided evidence of the targeting capability of aptamers for the delivery of cytotoxic agents. The concept for targeted cytotoxic drug delivery using aptamers was explored further by preparing physical conjugates of A10 aptamer and doxorubicin. Doxorubicin was found to intercalate into the CG sequence in the aptamer, improving the stability of this complex and also maintaining the targeting capability [113]. A10 aptamer is now being explored for the targeted delivery of several anticancer agents, including paclitaxel and cisplatin. These systems have been found to deliver the cytotoxic agents effectively to the tumor cells [32,114].

The A10 aptamer recognizes the PSMA antigen on PSMA-positive cancer cells. However, it cannot target PSMA-negative prostate cancer cells. To overcome this problem, DUP-1 peptide has been identified. By using A10 aptamer and DUP-1 peptide, doxorubicin nanoparticles were synchronously delivered to both PSMA-positive and PSMA-negative cells inducing cancer cell apoptosis [115]. Thus, by using the above system, targeted anticancer delivery would now be possible to both PSMA-positive and PSMA-negative prostate cancer cells.

Multiple chemotherapeutic agents are often administered in the treatment of cancer to enhance antitumor activity and to minimize dose-related toxicity. To investigate the possibility of targeting multiple anticancer agents using an aptamer-based targeting system, a nanoparticle–aptamer bioconjugate was designed to codeliver doxorubicin and docetaxel. It was observed that the dual-drug targeting provided more efficient cytotoxicity compared with the use of single anticancer agents [116]. This finding opens the possibility of delivering multiple anticancer agents, specifically to tumor tissues.

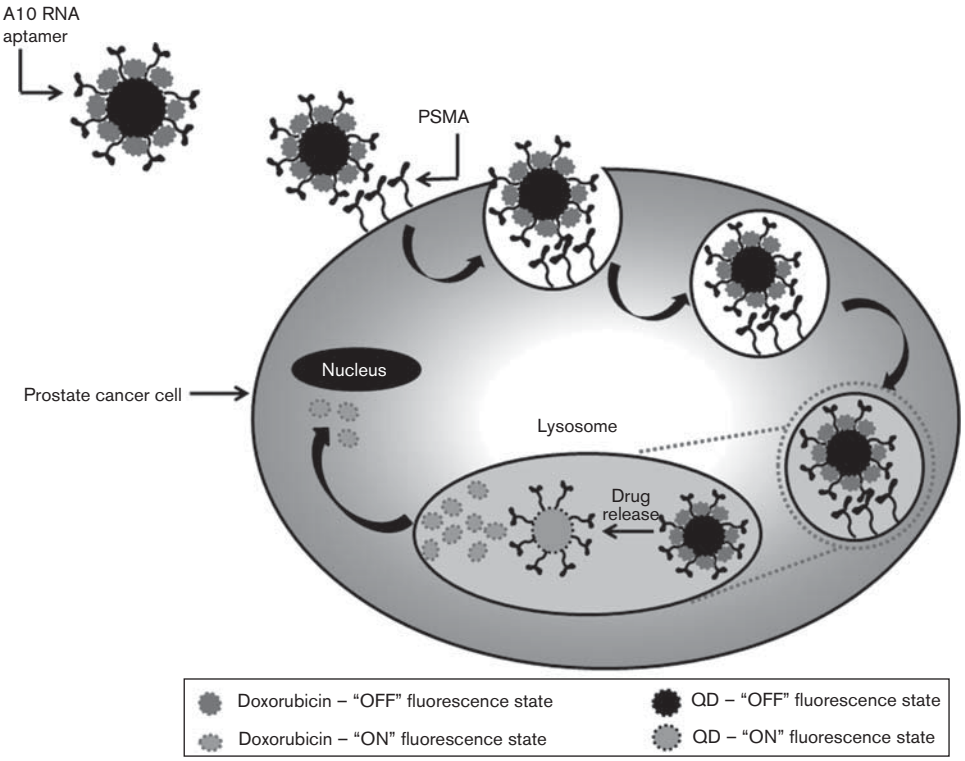
Several chemotherapeutic agents that are currently used in the clinical setting can induce a number of adverse effects in patients, and once the drug is administered, these cytotoxic actions cannot be reversed. Hence, antidotes for anticancer agent toxicities are of interest to regulate drug activity. Aptamers have been investigated as antidotes for regulating activity of anticoagulants, but such a system has not yet been reported for anticancer agents [117–119]. cDNA aptamer was recently designed for inhibiting cisplatin activity. The multifunctional carrier system consisted of cisplatin as the anticancer agent, which was encapsulated within a liposomal system and conjugated to AS1411-derived aptamer. In the absence of cDNA, the targeted nanoparticle showed cell-specific targeting and an improved cytotoxicity. When the cDNA aptamer was administered, it inhibited the cytotoxic activity of cisplatin. However, the interval between the administration of cDNA and nanoparticle seemed to be critical. It was observed that if cDNA was given immediately after the administration of the targeted nanoparticles, anticancer activity was terminated successfully. However, if there was a time lag between the administration of cDNA and nanoparticles, a complete inhibition of the cytotoxic effect was not observed as the anticancer agent was already endocytosed into the tumor cell [120]. Thus, aptamers can also be prepared as

antidotes for anticancer drugs to modulate anticancer effects.

Computed tomography is a diagnostic tool used for biomedical imaging, which includes magnetic resonance imaging, positron emission tomography, and ultrasound. These methods have been traditionally used for cancer imaging, but they have numerous limitations, including the inability to target tumor cells smaller than 1 μm, short imaging time, and risk of renal toxicity. Antibodies have been used for targeting imaging agents to tumor tissues. However, their slow kinetics, poor tissue penetration, relatively low affinity to receptors, and catabolism by RES cells have limited their application [121]. To overcome this problem, molecular imaging has been extensively explored to obtain information at a molecular and physiological levels [78,122–125]. Aptamers seem to be suitable for incorporation in imaging nanoparticles due to their small size and polyanionic nature [126,127].

Normally, a single aptamer is labeled with fluorescent dye, radioisotope or iron oxide for targeting imaging agents. A potential problem with this strategy is that as cancer is caused by multiple genetic changes, targeting a single biomarker may lead to lower efficiency of cancer diagnosis. To overcome this problem, Ko *et al.* [127] recently reported a multimodal nanoparticle called simultaneously multiple aptamers and RGD targeting cancer probe, to enhance targeting specificity and to signal sensitivity. These nanoparticles consisted of AS1411 and TTA1 aptamer and an arginine–glycine–aspartic acid (RGD) peptide. AS1411 binds to nucleolin protein (nuclear protein overexpressed in the cytoplasm and the surface of many cancer cells), and TTA1 binds to extracellular matrix protein tenascin-C [110,126]. In contrast, RGD peptide binds to integrin $\alpha_v\beta_3$, which is highly expressed during tumor angiogenesis and metastasis [128]. Fluorescence, radioisotope, and magnetic resonance imaging showed that the probe was able to target nucleolin, integrin $\alpha_v\beta_3$ and tenascin-C proteins, which improved cancer imaging. Aptamers have also been conjugated to gold nanoparticles [129–131] and superparamagnetic iron oxide nanoparticles [132,133] to obtain

Fig. 5



Schematic illustration of quantum dot (QD)-Apt (doxorubicin) Bi-FRET system. The system was designed by initially functionalizing the QD with A10 prostate-specific membrane antigen (PSMA) aptamer. Doxorubicin was then intercalated within the A10 PSMA aptamer, resulting in the formation of QD–Apt (doxorubicin) complex. Owing to the formation of this complex, the fluorescence of QD was quenched by doxorubicin absorbance and the fluorescence of doxorubicin was quenched by intercalation with A10 PSMA aptamer ('OFF' state). This mechanism was termed as the bifluorescence resonance energy transfer (FRET) system. In prostate cancer cells, PSMA-mediated endocytosis enabled the specific uptake of the QD–Apt (doxorubicin) complex. Inside the tumor cell lysosome, the acidic conditions would allow doxorubicin to be released. The release of doxorubicin would induce a recovery of fluorescence from both QD and doxorubicin ('ON' state). Thus, this system would not only allow targeted delivery of doxorubicin but it would also enable fluorescence imaging of the tumor cell [134]. (Modified with permission from *Nano Letters* (Bagalkot *et al. Nano Letters* 2007; **7**:3065–3070). Copyright 2007 American Chemical Society.

enhanced imaging of tumor tissues. When anticancer agents were incorporated with such targeted systems, it leads to greater antitumor activity. This targeting capability of aptamers for imaging agents was further explored by Bagalkot *et al.* [134] who reported the development of a novel quantum dot (QD; Fig. 5). In this system, A10 aptamer was intercalated with doxorubicin and covalently conjugated to QD for imaging purposes. The above system worked using the 'bi-fluorescence resonance energy transfer' mechanism. When the QD and aptamer complex were loaded with doxorubicin, the QD and doxorubicin were in an 'OFF' fluorescence mode. As the complex entered the acidic environment of the tumor cell endosome, the doxorubicin was released from the complex and the QD and doxorubicin switched to an 'ON' fluorescence mode. This allowed targeted doxorubicin delivery to tumor cells, an enhanced loading efficiency of doxorubicin, an improved imaging sensitivity, and an optimized anticancer activity. Hence, the investigators were able to design and engineer a system that could have research and medical applications.

Targeting from bench to bed

Drug delivery to tumor tissues using nanotechnology-based drug delivery systems continues to be an exciting topic in research. A number of studies have been reported on the delivery of anticancer agents to tumor tissues using albumin, FA, transferrin, and aptamers as the targeting ligands. On the basis of the plethora of research studies on this topic, it seems that we are now moving in a direction that would soon allow us to individualize anticancer therapy. Wang *et al.* [63] envisaged a situation in the near future where NBDS will be used to detect tumors, design individualized therapies, and target these therapeutic agents to tumor tissues. However, if we reflect on the outcomes of these targeted systems, we see that only a few NBDS have made it into clinical trials, with only a sparse number achieving clinical success. This limited clinical success could be due to lack of knowledge of the barriers that exists between the site of administration and the tumor tissue. These systems are complex in nature, with insufficient data on their safety, stability, and reproducibility. The industrial application of these targeted systems is limited due to its low scale-up properties and unavailability of specific safety and efficacy guidelines for approval by regulatory authorities, except Organization for Economic Cooperation and Development guidelines [135]. Hence, it is critical that more studies are now focused on taking the current delivery systems from 'the research bench' to the 'industrial and clinical setting'.

Conclusion

Delivery of anticancer agents to tumor tissues with the help of nanotechnology-based drug delivery systems is a topic that is pursued with great interest by research scientists. Nanoparticles can be designed to target the drug to the tumor tissue by passive or active mechanisms. To actively target nanoparticles to tumor tissues, several ligands have been identified and coated on the surface of these nanoparticles. Some of these actively targeted systems have made it past the research setting and have found clinical applications. However, in comparison with the research investment in these actively targeted systems, only a sparse number have achieved significant clinical success. Hence, more efforts should now be invested toward transforming the research outcomes into clinical benefits.

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Conflicts of interest

There are no conflicts of interest.

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