



Published in final edited form as:

J Cancer Res Clin Oncol. 2015 May ; 141(5): 769–784. doi:10.1007/s00432-014-1767-3.

Cancer active targeting by nanoparticles: a comprehensive review of literature

Remon Bazak,

Department of Otorhinolaryngology, Faculty of Medicine, Alexandria University, Alexandria, Egypt

Mohamad Hourii,

Department of Ophthalmology, Faculty of Medicine, Beirut Arab University, Beirut, Lebanon

Samar El Achy,

Department of Pathology, Faculty of Medicine, Alexandria University, Alexandria, Egypt

Serag Kamel, and

House Officer, Faculty of Medicine, Alexandria University, Alexandria, Egypt

Tamer Refaat

Department of Clinical Oncology and Nuclear Medicine, Faculty of Medicine, Alexandria University, Alexandria, Egypt; Department of Radiation Oncology, Robert H. Lurie Comprehensive Cancer Center, Northwestern University, Chicago, IL, USA

Tamer Refaat: tamer.refaat@northwestern.edu

Abstract

Purpose—Cancer is one of the leading causes of death, and thus, the scientific community has but great efforts to improve cancer management. Among the major challenges in cancer management is development of agents that can be used for early diagnosis and effective therapy. Conventional cancer management frequently lacks accurate tools for detection of early tumors and has an associated risk of serious side effects of chemotherapeutics. The need to optimize therapeutic ratio as the difference with which a treatment affects cancer cells versus healthy tissues lead to idea that it is needful to have a treatment that could act a the “magic bullet”—recognize cancer cells only. Nanoparticle platforms offer a variety of potentially efficient solutions for development of targeted agents that can be exploited for cancer diagnosis and treatment. There are two ways by which targeting of nanoparticles can be achieved, namely passive and active targeting. Passive targeting allows for the efficient localization of nanoparticles within the tumor microenvironment. Active targeting facilitates the active uptake of nanoparticles by the tumor cells themselves.

Methods—Relevant English electronic databases and scientifically published original articles and reviews were systematically searched for the purpose of this review.

Correspondence to: Tamer Refaat, tamer.refaat@northwestern.edu.

Conflict of interest There is no actual or potential conflict of interest with the production and publication of this work. No author has a direct or indirect commercial financial incentive associated with the publication of this article.

Results—In this report, we present a comprehensive review of literatures focusing on the active targeting of nanoparticles to cancer cells, including antibody and antibody fragment-based targeting, antigen-based targeting, aptamer-based targeting, as well as ligand-based targeting.

Conclusion—To date, the optimum targeting strategy has not yet been announced, each has its own advantages and disadvantages even though a number of them have found their way for clinical application. Perhaps, a combination of strategies can be employed to improve the precision of drug delivery, paving the way for a more effective personalized therapy.

Keywords

Cancer; Active targeting; Nanoparticles

Introduction

Cancer remains a leading cause of death in humans, and thus, great efforts have been put forward to improve the outcome of cancer management (Greish 2007). Although chemotherapeutics are able to kill neoplastic cells with high efficacy, they lack precision which results in drug-induced toxicity in non-neoplastic tissues (Greish 2010). Patients subjected to treatment paradigms with such nonspecific toxic compounds commonly develop serious side effects that can be debilitating in their own right (LaRocque et al. 2009; Wang et al. 2009). Paul Ehrlich, inspired by Karl Maria von Weber's opera “Der Freischütz,” introduced at least in theory the concept of the “magic bullet” into medicine at the turn of the twentieth century (Kreuter 2007). Since then, researchers worldwide have been searching for the “magic bullet” that would selectively target neoplastic cells with precision facilitating diagnosis and therapy. Recent progress in cancer nanotechnology has raised exciting opportunities for such an ideal platform by an emerging class of nanotherapeutics that can be targeted specifically to neoplastic cells, thereby offering a major advantage over conventional agents (Wang et al. 2009; Byrne et al. 2008). There are two ways by which targeting of nanoparticles can be achieved, namely passive and active targeting. In this report, we are presenting a comprehensive review of literatures focusing on the active targeting of nanoparticles to cancer cells.

Methods

Relevant English electronic databases and scientifically published original articles and reviews were systematically searched for the purpose of this review. At least two authors evaluated the relevant published scientific reports and agreed to include them in the study. A total of 157 reports are referenced in this review.

Results

Passive targeting facilitates the efficient localization of nanoparticles in the tumor interstitium but cannot further promote their uptake by cancer cells. This second step in uptake can be achieved by actively targeting nanoparticles to receptors or other surface membrane proteins overexpressed on target cells. The addition of targeting ligands allows the delivery of drug-encapsulated nanoparticles to uniquely identifiable cells or even

subcellular sites, thereby reducing the unwanted systemic exposure of cytotoxic drug. Specific interactions between the ligands on the surface of nanocarriers and receptors expressed on the tumor cells may facilitate nanoparticle internalization by triggering receptor-mediated endocytosis. Furthermore, active targeting of nanocarriers with small molecule therapeutic cargo has shown the potential to suppress multidrug resistance (MDR) via bypassing of P-glycoprotein-mediated drug efflux (Yu et al. 2010; Talekar et al. 2011; Wang et al. 2008). Recognizing that receptor-based active targeting of nanoparticles has the potential to be the optimal delivery strategy, there has been tremendous interest in developing novel-targeted nanoparticles for diagnostic and therapeutic applications (Wang et al. 2008). Numerous targeting ligands have been employed to actively target nanoparticles including antibodies, antibody fragments, aptamers, peptides and whole proteins (e.g., transferrin) and different receptor ligands (e.g., folic acid).

Antibody-based targeting

Antibodies are well-established target-specific reagents used in nuclear medicine for diagnostic and therapeutic purposes. Not surprisingly, antibodies were among the first agents used for targeting nanovehicles to specific cell types based on surface antigens they presented (Steinhauser et al. 2006). As targeting agents, antibodies have exceedingly high selectivity and binding affinity by virtue of the presence of two epitope binding sites in a single molecule (Yu et al. 2012). The first monoclonal antibody (mAb) able to bind to specific tumor antigen was developed in 1975 (Kohler and Milstein 1975), but potential role of mAbs in the cancer therapy was not explored until nearly 20 years later (Beduneau et al. 2007). The murine mAb Muromonab CD3 (OrthoClone OKT3[®]) was the first mAb to be approved by the FDA for clinical applications (Thistlethwaite et al. 1984). Currently, there are several FDA-approved mAb therapies, with hundreds more undergoing clinical trials (Debbage 2009; Waldmann 2003). Among the potential targets for mAb-mediated nanoparticle delivery, human epidermal growth factor receptor 2 (HER2) (Fig. 1), epidermal growth factor receptor (EGFR), transferrin receptor (TfR) and prostate-specific membrane antigen (PSMA) have been extensively investigated.

Human epidermal growth factor receptor 2 (HER2 receptor)—HER2 is overexpressed in approximately 25 % of invasive breast cancer (Nahta et al. 2006), but it is minimally expressed by normal adult tissues (Press et al. 1990). Targeted therapy with the humanized mAb trastuzumab which targets HER2 receptor has become a mainstay treatment of HER2 positive breast cancer (Spector and Blackwell 2009). As a result of its enhanced expression on tumor cells, its extracellular accessibility and its ability to internalize after antibody binding, HER2 has been envisioned as a suitable target for targeted nanoparticle delivery to breast cancer (Wartlick et al. 2004).

The ability of trastuzumab-conjugated nanoparticles to specifically target HER2 positive cells has been clearly demonstrated in vitro using different cell lines (Steinhauser et al. 2006; Wartlick et al. 2004; Day et al. 2010; Dilnawaz et al. 2010) and in vivo (Corsi et al. 2011; Chattopadhyay et al. 2012; Ruan et al. 2012). Being able to specifically target HER2-positive breast cancer cells, this antibody has been employed for enhancing radiological detection of breast cancer. Although mammography has improved early diagnosis of breast

cancer, it fails to detect 10–25 % of the tumors and is nonspecific for cancer (Destounis et al. 2004; Hathaway et al. 2011). Therefore, a tumor-specific imaging probe which is able to emit a detectable imaging signal from a malignant tumor in an early preclinical stage would be advantageous. Trastuzumab has been conjugated to supermagnetic iron oxide nanoparticles which serve as MRI contrast agents to detect HER2-positive tumors (Huh et al. 2005; Oghabian et al. 2011). Tumors overexpressing HER2 receptors exhibited enhanced signal intensities in the T(2)-weighted images improving cancer detection ability (Yang et al. 2010). Although both magnetic resonance imaging and magnetic relaxometry can be used to detect and locate targeted magnetic nanoparticles, magnetic relaxometry is theoretically more specific than MRI, because only target-bound nanoparticles are detected (Hathaway et al. 2011). Adolphi et al. (Adolphi et al. 2012) and Hathaway et al. (Hathaway et al. 2011) have demonstrated that HER2-targeted supermagnetic iron oxide nanoparticles can be easily detected by magnetic relaxometry. These results suggest that the trastuzumab-conjugated magnetic nanoparticles are promising diagnostic agents that can be used for early breast cancer detection (Hathaway et al. 2011; Adolphi et al. 2012). Besides targeting nanoparticles for imaging, trastuzumab has also been explored to for delivering chemotherapeutic drugs to tumors (Day et al. 2010; Dilnawaz et al. 2010).

Epidermal growth factor receptor (EGFR)—The epidermal growth factor receptor, a member of the ErbB family of receptors, is expressed on normal human cells but significantly higher levels of expression of the receptor have been shown to be correlated with malignancy detected in a variety of epithelial cancers. Cetuximab is a chimeric human-murine mAb that binds competitively with a high affinity to the EGFR, rendering it a potential target for anticancer therapy (Harding and Burtneis 2005).

Cetuximab has been shown to target gold nanoparticles specifically and effectively to EGFR-positive pancreatic and colorectal carcinoma cells lines in vitro (Glazer et al. 2010; Cherukuri and Curley 2010). Subsequent exposure of the targeted cells to nonionizing radiofrequency energy has resulted in generation of heat by the gold nanoparticles leading to thermal ablation of the malignant cells. Glazer et al. (2010) have investigated cetuximab-targeted gold nanoparticles in a pancreatic adenocarcinoma xenograft murine model. Exposure of tumor xenografts to radiofrequency after intraperitoneal injection of cetuximab-conjugated gold nanoparticles resulted in radiofrequency field-induced destruction of pancreatic carcinoma xenografts with no evidence of injury to healthy organs. Patra et al. (2008) conducted experiments with gold cetuximab-decorated nanoparticles both in vitro on pancreatic cancer cells expressing variable amounts of EGFR and in vivo on an orthotopic pancreatic cancer model. These particles were conjugated to chemotherapeutic gemcitabine as a cargo. This resulted in significant inhibition of pancreatic tumor cell proliferation both in vitro and in vivo in cells overexpressing EGFR.

In addition to targeting gold nanoparticles for therapeutic purposes, cetuximab has been investigated for its potential to target gold nanoparticles for cancer detection. Puvanakrishnan et al. (2012) have demonstrated that topical application of gold nanorods targeted specifically to EGFR results in a significantly higher image contrast for a skin surface growing cancer compared to nontargeted gold nanorods. These results demonstrate the possibility of using near-infrared narrow band imaging to image and demarcate tumor

margins during surgical resection following topical administration of targeted gold nanorods (Puvanakrishnan et al. 2012). Yang et al. (2008) have proposed that cetuximab-conjugated gold nanoparticles could be used for in situ detection of live cancer cells. The EGFR-targeted nanoprobe was able to detect EGFR-positive A431 cells with 54-times greater specificity and sensitivity in comparison with EGFR-deficient MCF7 cells. Choi et al. (2012) presented a new class of smart theranostic cetuximab-conjugated gold nanoparticles exploiting both the imaging and photothermal properties of gold nanoparticles.

Effective targeting of superparamagnetic iron oxide nanoparticles, excellent MRI contrast agents, to cells over-expressing EGFR has been demonstrated (Liu et al. 2011; Bouras et al. 2012). Liu et al. (2011) proposed MRI imaging of cetuximab-conjugated iron oxide nanoparticles for early detection of nasopharyngeal carcinoma as well as for defining the clinical target volume for the application of radiotherapy. Similarly, Bouras et al. (2012) have shown that cetuximab-conjugated iron oxide nanoparticles can provide MRI contrast enhancement as well as confer radiosensitivity to glioblastoma cell lines in vitro and in vivo tumors. Liao et al. (2011) suggested that cetuximab immunomicelles could serve as a useful delivery vehicle for doxorubicin and superparamagnetic iron oxide to EGFR over-expressing tumor cells. Cho et al. (2010) demonstrated the potential application of cetuximab-conjugated magneto-fluorescent silica nanoparticles for the detection of EGFR-expressing colon cancer using in vivo imaging approaches. Targeted delivery of nanoparticles loaded with chemotherapeutics to tumor cells has also been sought by using mAbs against EGFR (Deepagan et al. 2012). The results suggest that these targeted nanocarriers systems hold a promising role in management of cancers overexpressing EGFR. Pan et al. (2007) have reported the potential application of cetuximab immunoliposomes as a targeted delivery vehicle for boron. In this study, approximately an eightfold increase in cellular uptake of boron was obtained using cetuximab immunoliposomes in EGFR-positive glioma cells compared to nontargeted therapy.

Transferrin receptor (TfR)—Transferrin receptor is of particular interest in development of nanotherapeutics because it is expressed at levels several fold higher on malignant cells than on normal cells (Daniels et al. 2012). In addition, TfR is also expressed on the brain capillary endothelial cells (Jefferies et al. 1984). Blood–brain barrier can be an obstacle for the successful delivery of chemotherapeutics (Groothuis 2000), making transferrin receptor an attractive target for chemotherapeutic drug delivery to tumors beyond the blood–brain barrier.

Ulbrich et al. (2009) verified that loperamide which does not cross the blood–brain barrier can be transported into the brain if loaded on human serum albumin nanoparticles functionalized with TfR targeting mAbs (OX26 or R17217). Similarly, Gosk et al. (2004) have demonstrated that liposomes conjugated to OX26, an anti-transferrin receptor antibody selectively target brain capillary endothelial cells. This suggests that a specifically designed formulation of liposomes could be used so that they may undergo degradation within brain capillary endothelial cells. Following liposomal degradation, the liposomal cargo could travel further into the brain (Gosk et al. 2004). In fact, Huwyler et al. (1996) reported that specific OX26-mediated targeting of daunomycin to the brain could be achieved by the use of an immunoliposome-based delivery system. Xu et al. (1999) utilized a similar approach

by using anti-transferrin receptor mAbs to target liposomes encapsulating plasmids containing the p53 tumor suppressor gene to a radiation-resistant squamous cell carcinoma cell lines in vitro. Delivery of these plasmids resulted in a marked radiation sensitization of the p53 transfected cancer cells to ionizing radiation. These results indicate that target-specific delivery system for cancer gene therapy in concert with conventional radiotherapy can provide a new and more effective means for cancer treatment (Xu et al. 1999).

Prostate-specific membrane antigen (PSMA)—Prostate-specific membrane antigen is a tumor antigen expressed on prostate cancer cells and on the neovasculature of most non-prostate solid tumors (Hrkach et al. 2012). Thomas et al. (2004) synthesized PSMA-targeted dendimer-based nanoconjugates as a platform for targeted molecule delivery to PSMA expressing cells. The anti-PSMA antibody, J591, has also been explored in vitro by Serda et al. (2007) for enhancing T(1)-weighted MR images by targeting superparamagnetic iron oxide nanoparticles to LNCaP prostate cancer cells. Taylor et al. (2011) reported on the synthesis of polyethylene glycol-covered (PEGylated) superparamagnetic iron platinum nanoparticles that can be specifically targeted to human prostate cancer cell lines expressing PSMA using J591 mAb, and detected using both MRI and fluorescence imaging. Using the same nanoparticle platform, Taylor and Sillerud (2012) reported the encapsulation of superparamagnetic iron platinum nanoparticles and chemotherapeutic drug paclitaxel to create multifunctional micelles that were conjugated to an antibody against PSMA for the specific targeting, magnetic resonance imaging, and treatment of human prostate cancer xenografts in mice.

CD20—Anti-CD20 mAbs conjugated to polylactic acid nanoparticles has been shown to specifically target lymphoma cells overexpressing CD20, thereby providing a promising approach to achieve targeted cancer therapy (Cirstoiu-Hapca et al. 2007; Nobs et al. 2006). Recently, Bisker et al. (2012) reported on the in vitro use of gold nanospheres conjugated to rituximab, an anti-CD20 mAb-based drug, for carrying and releasing the drug upon irradiation of specifically tailored femtosecond laser pulses. The released anti-CD20 molecules retained their functionality and ability to trigger complement-dependent cytotoxicity.

Antibody fragments used for nanoconstruct targeting

Design and preparation of antibody fragments became possible with the advent of modern antibody technology (Lu et al. 2006). Antigen-binding fragments (Fab) and single-chain variable fragments (scFV) are the most common antibody fragments investigated for targeting of nanoparticles (Byrne et al. 2008; Yu et al. 2010). Considering small sizes of both the nanoparticles and antibody fragments, this approach allows that multiple targeting peptides be attached to a single nanoconstruct, which improves the targeting efficiency and specificity. The Fab fragments are composed of one constant and one variable domain of each of the heavy and the light chains (Fig. 1, IB), while scFVs are a fusion of the variable regions of the heavy and light chains (Fig. 1, IB) (Wang et al. 2008). The variable region of antibody fragments endows them with the binding specificity of the whole antibody (Byrne et al. 2008), while the absence of constant domains 2 and 3, the Fc fragment, makes them nonimmunogenic. Therefore, compared to whole mAbs, the use of antibody fragments as a

targeting moiety reduces construct uptake by the RES and improves the Fab and scFV pharmacokinetic profile, as well as that of nanoconstructs carrying them (Wang et al. 2008). In contrast to mAbs which are approximately 150 kDa, antibody fragments are smaller in size. Antigen-binding fragments are approximately 50 kDa, while scFv are closer to 25 kDa, a size small enough to allow them better penetration into solid tumors (Debbage 2009).

Single-chain variable fragments (scFV)—Liposomes loaded with chemotherapeutic agents that can be selectively targeted to specific cells represent an effective strategy for increasing the pharmacological efficacy of chemotherapeutic drugs. Single-chain antibody fragment with binding specificity to ED-B fibronectin, an isoform of fibronectin exclusively expressed in tumor tissues, has been successfully used to target liposomes to tumors and therefore represents a promising versatile drug delivery system (Marty et al. 2002; Marty and Schwendener 2005). Phage display technology has been used to isolate a single-chain antibody fragment (scFV-CM6) that specifically binds to the extracellular part of tumor endothelial marker 1 (TEM1), a protein predominantly expressed on newly developing blood vessels and tumor cells. ScFV-CM6 was further functionalized and coupled to liposomes. Such immunoliposomes loaded with a cytotoxic drug *N4*-octadecyl-1- β -D-arabinofuranosylcytosine-(5'-5')-3'-C-ethynylcytidine showed increased binding affinity and up to 80 % higher cytotoxic activity toward TEM1-expressing IMR-32 tumor cells compared to control liposomes (Marty et al. 2006). Using a similar approach, scFV antibodies that specifically bind to c-Met protein have been used to target doxorubicin-loaded liposomes (Lu et al. 2011). Protein c-Met is an aberrantly expressed receptor for hepatocyte growth factor and has been implicated in lung and other human cancers. In a tumor xenograft model, anti-c-Met immunoliposome was found to selectively increase tumor accumulation of a chemotherapeutic drug and enhance its antitumor activity (Lu et al. 2011). Zhou et al. (2007) investigated the binding and uptake of liposomal nanoparticles bearing the ScFV fragments directed against the EGFR. The liposomal single-chain anti-EGFR antibody (ScFV EGFR) has been shown to specifically bind to tumor cells overexpressing EGFR (Fig. 1, IB), leading to liposome internalization. Similar results were obtained when ScFV EGFR was used to functionalize quantum dots and superparamagnetic iron oxide nanoparticles (Yang et al. 2009). Efficient internalization of ScFV EGFR nanoparticles into tumor cells suggests that these nanoconjugates can be used as theranostic agents for targeted tumor imaging and simultaneous delivery of chemotherapeutic agents.

Ling et al. (2011) have described the use of single-chain antibodies against prostate stem cell antigen to target theranostic polymer nanoparticles loaded with both docetaxel and superparamagnetic iron oxide nanocrystals. These nanoconstructs were used for simultaneous targeting imaging, drug delivery and real-time monitoring of the therapeutic effect. Recently, Gao et al. (2012) used core-shell theranostic nanoparticles with a core of poly (D,L-lactic-coglycolic acid) (PLGA), docetaxel and hydrophobic superparamagnetic iron oxide nanocrystals, and a multilayer shell formed by poly allylamine hydrochloride and two polyethylene glycol (PEG) molecules of different sizes. Single-chain antibodies against prostate stem cell antigen were conjugated to these core-shell theranostic nanoparticles for targeted delivery to PC3M cells (Gao et al. 2012).

Antigen-binding fragments (Fab)—In addition to scFV, antigen-binding fragments of mAbs have been used to target several liposomal nanoparticles carrying chemotherapeutic cargos. Sterically stabilized liposomes loaded with doxorubicin have been conjugated to the antigen-binding fragments of a mAb specific for human beta1 integrins (Sugano et al. 2000). Since a number of beta1 integrins are expressed on the surface of human non-small cell lung carcinomas, liposomal nano-conjugates targeted toward beta1 integrins, demonstrated tumor-specific binding, efficient internalization and a marked enhancement of cytotoxicity compared to nontargeted doxorubicin (Sugano et al. 2000). Using a similar strategy, doxorubicin-loaded immunoliposomes have been prepared with a Fab of the mAb anti-GD(2), an antibody which targets disialoganglioside that is overexpressed on the surface of neuroblastoma cells (Brignole et al. 2003). In a metastatic model of human neuroblastoma in nude mice, these targeted nanoconjugates prevented metastatic growth of the tumor cells in all organs examined (Brignole et al. 2003). Drug-loaded immunoliposomes targeted using mAb anti-CD19 or its Fab fragments were compared in vitro as well as in vivo in a murine model of human B cell lymphoma (Sapra et al. 2004). Antigen-binding fragments had better therapeutic outcomes compared to mAbs for the drug doxorubicin but were equally efficacious for delivery of the drug vincristine. Although responses to anti-CD19-targeted liposomal doxorubicin were more modest, the longer circulation times of Fab accounted for the superior therapeutic effect for doxorubicin-loaded immunoliposomes (Sapra et al. 2004). Fab fragments of a humanized anti-HER2 mAb have been used to target PE38KDEL-loaded PLGA nanoparticles. A higher in vitro cytotoxicity of these targeted nanoconjugates has been demonstrated against HER2-overexpressing breast cancer cell lines. In a HER2-overexpressing tumor xenograft model, administration of these nanoconjugates showed an enhanced therapeutic efficacy in inhibiting tumor growth compared with nontargeted controls (Chen et al. 2008).

Aptamer-based targeting

Aptamers are short single-stranded DNA or RNA oligonucleotides that are folded into secondary and tertiary three-dimensional structures rendering them capable of binding to specific biological targets, most often proteins (Ni et al. 2011). Aptamers are considered equivalent to antibodies based on their high sensitivity and specificity as targeting agents (Ni et al. 2011). Aptamers are synthesized to be specific for a certain target through an iterative in vitro selective process called “systemic evolution of ligands by exponential enrichment” (SELEX) (Chiu and Huang 2009).

The classic SELEX method starts with a random sequence library of ssDNA or ssRNA that spans 20–100 nucleotides in length. The randomization of nucleic acid sequences provides a diversity of $\sim 10^{16}$. Each random sequence region is flanked by constant sequences required for aptamer capture and/or priming. The initial diverse pool of aptamers is then exposed to a target molecule, with the expectation that a portion of the aptamers can fold in such a way that they will specifically bind to the target molecule. Nonbinding aptamers are then washed away, while candidate aptamers with high target binding affinity are enriched at each selection round by PCR amplification (DNA aptamers) or RT-PCR followed by in vitro transcription (RNA aptamers) (Ni et al. 2011). Finally, when the best candidate aptamer(s) for a certain target gets selected, the same sequence of nucleobases gets

synthesized with a backbone different than either deoxy- or ribose-phosphate backbone, which makes the aptamers more resistant to DNases or RNases (Ni et al. 2011; Chiu and Huang 2009).

Farokhzad et al. (2004) first reported the use of the A10 RNA aptamer which recognizes the extracellular domain of the PSMA for targeted drug delivery of nanoparticle– aptamer bioconjugates (Fig. 1, II). This RNA aptamer has been used to target docetaxel encapsulated nanoparticles, and it showed significantly enhanced in vitro cytotoxicity compared to nontargeted nanoparticles (Farokhzad et al. 2006). A similar strategy was reported by Dhar et al. (2008) to deliver cisplatin to prostate cancer cells by constructing Pt(IV)-encapsulated PSMA aptamer-targeted nanoparticles. Bagalkot et al. (2007) reported targeting multifunctional quantum dot-doxorubicin using the A10 RNA aptamer. These nanoconjugates have been shown to deliver doxorubicin to the targeted prostate cancer cells and monitor the delivery of doxorubicin by activating concurrently tagged QDs allowing simultaneous imaging of the cancer cells. Recognizing that multiple chemotherapeutic agents administered in the treatment of cancer can enhance antitumor activity and minimize dose-related toxicity, Zhang et al. (2007) developed an A10 RNA aptamer-targeted nanoparticle to serve as a delivery system for both docetaxel and doxorubicin. It was observed that the dual-drug targeting provided more efficient cytotoxicity compared to the use of single anticancer agents. Similarly, Kolishetti et al. (2010) described a self-assembled polymeric nanoparticle platform for target and control precisely the co-delivery of cis-platin and docetaxel to prostate cancer cells employing the A10 aptamer as a targeting moiety. In vitro toxicity studies demonstrated superiority of the targeted dual-drug combination nanoparticles over nanoparticles with single-drug or nontargeted nanoparticles. Yu et al. (2011) utilized the PSMA-targeted RNA aptamer to synthesize cancer-specific theranostic nanoparticles composed of superparamagnetic iron oxide nanoparticles loaded with doxorubicin. These agents have been shown to allow simultaneous prostate tumor detection in vivo by magnetic resonance imaging and selective delivery of drugs to the tumor tissue.

Biomedical imaging is a cornerstone modality in cancer management; however, the lack of specificity and inability to visualize tumors smaller than 1 cm in size represents significant limitations to this day (Talekar et al. 2011). Aptamer-based nanoconjugates have been explored as a way to enhance imaging of a specific tumor tissue. Javier et al. (Javier et al. 2008) have investigated aptamer-based gold nanoparticles as contrast agents for reflectance imaging using A10 RNA aptamer as a targeting moiety for detection of PSMA. Employing a similar strategy, Kim et al. (Kim et al. 2010) targeted gold nanoparticles using an aptamer that binds to PSMA. The resulting PSMA aptamer-conjugated gold nanoparticles showed more than fourfold greater CT contrast for PSMA expressing (and therefore targeted) LNCaP cell than PSMA nonexpressing PC3 cell line. Furthermore, the PSMA aptamer-conjugated gold nanoparticles loaded with doxorubicin showed significantly more potent cytotoxicity against targeted LNCaP cells than PC3 cells. In a similar fashion, Wang et al. (2008) used the A10 RNA aptamer to develop a multifunctional superparamagnetic iron oxide nanoparticles-aptamer bioconjugates that can simultaneously detect prostate cancer and deliver doxorubicin directly to the prostate cancer cells.

In addition to A10 RNA aptamer, a multitude of different aptamers has been investigated for targeting nanoparticles. An anti-EGFR aptamer has been used to deliver gold nanoparticles specifically and quantitatively to cells expressing EGFR; this aptamer was shown to be able to trigger receptor-mediated endocytosis of gold nanoparticles (Li et al. 2010). Hwang et al. (2010) have reported the use of AS1411 aptamer that targets nucleolin, a cancer-specific cell membrane protein to functionalize a multimodal cobalt-ferrite nanoparticle capable of concomitant fluorescence, radionuclide and MRI imaging of malignant cells. Anti-MUC1 protein aptamer has been used to target adenocarcinomas such as breast cancer cells (Yu et al. 2011) conjugated to paclitaxel-loaded PLGA nanoparticles. Presence of the MUC1 aptamer increased the uptake of nanoparticles into the target cells as measured by flow cytometry. Furthermore, the paclitaxel-loaded nanoconjugates enhanced in vitro drug delivery and cytotoxicity of MUC1 positive cancer cells, as compared with nontargeted nanoparticles that lack the MUC1 aptamer (Yu et al. 2011). Zhao et al. (2011) have incorporated both anaplastic lymphoma kinase (ALK) siRNA and a RNA-based anti-CD30 aptamer onto nano-sized polyethyleneimine-citrate carriers. Cell binding assays revealed that presence of anti-CD30 aptamer selectively targeted nanocomplexes to anaplastic large cell lymphoma cells, and confocal fluorescence microscopy confirmed intracellular delivery of the nanocomplex. Moreover, exposure of these cells to nanocomplexes carrying both ALK siRNAs and anti-CD30 RNA aptamers specifically silenced ALK gene expression, leading to growth arrest and apoptosis of these cells.

Cell-based SELEX is a variant of classic SELEX where aptamers are generated based on their capability of recognizing complex molecular signatures of the cancer cells rather than a sole target on the cell membrane (Fig. 2) (Shangguan et al. 2006). One unique feature of cell-based SELEX is the possibility to isolate aptamers against cancer cells without prior knowledge of the number or identities of cancer-specific proteins on the cellular surface (Fang and Tan 2010). The combined selectivity of cell-SELEX-derived aptamers and the spectroscopic advantages of gold nanoparticles have been exploited to develop a direct colorimetric assay for cancer cell detection using aptamer-conjugated gold nanoparticles (Medley et al. 2008). Use of aptamer-conjugated gold nanoparticles and light scattering allowed detection of breast cancer cells by dark field microscopy as reported by Huang et al. (2009). Adopting a similar technique, Liu et al. (2009) described an aptamer– nanoparticle strip biosensor for the rapid, specific and sensitive detection of circulating cancer cells. Herr et al. (Herr et al. 2006) employed cell-based SELEX strategy to develop a method for rapid collection and detection of leukemia cells using aptamers as molecular recognition elements. Aptamer-modified magnetic nanoparticles were used for target cell extraction, while aptamer-modified fluorescent nanoparticles were simultaneously added for sensitive cell detection. Chen et al. (2008) developed aptamers that can recognize small cell lung cancer cells. In vitro studies demonstrated ability of these aptamers to specifically bind to small cell lung cancer cells. This selective and specific binding ability was also demonstrated with small cell lung cancer tissue from a patient as well as with small cell lung cancer cells circulating in the blood. Fluorescent and magnetic nanoparticles bound to these aptamers were then used both to magnetically isolate small cell lung cancer cells and to detect them using fluorescence. Use of the same approach by two different groups, one for leukemia and

one for lung cancer, underlines the potential of aptamer-targeted nanoparticles as a promising method for the recognition, and extraction of circulating tumor cells.

Estevez et al. (2010) used aptamers capable of recognizing acute leukemia cells (CCRF-CEM cells) and conjugated them with a dual-nanoparticle system, which combines magnetic nanoparticles and fluorescent silica nanoparticles. This approach allowed detection of as few as 250 malignant cells; the accuracy of detection was confirmed using confocal microscopy. Huang et al. (2008) were able to show that up to 80 molecules of sgc8c aptamer could be covalently linked to the surface of gold-silver nanorods. The avidity of the resulting sgc8c aptamer-nanorods toward the tyrosine kinase-7 PTK7 transmembrane protein on CCFR-CEM cells was shown to be 26-fold higher than the affinity of the nanoparticle free fluorescein-labeled aptamer sgc8c for the same cells. The fluorescence intensity signal of the aptamer-nanorods labeled cells by flow cytometry was 300-fold greater than the signals observed for CCFR-CEM cells labeled with the unconjugated fluorescein-labeled aptamer (Huang et al. 2008). Taghdisi et al. (2011) used the same aptamer to target single-walled carbon nanotubes and achieve controlled release of daunorubicin in a pH-dependent manner. Utilizing a similar strategy, Chen et al. (2011) achieved a pH-dependent release of doxorubicin from a porous hollow magnetite nanoparticles.

Advantages of aptamers—Aptamers possess several potential advantages over agents such as antibodies that are typically used in targeted delivery. First, aptamers are chemically synthesized without the need for biological systems, and hence, their production is easier to scale up with a low cost and low batch-to-batch variability (Zhang et al. 2011). In addition, in vitro production of aptamers confers the ability to select a wide range of targets, including toxic and nonimmunogenic molecules (Lee et al. 2010). Second, depending on their backbone, aptamers are much more stable to biological degradation as well as physical stresses such as heat, pH and organic solvents compared to antibodies (Lee et al. 2010). These properties enable aptamers to withstand the common production conditions encountered during nanoparticle preparation. After synthesis, aptamers can be transported at ambient temperatures and remain stable for long-term storage (Talekar et al. 2011). Additionally, aptamers have slow degradation kinetics and can be denatured and renatured multiple times without significant loss of activity (Liss et al. 2002). Chemical modification of aptamers with functional groups is simple and, dependent on their backbone, allows many routine synthetic chemistry approaches (Zhang et al. 2011). Fourth, lack of immunogenicity is another favorable advantage of aptamers over antibodies which can result in a better biodistribution (Zhang et al. 2011). Lastly, the smaller size of aptamers (~1–2 nm, <10 kDa) compared to those of antibodies (~10 nm and ~155 kDa) allows for better tissue penetration in solid tumors, although these size differences are less striking when one compares aptamers with antibody fragments (Lee et al. 2010).

Ligand-based targeting

A number of targeting moieties have been used to functionalize the surface of nanoparticles including peptides (such as transferrin) and short amino acid polymers, as well as small molecules (such as folic acid). Such targeting moieties have a very high selectivity and

avidity to their target receptors making them attractive tools for targeting cancer cells. In this section transferrin, and folic acid-based targeting will be discussed in more details.

Transferrin-based targeting—Transferrin is a serum glycoprotein that transports iron through the bloodstream and into cells by binding to cell-surface transferrin receptors, resulting in internalization of iron via receptor-mediated endocytosis (Fig. 1, III) (Qian and Tang 1995). The upregulation of transferrin receptors on metastatic and drug-resistant malignant cells (which may reach up to 100-fold higher than that in normal cells), the extracellular status of transferrin in the body and its internalization by cells make transferrin and transferrin mimicking agents suitable for delivery of cancer therapies (Byrne et al. 2008; Danhier et al. 2010).

Several liposomal nanocarriers have been utilized for intracellular delivery of doxorubicin using transferrin as a targeting ligand (Eavarone et al. 2000; Li et al. 2009; Fonseca et al. 2005; Kobayashi et al. 2007). The results of these studies show that transferrin-conjugated liposomes can efficiently deliver drug cargo into different neoplastic cells expressing transferrin receptor. Cell mechanistic studies indicate that these targeted liposomes bind specifically to transferrin receptors and are internalized via a receptor-dependent endocytotic pathway (Fonseca et al. 2005; Ishida et al. 2001), thus overcoming MDR by bypassing P-glycoprotein-mediated drug efflux. In an attempt to circumvent P-glycoprotein MDR, Wu et al. (Wu et al. 2007) developed transferrin-conjugated liposomes co-encapsulating doxorubicin and verapamil. The authors concluded that the combination of transferrin receptor targeting and co-encapsulation of doxorubicin and verapamil was highly effective in overcoming drug resistance in K562 leukemia cells (Wu et al. 2007). Because expression of transferrin receptor is higher in lung cancer cells (A549 cell line) compared to their alveolar counterparts, transferrin-conjugated liposomal doxorubicin was investigated as a potential inhalational therapeutic for lung cancer (Anabousi et al. 2006). Transferrin has been also explored as a targeting agent for liposomal cisplatin for management of peritoneal dissemination of gastric cancer. Intraperitoneal injection of Tf functionalized liposomes resulted in high uptake by the disseminated tumor cells in the ascetic fluid collection, while uptake by the liver and spleen was relatively low. These differences subsequently increased survival rates in nude mice. These results suggest that cisplatin-encapsulated transferrin-receptor-targeted liposomes may be useful nanotherapeutics for treatment of gastric carcinomas with peritoneal dissemination (Iinuma et al. 2002). Oxaliplatin is a less toxic cisplatin derivative; however, its low bioavailability in tissues limits its efficacy (Suzuki et al. 2008). Selective delivery of a relatively high concentration of this drug loaded into liposomal nanocarriers has been attempted using transferrin as a targeting ligand (Suzuki et al. 2008). Delivery using transferrin-conjugated liposomes significantly enhanced extravasation of liposomes into tumors resulting in a significant suppression of tumor growth (Suzuki et al. 2008). Besides use in delivery of chemotherapeutic cargos, transferrin-conjugated liposomes have been investigated as a tool for gene therapy (Nakase et al. 2005; Rudolph et al. 2002) and targeted photodynamic therapy (Derycke and De Witte 2002).

Several studies have used transferrin to target polymeric nanoparticles composed of PLGA loaded with paclitaxel to cancer cells (Sahoo and Labhasetwar 2005; Gan and Feng 2010; Shah et al. 2009; Pulkkinen et al. 2008; Sahoo et al. 2004). Significantly increased

antiproliferative activity of such constructs was observed in vitro (Sahoo and Labhasetwar 2005; Pulkkinen et al. 2008; Sahoo et al. 2004) and in vivo (Shah et al. 2009; Sahoo et al. 2004), which resulted in an increased survival of mice bearing prostatic carcinomas (Sahoo et al. 2004). This increased efficacy has been attributed to an increased cellular uptake and reduced exocytosis of paclitaxel by the P-glycoproteins. In this way, Tf-targeted nanoparticles offer a potential solution for overcoming MDR, which is a major drawback of paclitaxel as a chemotherapeutic agent (Sahoo and Labhasetwar 2005; Gan and Feng 2010; Shah et al. 2009).

Transferrin-conjugated lipid-coated PLGA nanoparticles carrying an aromatase inhibitor were also evaluated in SKBR-3 breast cancer cells in vitro. The results suggested that the aromatase-inhibiting activity of the transferrin-receptor-targeted nanoparticles was enhanced relative to that of the nontargeted nanoparticles. Explanation for this difference was attributed to the transferrin-receptor-mediated uptake (Zheng et al. 2010). Transferrin has been also employed to target PEG-coated polycyanoacrylate nanoparticles loaded with paclitaxel. These transferrin-conjugated nanoparticles have achieved higher concentration within the tumor, which translated into significant tumor regression and improved survival (Xu et al. 2005). In a similar fashion, transferrin has been used to target poly (ethylene) glycol-hydroxycamptothecin nanoconjugate, which improved its therapeutic efficacy (Hong et al. 2010). The targeted nanoconjugate achieved the highest tumor accumulation, which resulted in a significant reduction of tumor weight compared to nontargeted formulations in the in vivo S180 murine sarcoma model (Hong et al. 2010).

Superparamagnetic iron oxide nanoparticles (SPIONs) have been extensively investigated as MRI contrast agents. Transferrin-based targeting of this contrast agent has been attempted to improve the diagnostic specificity of SPIONs (Jiang et al. 2012; Fan et al. 2012). Implementing this strategy, a significant contrast enhancement could be clearly detected up to 48 h after injection, due to the retention of the nanoparticles in the cytoplasm of tumor cells (Jiang et al. 2012).

Recently, Fan et al. (2012) have shown that magnetoferritin nanoparticles can be used to target and visualize tumor tissues without the use of any targeting ligands. Iron oxide nanoparticles encapsulated inside a recombinant human heavy-chain ferritin protein shell were able to bind to tumor cells overexpressing transferrin receptor. In tissue specimens, the iron oxide cores catalyze the oxidation of peroxidase substrates in the presence of hydrogen peroxide to produce a color reaction that can be used to highlight the tumor tissue. The authors examined 474 clinical specimens from patients with nine types of cancer and verified that these nanoparticles can distinguish neoplastic cells from normal cells with a sensitivity of 98 % and specificity of 95 %.

Gold nanoparticles have been widely investigated as potential therapeutic as well as diagnostic agents in the management of cancer. Transferrin gold nanoconjugates have been shown to have a significantly higher cellular uptake in cells overexpressing transferrin compared to controls (Parab et al. 2011). This suggests that transferrin-receptor-targeted nanoparticles can improve intracellular delivery of therapeutic agents to the neoplastic cells;

when carrying chemotherapeutic cargos, Tf-targeted nanoparticles can be expected to improve the efficiency of cancer chemotherapy (Choi et al. 2010).

Folate-based targeting—Folic acid is a small molecular weight molecule (441 Da), a vitamin that is required by eukaryotic cells for the biosynthesis of purines and pyrimidines (Talekar et al. 2011). Cellular uptake of folates is facilitated by either a low-affinity-reduced folate carrier, which is present in virtually all cells of the body, or via high affinity glycosylphosphatidyl-inositol-linked folate receptor (FR), which has a very limited distribution (Hilgenbrink and Low 2005). Although membrane-associated forms of folate receptor (FR- α and β) are capable of transporting folate into the cells, most adult tissues depend on the reduced folate carrier for uptake of this vitamin (Matherly and Goldman 2003). While the reduced folate carrier facilitates transport of reduced forms of folic acid, FR is capable of transporting folic acid, nonphysiologic forms of the vitamin, as well as folate-linked nanoconjugates (Hilgenbrink and Low 2005).

The FR is significantly upregulated on many human tumors while it is minimally expressed in most normal tissues (Byrne et al. 2008; Yu et al. 2010); therefore, FR is an attractive target for selective delivery of anticancer agents (Yu et al. 2010). All FRs have a high binding affinity for folic acid. FR- α , the most widely expressed receptor isoform in normal adult tissues, bind to the physiologically reduced folate coenzyme *N*5-methyltetrahydrofolate as strongly as to the folic acid, while FR- β expressed in normal hematopoietic cells has >50-fold lower affinity for the compound (Elnakat and Ratnam 2004). The alpha isoform is overexpressed on 40 % of human cancers, whereas FR- β is expressed on activated macrophages and malignant hematopoietic cells (Low and Kularatne 2009). These differences in receptor distribution and specificity for different ligands can be exploited for designing folate-based selective tissue-targeting devices (Elnakat and Ratnam 2004; Pan et al. 2002). A wide array of nanoplatforms including liposomes, gold nanoparticles, dendimers, iron oxide and quantum dots has been targeted to cancers using folic acid as a targeting moiety (Fig. 1, III).

Folic acid has been used for targeting liposomal nano-carriers to various tumors, often helping with bypassing of MDR (Torchilin 2010). Ni et al. (2002) have shown that FR-targeted liposomal daunorubicin to FR-expressing cells resulted in an increased daunorubicin cellular uptake and cytotoxicity in vitro (Ni et al. 2002). However, in vivo studies using folic acid as a targeting ligand revealed that these targeted nanocarriers tend to be cleared rapidly from the circulation, and this accelerated clearance was associated with the folate itself (Gabizon et al. 2003). Folate-targeted liposomal nanocarriers administered systemically can potentially enhance the delivery of anticancer drugs in vivo; however, their uptake by FR-expressing normal tissues must be blocked in order to improve tumor targeting (Riviere et al. 2011).

Folic acid targeting of liposomes loaded with doxorubicin or daunorubicin has demonstrated therapeutic efficacy in vivo in murine ascites tumor models and seems to be a promising tool for in vivo intracavitary drug targeting (Shmeeda et al. 2006; Gabizon et al. 2010). Upregulation of FR- β in acute myelogenous leukemia cells by all-transretinoic acid combined with folate-modified doxorubicinloaded liposomes has been shown to provide an

alternative effective strategy for rapid elimination of the cells upon systemic administration (Pan et al. 2002). Recently, intraperitoneal administration of FR- α -targeted high-density lipoprotein fluorescent nanoparticles have proved to result in a high fluorescence signal in ovarian tumors, surpassing that detected in the rest of the host tissues (Corbin et al. 2012). The authors concluded that intraperitoneal FR- α -targeted nanoparticles could provide an effective approach for selective targeting of ovarian cancer (Corbin et al. 2012).

A significant amount of research has been devoted to the preparation of functionalized gold nanoparticles conjugated to folic acid to exploit the advantages of both of these systems simultaneously. Folate-functionalized gold nanoparticles are selectively internalized into cells expressing folate receptor, a process which is significantly inhibited by excess free folic acid (Li et al. 2009). Several studies have employed folate as a targeting ligand for gold nanoparticles loaded with a chemotherapeutic cargo (Prabaharan et al. 2009; Asadishad et al. 2010; Patra et al. 2010; Manju and Sreenivasan 2012). The enhanced cytotoxicity for FR-targeted gold nanoparticles loaded with doxorubicin toward FR-expressing cell lines has been attributed to FR-mediated endocytosis (Prabaharan et al. 2009; Asadishad et al. 2010). Taking advantage of the increased cellular uptake of FR-targeted gold nanoparticles, fabrication of folate-functionalized gold nanoparticles loaded with cisplatin (Patra et al. 2010) or curmin (Manju and Sreenivasan 2012) as a chemotherapeutic cargo has been reported. There has been considerable interest in dendimer-based nanotechnology for targeted cancer imaging and therapy. Folatefunctionalized dendimer-entrapped gold nanoparticles have been reported as a unique nanoplatform for targeting and imaging of a variety of biological systems (Shi et al. 2009). These nanoplatforms have been shown to specifically bind to FR-expressing cells followed by their internalization into lysosomes (Shi et al. 2007). Internalization kinetics has been shown to be not dependant on the gold nanoparticles denoting that this platform can be extrapolated using a variety of metal or inorganic nanoparticles (Shi et al. 2009). Recently, folic acid-modified dendimer-entrapped gold nanoparticles proved to have a good biocompatibility as an imaging probe for targeted CT imaging of human lung adenocarcinoma in a xenograft animal model (Wang et al. 2013).

Superparamagnetic nanoparticles functionalized with folate demonstrated better uptake by cancer cells (Zhang et al. 2002), a finding that has been explored for targeted MRI imaging and therapy (Choi et al. 2004; Chen et al. 2008). Recently, FR-targeted doxorubicin-loaded magnetic core-shell nanoparticles have been envisioned as a useful nanomedical carrier system for cancer diagnosis and treatment (Wang et al. 2012). Moreover, folate-targeted quantum dots have also been shown to be taken specifically by cells bearing FR, thereby constituting an attractive fluorescent nanobioprobes for selective tumor cell labeling (Schroeder et al. 2007; Song et al. 2009).

Advantages of folic acid as a targeting ligand—The inherent properties of FA confer distinctive advantages and make it suitable ligand for nanoparticle targeting. First, it is a stable and relatively inexpensive vitamin, which facilitates its processing (Talekar et al. 2011). Second, as with other low molecular weight ligands, folic acid has advantages of simple conjugation chemistry and nonimmunogenicity (Byrne et al. 2008; Yu et al. 2010). Third, at the tumor site, it has a very high affinity for FR on tumor cell surface with rapid

internalization into tumor cells (Talekar et al. 2011). Folic acid being essential for executing a cellular function, when used as a targeting ligand, its cargo is retained within an endocytotic vesicle or released into the cytoplasm. This offers an advantage compared to antibodies and other related ligands which after internalization are shuttled to the lysosome for destruction (Byrne et al. 2008). Finally, FR expression in most proliferating nonmalignant epithelial tissues is restricted to the luminal surface of the epithelial cells, which is inaccessible to the circulation. On the other hand, it is consistently expressed in specific types of major malignant tumors and leukemic cells making them accessible to the targeted nanoparticles via the circulation, thereby eliminating nonspecific targeting and minimizing toxicity (Hilgenbrink and Low 2005; Elnakat and Ratnam 2004).

Conclusion

Receptor-based active targeting of nanoparticles has a great potential to become an optimal delivery strategy. Tumor-targeted nanovehicles are being employed for early tumor diagnosis, therapy and even post-therapeutic follow-up. Furthermore, active targeting has allowed overcoming a number of obstacles such as bypassing the blood–brain barrier and multi-drug resistance in tumors.

The present manuscript has discussed some of the most important active targeting strategies explored to date. Monoclonal antibody-based targeting involves the use of whole antibody fragments that have a high selectivity and binding affinity to their target receptors. However, they have shown high immunogenicity when applied in vivo, which triggered the use of antibody-based fragments such as scFv and Fab fragments. These approaches have little immunogenicity, resulting in reduced uptake of nanovehicles by the RES, improving the bioavailability of the NPs. Aptamers are similarly nonimmunogenic, but are also chemically synthesized making them highly stable compounds in vivo. They can also be synthesized to target a wide range of targets including toxic and immunogenic molecules. Finally, targeting ligands such as transferrin and folic acid have been utilized to target transferrin receptors and FR- α , respectively, that are overexpressed not only on tumor cells, but even metastatic and drug-resistant malignant cells. To date, the optimum targeting strategy has not yet been announced, each has its own advantages and disadvantages even though a number of them have found their way for clinical application. Perhaps, a combination of strategies can be employed to improve the precision of drug delivery, paving the way for a more effective personalized therapy.

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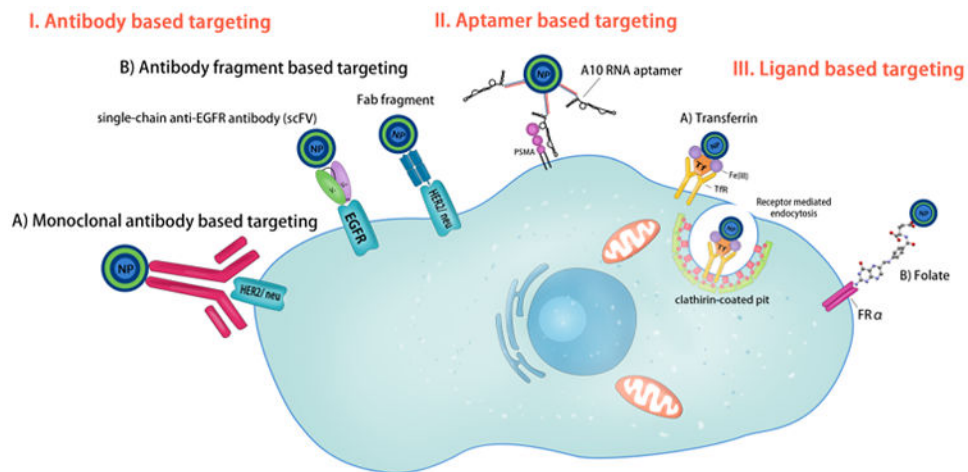


Fig. 1.

A schematic illustration showing methods used for active targeting of nanoparticles. *I* Antibody-based targeting, which involves the use of *A* monoclonal antibodies such as anti-Her2/neu antibody directed toward Her2/neu receptors on the target cell membrane, (*B*) antibody fragments: single-chain variable fragments (scFV) such as single-chain anti-epidermal growth factor receptor (EGFR) antibody directed toward EGFR, or antigen-binding fragment (Fab) such as anti-Her2/neu Fab. *II* Aptamer-based targeting such as the A10 RNA aptamer directed toward prostate-specific membrane antigen (PSMA) on the surface of the target cells. *III* Ligand-based targeting such as (*A*) transferrin-based targeting of nanoparticles toward transferrin receptors where uptake of the nanoparticles takes place through receptor-mediated endocytosis through clathrin-coated pits, (*B*) folate-based targeting using folic acid to target folate receptor alpha (FR α), which is upregulated on the surface of neoplastic cells

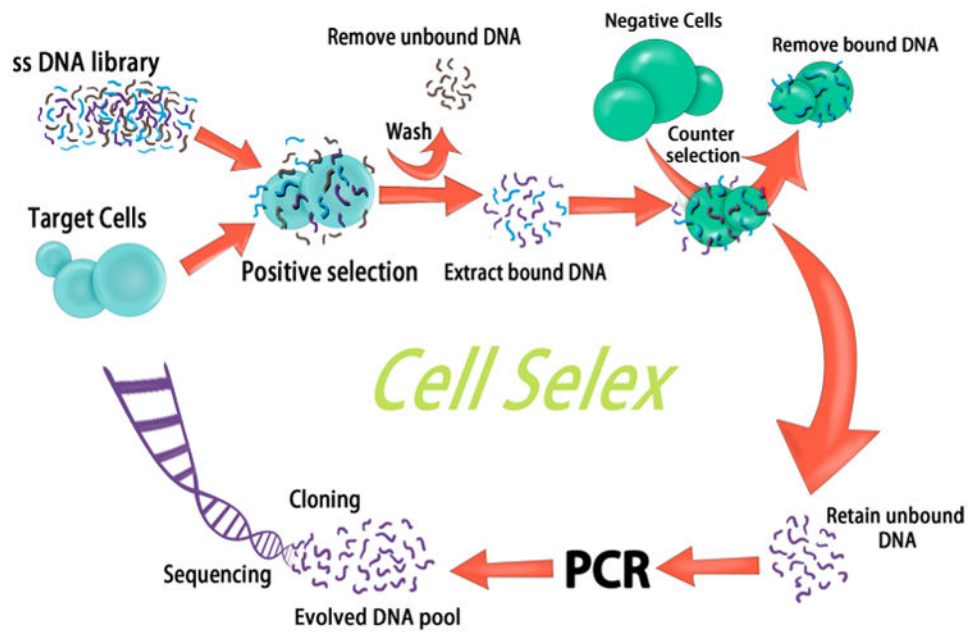


Fig. 2.

A schematic illustration showing the principle of cell-based systemic evolution of ligands by exponential enrichment (SELEX) for generation of aptamers based on their capability of recognizing complex molecular signatures of cancer cells rather than a single target on the cell membrane. Initially, a single-stranded nucleic acid (RNA/DNA) library is prepared (30–40 nucleotides in length flanked by primer sequences) followed by incubating the oligonucleotide library with a target cancer cell. The unbound DNA probes are washed out, and the bound ones are then collected and incubated with negative control cells for counter selection. All unbound probes are collected and amplified using polymerase chain reaction (PCR), and the evolved DNA pool is cloned and sequenced to determine the sequence of specific aptamers