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Ligand-targeted theranostic nanomedicines against cancer

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Abstract

Nanomedicines have significant potential for cancer treatment. Although the majority of nanomedicines currently tested in clinical trials utilize simple, biocompatible liposome-based

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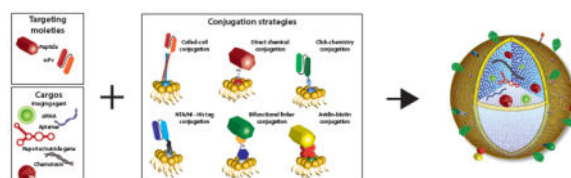
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nanocarriers, their widespread use is limited by non-specificity and low target site concentration and thus, do not provide a substantial clinical advantage over conventional, systemic chemotherapy. In the past 20 years, we have identified specific receptors expressed on the surfaces of tumor endothelial and perivascular cells, tumor cells, the extracellular matrix and stromal cells using combinatorial peptide libraries displayed on bacteriophage. These studies corroborate the notion that unique receptor proteins such as IL-11R α , GRP78, EphA5, among others, are differentially overexpressed in tumors and present opportunities to deliver tumor-specific therapeutic drugs. By using peptides that bind to tumor-specific cell-surface receptors, therapeutic agents such as apoptotic peptides, suicide genes, imaging dyes or chemotherapeutics can be precisely and systemically delivered to reduce tumor growth *in vivo*, without harming healthy cells. Given the clinical applicability of peptide-based therapeutics, targeted delivery of nanocarriers loaded with therapeutic cargos seems plausible. We propose a modular design of a functionalized protocell in which a tumor-targeting moiety, such as a peptide or recombinant human antibody single chain variable fragment (scFv), is conjugated to a lipid bilayer surrounding a silica-based nanocarrier core containing a protected therapeutic cargo. The functionalized protocell can be tailored to a specific cancer subtype and treatment regimen by exchanging the tumor-targeting moiety and/or therapeutic cargo or used in combination to create unique, theranostic agents. In this review, we summarize the identification of tumor-specific receptors through combinatorial phage display technology and the use of antibody display selection to identify recombinant human scFvs against these tumor-specific receptors. We compare the characteristics of different types of simple and complex nanocarriers, and discuss potential types of therapeutic cargos and conjugation strategies. The modular design of functionalized protocells may improve the efficacy and safety of nanomedicines for future cancer therapy.

Graphical abstract

The modular functionalized protocell is targeted to tumors via peptide ligands or recombinant human single chain variable fragments and contains imaging or therapeutic agents embedded in the mesoporous silica core.



1. Introduction

Limitations of conventional cancer drug efficacy include insolubility, systemic toxicity and drug resistance compounded by debilitating side effects such as nausea, fatigue, neuropathy, and organ failure. An effective solution to circumvent these limitations is to deliver cancer drugs within biocompatible nanocarriers. Simple nanocarriers span diverse materials such as magnetic or colloidal metals, carbon-based structures, silica, liposomes or polymeric formulations. These materials differ in size, shape, loading capacity, payload release, stability, retention and clearance from the body, which impose further restrictions on their efficacy as cancer therapeutics. For example, nanocarrier size is a critical determining

parameter since particle sizes <5 nm are cleared in the urine [1] although particles up to 50 nm have been detected as well, and nanoparticles >100 nm are cleared by the mononuclear phagocyte system (MPS), respectively. Ideally, an optimally loaded nanocarrier would be stable in the circulation to protect and deliver its therapeutic cargo to the target site, have good penetrance and retention within the target site so that measured cargo release occurs within a therapeutic window, and ultimately be organically cleared to prevent toxicity from long-term accumulation [2]. By combining features from simple nanocarriers, complex nanocarriers have improved biocharacteristics so that delivery of cancer therapeutics is clinically efficacious.

Although nanocarrier technology has improved, their lack of target specificity limits their widespread use. In solid tumors however, large fenestrations at endothelial cell borders and numerous, loose pericyte attachments are characteristic of rapidly growing tumor blood vessels that allow nanocarriers to passively exit the circulation within tumors and accumulate non-specifically [3–5]. This phenomenon is referred to as the enhanced permeability and retention (EPR) effect [6, 7]. Nevertheless, the EPR effect does not significantly increase payload concentrations at the target site and in fact, increased circulation times dissipate accumulation [8]. So, how could nanocarrier targeting and retention be improved for efficacious tumor treatment?

Since 1996, we and others have used, modified and adapted *in vivo* and *in vitro* phage display to identify ligand-receptor or scFv-epitope pairs as a means to specifically deliver a covalently linked apoptotic peptide, chemotherapeutic drug, reporter or suicide gene or imaging agents directly to tumors by intravenous administration [9–30]. Unlike other targeting moieties, peptides identified by *in vivo* phage display bind only to physiologically accessible receptors and, depending on the selection constraints, can enrich for targeting moieties that are internalized into cells subsequent to ligand binding. Thus, functional selection of targeting peptides embedded within the experimental design circumvents issues such as the EPR effect and non-specific uptake and obviates the need to reassess internalization of tumor-targeted therapeutics during downstream drug development. Additionally, depending on receptor location, i.e., tumor vs. tumor endothelial cells, internalization of nanomedicines will minimize or maximize, respectively, their distribution within the tumor via the bystander effect [31]. Off-target effects are minimized by using targeted liposomes loaded with doxorubicin to treat neuroblastoma [32–35]. Targeting liposomal doxorubicin to cultured human breast cancer or pancreatic adenocarcinoma cells was improved by inserting different targeting peptides purified as fusion proteins of the bacteriophage pVIII major coat proteins [36]. Consequently, one could envision a modular design of a targeted, stable complex nanocarrier consisting of a peptide ligand or monoclonal antibody targeting moiety conjugated to the lipid bilayer coated mesoporous silica nanocarrier, termed a functionalized protocell, which can specifically deliver a protected therapeutic cargo intravenously or locally by peritumor injection or inhalation. The term protocell (also known as a protobiont) is utilized in evolutionary biology to describe a self-organized spherical collection of lipids proposed as a stepping-stone to the creation of life. In the context of nanomedicine (and throughout this review) we use the term protocell to refer to a cell-like nanocarrier composed of a high surface area mesoporous silica nanoparticle core enveloped within a supported lipid bilayer [37–39]. In this construct, the

core can be loaded with high concentrations of disparate cargos. The lipid bilayer serves to seal and protect the cargo and provides a biocompatible interface that can be conjugated with polymers to enhance stability and peptides or antibodies to direct specific targeting and intracellular trafficking.

The modular design of functionalized protocells will permit the targeting moiety to be exchanged depending on the tumor of interest. For instance, the targeting moiety can be a peptide or antibody-like moiety such as a single chain variable fragment (scFv) that binds to overexpressed receptor proteins such as interleukin-11 receptor alpha (IL-11R α) [23, 40–42], or the 78-kDa glucose-regulated protein (GRP78) [43–47] in prostate or breast tumors. EphA5 would be an appropriate surface receptor to target in non-small cell lung tumors due to its high expression [18, 29]. scFvs that exhibit distinct receptor affinities or bind to different epitopes can be used as the binding moiety to elicit a specific therapeutic effect. For example, scFvs can be used to inhibit or modulate receptor function or act synergistically with the delivered therapeutic cargo [48]. Alternatively, binding of the functionalized protocell can elicit receptor internalization for cargo release within the cell. Table 1 lists targeting peptide ligands that have been identified by *in vivo* and/or *in vitro* phage display, whereby binding to their target receptor elicits receptor-mediated internalization.

Other examples of targeting peptides include tumor-targeting peptides derived from luteinizing hormone/chorionic gonadotropin conjugated to membrane-disrupting lytic peptides to effectively inhibit human breast and prostate xenograft tumor growth and metastases [49–52]. In addition to peptides or antibodies, aptamers, short, single stranded RNA or DNA oligonucleotides have been developed for targeted cancer therapy to treat a variety of tumors in clinical trials by delivering intercalated chemotherapeutics or conjugated directly to nanocarriers containing therapeutic cargos (reviewed in [53, 54]). Combinations of aptamers containing intercalated doxorubicin or a NF- κ B decoy were effectively used *in vitro* to inhibit growth of cultured pancreatic tumor cells by inhibiting nuclear translocation of NF- κ B [55].

Similar to chemotherapeutic drugs, targeted therapies are designed to inhibit tumor growth via a dynamic, progressive process. This ensures that toxic cellular byproducts are within physiological limits that can be effectively cleared. Due to the leakiness of tumor blood vessels, there is no doubt that targeted nanocarriers will accumulate in tumors partly due to the EPR effect. Nevertheless, once passive accumulation of targeted nanoparticles occurs, specific binding to tumor-specific receptors, internalization and retention in cells within the tumor microenvironment will ensure effective cargo release and higher, localized therapeutic indices with decreased systemic, collateral damage. Targeted delivery of functionalized protocells may also circumvent problems associated with “binding site inhibition” as this model does not take into account variability in receptor concentrations or turnover at the tumor site [56]. For instance, unless locally administered, intravenous infusion of targeted nanomedicines will be diluted in the circulation so that target site accumulation occurs over time. Unlike passive accumulation, targeted therapies, by definition, can be administered at lower doses due to their increased, effective concentration at the target site as confirmed both experimentally and by modeling and simulation [37, 38, 57]. Furthermore, functionalized protocells have a high cargo loading capacity, so that saturating receptor

concentrations are avoided. Finally, the concentration of the targeting moiety can be modulated by varying the composition of functional groups available for conjugation in the protocell lipid bilayer. Given these considerations, selective targeting by functionalized protocells can successfully circumvent binding site inhibition.

Below, we will discuss in detail the advantages of the protocell over other types of nanocarriers. In a similar fashion, a variety of payload cargos or payload combinations will be discussed including non-invasive imaging agents and/or therapeutics, alone or in combination depending on the application. Thus, we envision the modular design of a functionalized protocell may be tailored for a particular tumor type or tumor subtype whose therapeutic payload can be personalized to accommodate a prescribed clinical treatment plan. The objective of this review is to 1) describe how targeting peptides and scFvs are selected using *in vivo* and *in vitro* phage or antibody display and examine their clinical utility, 2) compare a variety of simple and complex nanocarriers and types of therapeutic cargoes and 3) review various conjugation strategies to functionalize nanocarriers and optimize therapeutic efficacy. Ultimately, optimization and personalization of targeted nanomedicines developed as cancer therapies will have to be empirically determined.

2. Targeting Strategies

2.1. Peptide phage display

In vitro phage display was originally reported as a novel method to clone genes by using a known antibody to probe phage clones that display peptide epitopes as a fusion protein of the pIII minor coat protein [58]. These studies showed that the correct peptide epitope was enriched by a thousand-fold after a single selection round. Since this initial study, phage display of random peptide libraries displayed on pIII has been used as an unbiased *in vivo* screening tool [59, 60] to identify numerous ligand-receptor pairs within the physiological context of normal brain, kidney, adipose tissue, lung, skin, pancreas, retina, intestine, uterus, prostate, and adrenal glands [24, 61, 62] and in disease tissues, both in humans and animal models [19, 27, 40, 61, 63–66]. Moreover, *in vivo* phage display combined with fluorescence laser pressure catapult microdissection revealed endothelial receptors are differentially expressed within specialized sub-cellular regions, such as pancreatic islets, and can be overexpressed in pancreatic islet tumors [67]. These results demonstrate receptors expressed by the vascular endothelia of normal or disease tissues have an inherent and distinctive molecular heterogeneity. This highlights the limitations of other methods to identify clinically relevant cell surface receptors by systematically profiling protein expression, as they do not take anatomical context into account despite the fact that some clinically important endothelial proteins are expressed in restricted locations or become accessible only under specific biologic, physiologically- or pathologically-induced, circumstances.

2.1.1. Selecting peptides by *in vivo* phage display—In a typical *in vivo* phage display experiment, a linear or circular peptide library with up to 10^9 diversity, expressed on the bacteriophage pIII minor coat protein, is injected intravenously so that circulating ligands can preferentially bind to physiologically accessible cell surface receptors (Fig. 1).

The organ or tissue of interest is subsequently removed after a period of time and tissue-specific bound phage are recovered by bacterial infection so that the peptide coding sequences can be identified by DNA sequencing [40]. Alternatively, recovered phage from multiple tissues of interest are tagged using PCR-assisted bar-coding followed by high-throughput DNA sequencing [12, 20, 27].

After several iterative rounds of selection, peptide ligands are enriched in the tissue of interest, since each successive round selects for phage recovered from internalized, bound receptors. Bioinformatic analyses of recovered peptide sequences in the forward and reverse directions [68, 69] reveals enriched tripeptide motifs, which define specific protein-protein interactions [70, 71]. Enriched peptide sequences or consensus sequences are cross-referenced against the NIH National Center for Biotechnology Information (NCBI) protein database using the **Basic Local Alignment Search Tool (BLAST)** to search for putative protein “ligand” identities which in turn may identify their corresponding receptor. Candidate ligand:receptor pairs are verified using *in vitro* phage binding assays and by ELISA if the putative receptor and appropriate antibodies are commercially available. Binding of individual phage displaying a single peptide ligand is validated *in vivo* and novel receptor proteins are subsequently isolated by affinity chromatography using purified, synthetic peptide ligands [72].

Thus far, we showed that screening libraries of phage-displayed peptides by intravenous injection into mice, rats, swine, non-human primates or brain-dead patients selects for peptides that bind specifically to normal or diseased organs/injured tissues. Importantly, and unlike untargeted nanocarriers whose fate is affected by serum proteins [8], synthetic peptides bind to the same receptors in these tissues as peptides displayed on phage particles. Moreover, peptides displayed on phage are capable of binding to post-translationally modified receptors that are expressed on cell surfaces as molecular signatures intrinsic to the microenvironment [73–76]. Not only do peptide-displaying phage bind to physiologically accessible receptors, successive rounds of selection enriches for peptides that bind to cell-surface receptors and are internalized. Enrichment of phage in the target tissue increases from 3–35 fold compared to untargeted, control phage [60]. Since 1998, considerable progress has been made in the delivery of targeted peptidomimetic drugs or imaging agents by our group and others as well as the isolation of novel peptide ligands by improvements of *in vivo* phage display technology and bioinformatic analyses [9, 12, 17, 18, 20, 27–29, 43, 63, 77–82]. Indeed, tissue-specific and angiogenesis-related vascular ligand-receptor pairs have been identified and exploited for targeted delivery of cytotoxic drugs, proapoptotic peptides, fluorophores, or cytokines to tumors, which generally improves selectivity and/or therapeutic windows in preclinical animal models.

2.1.2. Applications of peptide targeting in cancer—The application of *in vivo* phage display technology is particularly suitable for identifying and exploiting unique vascular receptors in human diseases such as cancer, where tumor cell growth and proliferation are highly dependent upon robust tumor blood vessel growth despite their abnormal molecular signatures and structural morphologies. Due to the leakiness of tumor blood vessels, *in vivo* phage display has identified unique receptors expressed on tumor endothelial cells as well as on receptors expressed on stromal cells, the extracellular matrix, pericytes, lymphatic

endothelial cells and tumor cells [29, 41, 42, 44, 45, 59, 76, 79, 83–85]. Moreover, angiogenic blood vessels acquire unique molecular signatures that can be exploited for specific, targeted delivery of therapeutic agents [9, 19, 22, 23, 28, 42, 43, 45, 86, 87]. Receptors on cultured tumor cells have also been identified using a modified *in vitro* phage display technique called BRASIL in which phage bound to receptors expressed on the surfaces of cultured cells are separated from unbound phage by centrifugation from a miscible organic phase into an aqueous phase [18, 88]. Analyses of peptide ligands recovered by the BRASIL method identified EphA5 as a putative receptor expressed on the surface of cultured human non-small lung tumor cells [18, 89]. EphA5 expression was subsequently verified as a physiologically accessible, overexpressed receptor in human lung cancer, and its expression correlates with radioinsensitivity. Treatment of lung cancer cells or human lung xenografts with an EphA5 monoclonal antibody (mAb) improved tumor sensitivity to irradiation and prolonged survival in tumor-bearing mice [29].

Phage display in a brain-dead human cancer patient revealed that peptide motifs localize non-randomly to different organs [40, 66]. One selected peptide motif, GRRAGGS, was identified from a prostate biopsy sample that exhibited sequence homology to interleukin 11 (IL-11). Our group as well as others confirmed IL-11 binds to its cognate receptor, IL-11R α [41, 90, 91], which is overexpressed during tumor progression and metastases in a large cohort of prostate cancer patients [42]. Similar to prostate cancer, expression of IL-11 and IL-11R α are significantly higher in breast cancer samples compared to healthy mammary tissue [92]. Moreover, IL-11 and IL-11R α transcript levels are approximately 3-fold greater in node-positive tumor samples compared to node-negative tumor samples [92], indicating that their expression directly correlates with the clinical and pathologic progression of breast cancer. Taken together, the vascular accessibility of overexpressed IL-11R α and its role in human prostate and breast cancer make it a clinically relevant therapeutic target.

Our studies with IL-11R α expression in prostate cancer led to the design of a ligand-directed agent, Bone Metastasis Targeting Petidomimetic-11 (BMTP-11), which consists of the IL-11R α binding peptide motif, CGRRAGGSC (Table 1), conjugated to the apoptotic peptide, $D(KLAKLAK)_2$. $D(KLAKLAK)_2$ is non-immunogenic and nontoxic outside cells but disrupts mitochondrial membranes when internalized [93, 94]. We validated the efficacy of BMTP-11 in pre-clinical models of prostate cancer, and in murine and human osteosarcomas [95]. Mice bearing DU-145, LNCaP prostate tumors or implanted with a patient-derived MDA-PCa-118b tumor, an osteoblastic, androgen receptor-independent prostate tumor [96], and treated with BMTP-11 had significantly smaller tumors compared to tumor-bearing control mice treated with untargeted $D(KLAKLAK)_2$ [23]. Toxicology studies of BMTP-11 in cynomolgus monkeys showed good stability, linear accumulation over time and predictable metabolism [23]. A phase zero clinical trial testing BMTP-11 as an investigational new drug in castrate-resistant prostate cancer patients indicated that BMTP-11-induced apoptosis of secondary bone metastasis. These results illustrate how the IL-11R α targeting peptide discovered by *in vivo* phage display in a human subject was translated into a tumor-specific, clinically relevant drug [23] (Fig. 1).

Unlike IL-11R α expression in tumors, the identification and validation of GRP78 (reviewed in [44, 97]), in breast and prostate tumors was more circuitous due to its association with the

unfolded protein response [98–100]. We and other groups showed that WDLAWMFRLPVG, WIFPWIQL and SNTRVAP-displaying phage (Table 1) bind specifically to GRP78 [43, 45, 101]. Expression of GRP78 in the endoplasmic reticulum (ER) or cell surface is induced by acidosis, hypoxia and imbalanced glucose metabolism; its expression serves as a sentinel of ER-related stress in various pathological conditions, including cancer [102, 103]. Anti-GRP78 antibodies were identified in serum from prostate cancer patients by *in vitro* phage display [46], and retrospective immunohistochemistry (IHC) studies showed that GRP78 expression predicts recurrence in prostate cancer patients [104] and poor survival in advanced breast cancer [102, 105]. Silencing GRP78 expression restored cancer cell sensitivity to chemotherapeutic regimens and established a functional role for GRP78 in cancer cell survival [102, 105]. GRP78 expression correlates with metastatic disease in inflammatory breast cancer, revealing a potential therapeutic target for a disease that currently lacks an effective treatment. mAbs against GRP78 show promise in pre-clinical studies and in early stage clinical trials, thus substantiating the development of anti-GRP78 based therapies [44, 106]. Similar to IL-11R α , cell surface overexpression and the role of GRP78 in human breast and prostate cancer make it an ideal therapeutic target [45, 47].

2.2. Antibody display

In 1986, the FDA and EMA approved the first therapeutic antibody, the CD3 OKT3 mAb, to prevent organ rejection in kidney transplants. Since then, the clinical use of antibody products has grown steadily, with 38 antibody-based biotherapeutics as of May 2015 and more expected to be approved by the end of 2015 [107]. Antibody-mediated therapeutic interventions have been successful because of their high specificity and because they share the same structural features and catabolic pathways of endogenous circulating antibodies, thereby mitigating potential safety issues in drug development.

2.2.1. Current applications of tumor-targeting antibodies—Antibody based therapies exert anti-tumor effects through a wide variety of mechanisms. Antibody binding to a cell surface receptor is sufficient to trigger antibody-dependent cell-mediated cytotoxicity [108], the effective mechanism of action for rituximab, the anti-CD20 mAb used to treat non-Hodgkin's lymphoma [109]. Bevacizumab, an anti-VEGF mAb used to treat breast, metastatic colon and rectal carcinoma, arrests tumor angiogenesis by sequestering soluble VEGF and inhibiting its binding to VEGFR-2 [110]. Antibodies that target different epitopes of the same molecule show a potentiated therapeutic effect. For example, binding of trastuzumab and pertuzumab to different domains of the receptor tyrosine kinase HER2 prevents its dimerization and results in enhanced antitumor activity [111, 112]. The development of HER2 antibody-drug conjugates (ADC, reviewed in [113]) represents a step toward personalized medicine. HER2 breast cancer patients who eventually develop resistance to trastuzumab or pertuzumab, possibly due to activation of compensatory mitogenic signaling pathways, can now be treated with the ADC, trastuzumab-DM1, that exploits the tumor-targeting capability of the HER2 mAb to deliver a microtubule-depolymerizing agent (DM1) with improved efficacy, pharmacokinetics and reduced toxicity [114].

More recently, antibodies that engage and activate the host immune system against melanoma cells have pioneered clinical immunotherapy treatment. Antibodies activate the immune system by targeting the T-cell surface receptors CTLA4 (ipilimumab) or PD-1 (nivolumab) and blocking negative regulators of T-cell activation. Combining these separate immunotherapies in a phase 1 trial of advanced stage disease resulted in tumor regression in some, but not all, patients [115]. The efficacy of antibody-based immune checkpoint therapy has been proven in a variety of cancers [116]. Other antibody-based therapies involve conjugating cytokines [117] or bacterial toxins [118] to an antibody component have shown varying degrees of efficacy in clinical trials.

In the following sections, we describe how antibodies that bind to cancer-specific proteins are selected by screening naïve antibody libraries. The design of new cancer therapeutics that utilize targeted antibodies, validated *in vivo*, to deliver therapeutic cargos in nanocarriers will be discussed, as well as exploiting synergistic combinations of antibodies with their delivered cargos as possible strategies for clinical applications.

2.2.2. Selecting targeting antibodies—In principle, antibodies can be produced from cultured cells [119], and can be engineered [120] or selected [121, 122] against any target protein to regulate its downstream effect. Nevertheless, for decades the hybridoma technique developed by Köhler and Milstein was the only reliable method to produce mAbs from splenocytes isolated from mice immunized with a target antigen. The murine origin of these mAbs however, elicited an immunogenic response in humans and made them unsuitable for therapeutic use. Recombinant antibody technology initially enabled progressive reduction of immunogenicity by producing human/mouse chimeric antibodies by engrafting the mouse-specific complementarity determining regions (CDR) onto a human antibody backbone (see [123] for a comprehensive review). Finally, generation of the genetically engineered Xenomouse [124] allowed producing target-specific human antibodies following antigen immunization.

Parallel paths to generate human antibodies were established through a combination of recombinant antibody and *in vitro* display technologies to select target-specific human recombinant mAbs (rhAbs) using a high-throughput approach. The concept of rhAb display selection is based on the exploration of large antibody-like diversity spaces (libraries) to obtain target-specific binders. To achieve this, the complexity of antibodies - a molecular complex of 4 polypeptide chains - is reduced to its essential target-binding regions such as the scFv [125], Fab [126] or nanobodies (camelid single variable domains) [127]. These variations of full-length rhAbs are fully capable of binding to target antigens, and are therefore called “antibody-like binders”. All types of antibody-like binders have been explored for antibody display and selection, and can eventually be engineered into full-length immunoglobulin (Ig)-like molecules (reviewed in [128]).

One of the most challenging aspects in antibody display technology is the creation of large, diverse antibody-like libraries so that ideally, virtually any antibody-like binder against any given target molecule can be found. As opposed to peptide libraries, where short random sequences can be generated using degenerate oligonucleotides, antibody libraries suffer from several structural constraints (chain complexity, intra-/inter-chain disulfide bond formation,

proper folding of domains and hydrophobic surface interactions) [129], which makes the creation of large, functional libraries a daunting task. Nonetheless, several successful diverse antibody libraries have been produced from naïve human repertoires [25, 130, 131], restricted human antibody scaffolds with natural diversities [126, 132] or by designing synthetic diversities [133]. In general, antibody libraries are created by cloning however; approaches using site-specific recombination [134] facilitate the creation of larger libraries with less effort. Although phage display [135] and its variations (reviewed in [10]) have been the most popular display platform for recombinant antibody libraries to date, ribosome [136] or yeast display [137–139] have also been successfully used to select high-affinity antibody binders against desired target proteins.

By combining *in vitro* antibody phage display and antibody yeast display [13, 14], a pool of hundreds to thousands of specific antibody binders from a large naïve human library can be fine-tuned to select against a protein of interest. In these studies, pre-selection of a naïve antibody-like library on the desired protein using antibody phage display is followed by antibody yeast display in which limiting amounts of the protein in the 2nd selection phase enriches for high affinity antibody-like binders. We commonly have used *in vitro* antibody phage and yeast display in successive selection rounds to identify antibody binders to a tumor-specific receptor.

Once the pool of antibody-like binders is selected and enriched from a library, choosing an antibody for tumor targeting presents yet another challenge. Specificity is clearly the driving aspect, but factors such as high tumor interstitial pressure, which influence the distribution of the targeting antibody within the tumor need to be considered as well. For instance, suboptimal concentrations of the targeting antibody to some tumor regions may lead to ineffective treatment and instigate a potential source of resistant, mutant tumor cell populations. We have found that next generation sequencing can identify the best antibodies in a selected pool, providing the selection step is appropriately designed [140].

Affinity and size play another important role in antibody-based targeting strategies. Although tumor uptake of large molecules (full-length antibodies, nanoparticles) is mostly influenced by the EPR effect [7, 141], retention of smaller molecules (peptides, scFvs, Fabs, alternative binding scaffolds) at the tumor site is highly dependent on their binding kinetics [142–146]. Tumor targeting can be achieved either by using small antibody-like binders with very high affinities (pM to low nM) or large antibodies with relatively lower affinities (high nM range) and longer half-lives in the bloodstream. Multivalency (more than one antigen binding site per antibody or antibody-like molecule) is another parameter that favors retention of antibody-like binders or antibodies in tumors and increases their functional affinity [147–149].

Antibody penetration refers to the homogeneous distribution of antibodies within the tumor and is another key factor to consider in selecting solid tumor targeting antibodies for clinical application. Factors that retard tumor penetration are high affinity and internalization. Fujimori and colleagues introduced the concept of “binding site barrier” in which high affinity antigen/antibody interactions reduce the amount of free antibodies available to diffuse into the tumor interstitium, thus spatially limiting the therapeutic effect of the

targeting antibody [56]. Additionally, Wittrup and colleagues extensively demonstrated that fast internalization rates and catabolism retard antibody penetration [150]. Despite these studies, internalization following receptor binding has been successfully exploited for delivery of ADCs (reviewed in Section 2.2.1). Furthermore, cell-internalizing antibodies exist for a variety of tumors, and are selected to bind to specifically identified antigens or, using an unbiased approach, to unknown cell surface receptors by screening cancer cells with antibody display libraries [151–153]. The evolution of recombinant antibody and display technologies enable the selection of antibody-like binders with desired properties that can overcome the binding kinetics and distribution hurdles described above.

2.2.3. Validation of antibody-like binders *in vivo*—Unlike *in vivo* peptide phage display, which has been used to screen diverse peptide libraries in terminal wean patients or animal models [19, 24, 27, 40, 67, 76, 84], *in vivo* antibody display has proven to be a more challenging task. To date, only Shukla, Krag and coworkers have shown some degree of success by injecting naïve antibody phage libraries in cancer patients [21, 26], although, the selected antibodies tend to be patient specific. Nevertheless, antibody-like binders to receptor targets identified by *in vivo* peptide phage display can be isolated by screening a naïve human scFv library using the purified receptor protein. Unpublished data from our group indicate that a combination of *in vitro* and *in vivo* antibody display yields tumor-specific antibodies when recombinant tumor receptors (identified by *in vivo* or *in vitro* peptide phage display) are used as selection targets. In these studies, an *in vitro* preselected, enriched antibody phage sub-library containing antibody clones that specifically bind to a known, overexpressed cell surface tumor-specific protein was injected into tumor-bearing mice. Several tumor-localizing rhAbs were recovered from the tumor after assessing their biodistribution using next generation sequencing [140, 154] and immunohistological analyses of tumor sections relative to control organs. Specific therapeutic characteristics such as receptor inhibition by direct or allosteric binding or conjugation to imaging dyes or therapeutic drugs can be evaluated *in vivo* so that tumor-specific receptors can be fully exploited for treatment by functionalized protocells.

3. Nanocarriers

The experimental design by which tumor-specific peptides and recombinant human scFvs are selected within the physiological setting is a significant improvement for targeted drug delivery. By using tumor-targeting peptides, we and others have successfully demonstrated *in vivo* tumor growth inhibition using tumor-targeting peptides to deliver: $D(KLAKLAK)_2$ [23, 94], doxorubicin to treat tumor-bearing mice [9], TNF- α [11, 15, 16, 22, 30], and reporter or suicide genes [17, 28]. In this section, the advantages and disadvantages of noteworthy simple and complex nanocarriers will be examined including a discussion of different types of therapeutic cargos.

3.1. Simple nanocarriers

A wide variety of nanoparticles have been developed for the delivery of therapeutic cargos including magnetic and metallic nanoparticles, such as iron oxide or gold nanoparticles, carbon based structures, such as graphene sheets and carbon nanotubes, polymer

nanoparticles, dendrimers, quantum dots, hydrogel-based delivery systems, liposomes and silica-based nanoparticles (Fig. 2). Each nanocarrier type has strengths and weaknesses, which can be exploited for specific applications. For example metallic nanoparticles act inherently as contrast agents for imaging while other nanocarriers would require the addition of an imaging agent. The advantages and disadvantages of nanocarriers for therapeutic delivery and repetitive dosing are summarized in Table 2.

Magnetic-based nanoparticles, most commonly iron oxide nanoparticles, have the theoretical advantage of precise therapeutic delivery to the region of interest using a magnet [155–157]. Additionally, metallic nanoparticles have the potential for multimodal, theranostic applications [158–161]. The theranostic potential of magnetic iron oxide particles is supported by FDA approval of a number of iron oxide nanoparticle imaging agents [157, 162]. The non-degradable nature of magnetic and metallic nanoparticles however limits repeated applications for therapeutic efficacy due to their accumulation [156, 163]. For example, iron based nanoparticles degrade slowly in biological systems such that even a single dose of iron oxide nanoparticles shows significant accumulation in the liver, spleen and lungs 90 days post-injection [164], and elimination of accumulated iron through the urine and feces occurs slowly [157, 165]. Finally, the solid nature of the magnetic nanoparticle limits the amount of therapeutic cargo that can be delivered. In addition to iron oxide nanoparticles, the other most commonly proposed metallic therapeutic nanoparticle is gold (Au).

Although Au nanoparticles have the potential to work as imaging agents or in photothermal therapies, are biocompatible, and show no significant toxicity, they accumulate, typically for months post-injection, particularly in the liver and spleen [157, 163, 166–168]. Although the accumulation of Au nanoparticles has not been associated with adverse effects, their lack of biodegradation is of concern for development as therapeutic delivery nanocarriers. Furthermore, the therapeutic loading potential of Au nanoparticles is constrained by their solid structure thereby limiting the therapeutic dose per particle.

To increase therapeutic loading, nanocarriers with very high loading potential such as carbon and silica framework structures including mesoporous silica nanoparticles (MSNP), carbon nanotubes and graphene sheets have been explored. The primary interest in the carbon-based structures is that their extremely large surface area has every atom exposed leading to the possibility of ultra-dense functionalization and therapeutic loading [159]. Despite these advantages, the major disadvantage of carbon-based structures is their limited biodegradability, which causes systemic buildup upon repeated use and potential pulmonary and immune toxicity [159, 169–175]. Similar to carbon-based nanocarriers, MSNPs are characterized by exceptionally high internal surface areas ranging from 500 to over 1200 m²/g due to periodic arrangements of uniformly sized mesopores (ranging in diameter from 2 to >20 nm) embedded within an amorphous silica framework [176]. Methods to synthesize MSNPs allows for a variety of sizes ranging from 25 nm to over 250 nm, and the MSNP shape can vary from prismatic to spherical to toroidal to rod-like [177–182]. Additionally, the MSNP pore diameter can range from 2 nm to over 20 nm and, by using silane coupling chemistry, the pore surface chemistry can be altered to accommodate high concentrations of disparate cargos [183–186]. Another major advantage of MSNPs is that amorphous silica is

Generally Recognized As Safe (GRAS) by the FDA and recently, a silica-based nanoparticle was approved for diagnostic applications in a stage I human clinical trial [187]. Although amorphous silica is GRAS, biocompatibility testing of MSNPs has been variable. Occasionally, MSNPs test positive for toxicity, which is most likely due to incomplete removal of residual surfactant used to template the pores [188]. Confirmed removal of the surfactant prior to toxicity testing has shown that very large doses of MSNPs do not adversely affect survival in mice [189]. In addition to its low toxicity, the porous silica framework of MSNPs promotes a high rate of dissolution into soluble, non-toxic silicic acid species that are easily cleared from tested *in vivo* systems [178, 188, 190, 191]. Nonetheless, disadvantages of MSNPs include instability in physiological buffers and rapid clearance by the mononuclear phagocyte system (MPS) after injection [178, 190, 192–194], which can be mitigated by coating or encapsulation with polymers or lipids *vide infra*.

To avoid bioaccumulation and uptake by the MPS, highly biocompatible systems such as polymer and lipid based nanostructures have been employed. One of the most successful nanoparticle formulations is a liposomal nanoparticle-based drug delivery of which several FDA approved formulations exist [195–197]. The advantages of liposomes are their high biocompatibility, flexible formulation and easy synthesis [33, 198–201]. Moreover, liposomal formulations can be targeted specifically to tumors by incorporating antibodies such as the GAH, anti-EGFR or anti-HER2 mAbs, small molecules such as folate, transferrin or tumor-targeting peptides such as cyclic RGD [33, 196, 202, 203]. Unfortunately, the success of liposomal formulations varies with the encapsulated drug. Stable lipid formulations of some common chemotherapeutics have been difficult to determine, in particular to limit drug leakage, making liposomes a poor universal carrier [204, 205]. Nevertheless, liposomal encapsulation significantly reduces toxic off-target effects while retaining clinical efficacy for chemotherapeutics such as doxorubicin [206–209], cisplatin [210], camptothecin [211], irinotecan and floxuridine [212]. Polymeric based nanocarriers have also been developed and several novel formulations are currently undergoing clinical trials [196]. In one example, the small cell lung cancer targeting peptide, AHSGMYP, was used to deliver docetaxel loaded into a polylactic acid polymer nanocarrier [213]. Treatment of nude mice bearing small cell lung cancer tumors with AHSGMYP-conjugated docetaxel nanoparticles resulted in higher tumor docetaxel accumulation and survival compared to tumor-bearing mice treated with untargeted docetaxel nanoparticles. Antibodies have also been used to target liposomal drugs [214–216], an approach that requires the addition of lipid tails to the C-terminus of the antibody [215, 217] in order to incorporate into liposomes.

Similar to lipid formulations, many polymer-based nanoparticles are highly biocompatible and easy to produce however, they also suffer from limited stability in *in vivo* systems and dose-dependent toxicity [159, 218]. Furthermore, both liposomes and polymer-based nanocarriers are subject to invariant size and shape, poorly controllable release profiles and highly interdependent factors whereby altering one parameter, such as size, affects loading efficiency, charge and stability [200, 219, 220].

3.2. Complex nanocarriers

To address the specific limitations described above, complex nanocarriers combine multiple features of simple nanocarriers to exploit their strengths, as well as reduce or eliminate their limitations [37, 38, 194, 221–231] (Fig. 2). For example, both liposomes and polymer-based nanoparticles have good circulation half-lives and biocompatibility but limited stability and drug retention. These limitations can be improved by the inclusion of a stable nanoparticle core within polymeric or liposomal carriers. The combination of polymers or lipids with a stable core can be accomplished with magnetic nanoparticles, Au nanoparticles, carbon based nanocarriers and MSNPs. For example, polygalacturonic acid was used to coat magnetic cobalt spinel ferrite nanoparticles and then conjugated to an EphA2 binding peptide. The resultant complex nanocarrier was used to extract metastatic ovarian cancer cells from the abdominal cavity and circulation [232]. For delivery of therapeutic cargos, the nanoparticle core should have good biocompatibility and biodegradation to allow repeated dosing, a high surface area for high therapeutic loading and a tunable nature to permit loading with a variety of cargos. As described above, MSNPs are biodegradable, biocompatible, stable and porous. Moreover, their facile chemistry allows them to act as a tunable base to load a variety of cargos, as well as a number of covalent and non-covalent coatings. The simple addition of a polymer to the surface of MSNPs, such as PEG, PEG-PEI or NIPAM-co-MAA, greatly increases their circulation time and allows significant accumulation in tumors by utilizing the EPR effect [233, 234] and demonstrates significant delivery of therapeutic drugs in preclinical *in vivo* models of cancer [233–236]. In addition to polymers, their facile chemistry allows the addition of targeting agents, such as transferrin or folic acid, to their surface [189, 190, 237, 238]. Polymer-coated and -targeted MSNPs particles are currently being used to reassess chemotherapeutics such as selenocysteine, whose clinical efficacy was previously hindered by low selectivity, solubility and stability [189].

Increased flexibility and versatility were achieved by combining liposomes and MSNPs to create a “protocell” [37–39, 176, 224, 239]. Protocells are formed by the encapsulation of the MSNP core within a supported lipid bilayer (SLB), followed by the optional conjugation of polymers, such as PEG, and targeting or trafficking ligands to the surface of the SLB [37, 38, 177, 194, 222, 224, 240–245]. Protocells synergistically combine the advantages of liposomes, low inherent toxicity and immunogenicity, and long circulation times, with the advantages of MSNPs, stability and enormous capacity for multiple cargos and disparate cargo combinations [38, 176]. The adhesion energy between the MSNP and the lipid layer suppresses large-scale membrane bilayer fluctuations, resulting in reduced liposome instability and leakage, and the lipid bilayer permits retention of soluble cargos. Since its inception, the facile chemistry of the MSNP and variability of lipid bilayer formulations have led to a wide variety of protocell designs to include: lipid monolayer encapsulated hydrophobic MSNPs to load hydrophobic cargos [194, 240], covalent attachment of lipids to enable triggered cargo release [246], polymer additives to the lipid layer to enhance circulation times and the EPR effect [240, 245], and native cell membrane encapsulated particles to improve biocompatibility [240, 247] including lipid compositions that mimic red blood cells [246].

3.3. Therapeutic payloads

3.3.1. Imaging agents—The highly modifiable nature and large cargo capacity of MSNPs enables the inclusion of an imaging modality with therapeutics. For example, inclusion of a near infrared (NIR) [242] or fluorescent dyes such as fluorescein isothiocyanate (FITC) [190] in MSNPs permits imaging *in vivo* for real time biodistribution analyses of novel nanocarriers after intravenous injection. Labeling MSNPs with NIR dyes enabled the analysis of their biodistribution over time, and the evaluation of various surface coatings such as polyethylene glycol (PEG) or PEG-polyethylenimine (PEI) on their biodistribution and clearance *in vivo* [234]. Inclusion of fluorescent dyes provides the added advantage of visualizing MSNP localization in tissues after animal dissection to confirm MSNP biodistribution studies [190, 248, 249]. Other encapsulated visualization agents include radioactive nuclides for positron emission tomography (PET) [248] or superparamagnetic iron oxide particles as an MRI contrast agent [250–252]. The structure and facile chemistry of the MSNP platform even allows multiple labels to be incorporated into the same particle, thereby enabling simultaneous confirmation of nanoparticle biodistribution in real time [251]. A similar approach [253] has been proposed to identify patients who may benefit from antibody targeted liposomes, whereby tumors are first tested for their ability to internalize fluorescent liposomes displaying specific antibodies. If internalized fluorescence is noted, liposomes bearing cytotoxic payloads will be similarly internalized, and consequently therapeutic.

In addition to their use in research, MSNPs have a potential benefit in the clinical setting as imaging agents. The ability to readily incorporate imaging agents such as NIR dyes or radioligands makes them ideal for image-guided removal of sentinel lymph nodes or small metastatic foci [254]. This approach was demonstrated using the Cornell dot or “C dot”, which was recently used in a melanoma first-in-human trial [187]. The C dot is an ultra small, 6–7 nm, silica nanoparticle containing Cy5 fluorescent molecules in the core particle, coated with PEG and further modified with radioactive iodine for PET and the cRGDY peptide for integrin-mediated targeting [255]. The dual functionality of C dots facilitated whole body PET imaging and fluorescence optical imaging during sentinel lymph node surgery [256]. In a similar fashion, MSNPs can be targeted using tumor-specific peptides or antibody-like binders, can contain imaging agents, radioligands or therapeutic agents, and can be easily coated with polymers or engineered into functionalized protocells to create biocompatible, targeted imaging agents and/or theranostics [37, 38].

3.3.2. Chemotoxins—The most common area of research for therapeutic MSNPs is chemotherapeutic delivery. Although most studies have focused on systemic delivery, an inhalation delivery study reported significant localization of a luteinizing hormone-releasing hormone peptide targeted-MSNP in a model of lung cancer compared to intravenous administration [257]. These studies demonstrated that localized delivery might be advantageous depending on the location of the tumor.

Many current studies involving systemic delivery of therapeutic MSNPs to tumors have focused on taking advantage of the EPR effect. For example, even bare uncoated MSNPs show therapeutic advantages over free drug in tumor xenografts [190]. Surface modification

of MSNPs with PEG, or PEG and PEI enhanced EPR-based accumulation in tumors and resulted in increased drug efficacy and reduced toxicity [234, 235, 258]. In a similar fashion, modifications of protocell constructs have also been utilized to enhance drug delivery via the EPR effect [242, 244, 245]. The addition of the lipid bilayer allows delivery of a hydrophilic drug within the MSNP core and a hydrophobic drug within the lipid bilayer [242]. Polymers associated with the protocell can also exert a therapeutic effect. For instance, the inclusion of Pluronic 123 blocks the action of the breast cancer resistance protein pump and increases the efficacy of the chemotherapeutic cargo in a xenograft breast cancer model [245].

Although the EPR effect can be used by modified nanocarriers to improve drug delivery, recent studies indicate a trend towards adding tumor-targeting moieties to MSNP surfaces [190, 237, 238, 244, 257]. Tumor targeting ensured delivery for tumor types and in patients for which the EPR effect is insufficient for treatment [259, 260]. When directly compared to non-targeted MSNPs, targeted MSNPs showed enhanced therapeutic efficacy and decreased toxicity over non-targeted MSNPs [189, 190, 237, 238]. For example, the addition of the targeting moiety hyaluronan to protocells enhanced their delivery of docetaxel to a xenograft breast cancer model [244].

The use of polymer coated MSNPs has even been explored to treat non-cancer cells to improve vascular access of drugs in difficult cancer types such as pancreatic ductal adenocarcinoma (PDAC). PDAC elicits a dense stromal response that limits vascular access due to pericyte coverage of vascular fenestrations and is a contributing factor to chemotherapy resistance. Tumor-bearing mice treated with an initial delivery of MSNPs containing the TGF- β inhibitor, LY364947, to decrease vascular pericyte coverage, followed by treatment with liposomes containing gemcitabine showed reduced tumor burden compared to treatment with free drug or gemcitabine-loaded liposomes only [236].

Thus, MSNPs show promise for delivering a wide variety of chemotherapy agents with decreased toxicity [233, 235, 238, 244, 245], and may resurrect shelved drugs such as selenocystine [189], whose clinical use has been hindered by low stability or solubility. MSNPs also provide the capability for combinations of therapy agents to be delivered either individually [244] or within a single nanocarrier [242, 258]. New functionalities, such as pH-responsive nanovalves on multifunctional transferrin-modified MSNPs loaded with fluorescent molecules show effective cargo release *in vitro* and *in vivo* [237]. These technological advances show the versatility and tunability of MSNPs in biological systems that capitalize their high loading capacities to ensure targeted, high chemotoxin therapeutic indices at the tumor site.

3.3.3. Reporter and/or suicide genes—Gene delivery to cells in biological systems has been explored using both viral and non-viral vectors. Despite its potential benefit, gene therapy is limited since modified adenoviral vectors may elicit an immune response and cell transduction may be inefficient. To construct a targeted adenoviral vector, we introduced an adeno-associated virus (AAV) bacteriophage chimeric vector termed AAVP [17]. We used the RGD-4C peptide (Table 1) to target AAVP containing the Herpes simplex virus thymidine kinase gene (HSV *tk*) to human DU145 prostate tumors in tumor-bearing mice. The RGD-4C AAVP-HSV *tk* vector successfully transduced tumor cells since expressed

thymidine kinase enabled tumor imaging by positron emission tomography in the presence of the substrate [^{18}F]-FEAU. Moreover, tumor growth was inhibited in the presence of the thymidine kinase substrate, ganciclovir, compared to tumor-bearing mice treated with untargeted AAVP-HSV *tk*. By utilizing a MSNP carrier, plasmid DNA is protected from enzymatic degradation in the biological environment [261, 262], facilitating entry of plasmid DNA into cells. *In vitro* delivery of green fluorescent protein (*gfp*) reporter plasmids and therapeutic plasmids by polymer-coated MSNPs has been reported [263–268]. Modifying nanocarriers containing genetic material with tumor-targeted peptides or scFvs that are internalized upon binding improves specificity and safety by ensuring only targeted cells will be transduced.

3.3.4. siRNA—Small interfering RNAs (siRNAs) can be targeted to any number of currently undruggable genes and, for instance, to amplified genes in cancer [159, 269]. Given the potential for siRNA to arrest growth of a variety of tumors [270, 271], a number of clinical trials are currently underway which utilize siRNA technology [159, 272]. Unprotected, naked siRNAs are subject to rapid degradation, on the order of 5 minutes, in the extracellular environment and can also lead to systemic inflammation, making the use of a carrier vehicle essential for effective siRNA delivery [159, 269, 272].

A variety of nanocarriers including MSNPs have been utilized to deliver siRNA *in vitro* and *in vivo* [159, 218, 273, 274]. The earliest studies utilized the MSNP surface and a protective polymer coating to encapsulate and protect siRNA [268, 275–277]. Surface association limited the amount of siRNA that could be delivered to a level similar to other solid nanocarriers, and the porous MSNP structure could be filled with other therapeutics for dual delivery. Later studies focused on loading siRNA into the MSNP pore structure to facilitate greater loading [278]. Therapeutic delivery of siRNA utilizing MSNPs with polymer coatings has been demonstrated in a variety of *in vivo cancer* models [250, 258, 279–283]. Co-delivery of therapeutic drugs with siRNA within a single MSNP has been shown both *in vitro* [277] and *in vivo* [258]. While the majority of the studies to date have utilized the EPR effect to deliver siRNA to the tumors, targeted delivery has also been demonstrated *in vitro* [37, 277] and *in vivo* [282]. We recently reported *PCA3*, a prostate cancer biomarker, is an antisense intronic long noncoding RNA that controls PRUNE2 levels via a unique regulatory mechanism by forming a PRUNE2/*PCA3* double-stranded RNA that undergoes RNA editing [271]. These results established *PCA3* as a dominant-negative oncogene and PRUNE2 as an unrecognized tumor suppressor in human prostate cancer. LNCaP prostate tumor-bearing mice treated with a stabilized anti-sense *PCA3* siRNA resulted in significant tumor growth inhibition and concomitant decreased serum PSA concentrations compared to tumor-bearing mice injected with a scrambled siRNA control. These studies show that siRNA against *PCA3* represents a promising effective nanocarrier cargo to inhibit *PCA3* activity and treat prostate tumors.

Nanocarrier cargos can become entrapped in the endosome, and endosomal escape is particularly important for nucleic acid delivery. Endosomal escape can be achieved by adding fusogenic lipids, endosomal escape peptides, membrane disruptive polymers or lysosomotropic agents to the surface of the nanocarrier or with the cargo [284, 285]. For example, efficient delivery of siRNA or DNA during cell transfection is attained by

incorporating fusogenic lipids, commonly cationic lipids, into liposomes. Other helper lipids, such as dioleoylphosphatidylethanolamine, are often added to promote fusion of liposomes to endosomal membranes and enhance nucleic acid release into the cytosol [284]. Liposomes containing cationic lipids have successfully delivered siRNA [286–289] and plasmid DNA [290–292] both *in vitro* and *in vivo*. In addition to their use in liposomes, fusogenic lipids can also be included into the lipid bilayer of protocells [39, 222, 241].

3.3.5 Aptamers—Although the majority of the aptamer research has focused on extracellular targets, the interactions of aptamers with intracellular proteins may prevent binding of a secondary molecule or alter enzymatic activity or gene expression and therefore, has potential for therapeutic applications. The use of targeted, internalized nanocarriers can transport aptamers into cells and permit clinical development of intracellular aptamers. Additionally, loading aptamers within nanocarriers would alleviate two other challenges, namely nuclease-mediated degradation and rapid renal clearance [293, 294]. Due to their small size, aptamers are rapidly degraded with half-lives as fast as 10–15 minutes [293]. To reduce renal clearance rates, aptamers are currently conjugated to high molecular weight molecules like PEG or cholesterol which increases their half-lives to as long as 12–24 hours [293]. Nevertheless, their protection within a nanocarrier would avoid conjugation to a secondary moiety, which may potentially alter target binding.

RNA based aptamers have been developed that can bind to T-cell factor 1, WT1 and β -catenin and alter their transcriptional activity in colon cancer and Wilms' tumors [295–297]. Binding of an RNA aptamer to β -catenin inhibits the β -catenin dependent transcription of cyclin D1 and *c-myc* in colon cancer cells that results in cell cycle arrest and reduced tumor forming potential in colony formation assays [296]. DNA based aptamers have also been designed to interact with transcription factors, such as Amplified in Breast Cancer 1 which is a transcriptional activator and oncogene that is over expressed in a number of human cancers [298]. In addition to transcription factors, DNA based aptamers have been designed to functionally bind to the eIF4e eukaryotic translation initiation factor. Transfection of HeLa and HEK293 cells with eIF4e aptamers reduced cell proliferation that was concentration-dependent [299]. Non-faithful recombination by BCR-ABL1-mediated tyrosine phosphorylation of RAD51 at residue 315 (pY315) may play an important role in the accumulation of chromosomal aberrations and lead to chronic myelogenous leukemia relapse and progression. Peptide aptamers were developed to act as a decoy for RAD51(pY315), and treatment of BCR-ABL1–32Dc13 cells inhibited non-faithful homologous recombination by approximately 2-fold [300]. Peptide aptamers have also been designed to modify the activity of heat shock proteins 27 (HSP27) and 70 (HSP70) [301, 302]. Over-expression of HSP27 and HSP70 has been associated with chemotherapy insensitivity and decreased tumor cell apoptosis. Treatment of mice bearing B16F10 subcutaneous melanoma with peptide aptamers increased chemosensitivity and reduced tumor burden *in vivo* [301].

4. Conjugation strategies to functionalize nanocarriers

As discussed above, the efficacy of innovative nanocarriers to treat tumors can be significantly improved by targeting. Conjugation strategies that covalently or non-covalently

link targeting moieties such as peptides, antibody scFvs or fluorescent molecules to nanocarrier surfaces are detailed in Fig. 3. The selection of an appropriate conjugation strategy is not trivial since the function of the targeting moiety has to be preserved and may be sensitive to alterations in secondary structure integrity during the conjugation process. Other considerations include proper orientation, and density per nanoparticle. As detailed below, direct conjugation strategies utilize existing surface functional groups and a single step process, whereas multistep conjugation strategies employ the addition of a new chemical entity to attach targeting moieties to functional groups on nanocarriers [303, 304].

4.1. Direct chemical conjugation strategies

4.1.1. Basic conjugation chemistries—Direct conjugation of a targeting moiety or an imaging agent to a nanocarrier may require the addition of functional groups to the surface of the nanocarrier. Surface functional groups on MSNPs can be added by co-condensation during nanoparticle preparation or by post-modification of surface silanols after nanoparticle preparation. This same strategy can be employed for conjugation of targeting moieties to complex nanocarriers such as protocells. Specific lipid compositions can be selected or synthesized to allow direct conjugation of targeting moieties onto liposome or protocell surfaces [305]. For example, amine groups present on the MNSP surface, added either during synthesis or as a post-modification, have high reactivity with isothiocyanates and are used to attach fluorescent probes, such as FITC or rhodamine isothiocyanate (RITC), [306]. Adding thiol functional groups, can also be used to conjugate targeting moieties. A thiol group on the nanocarrier surface can be conjugated to a second thiol group present in the targeting moiety to form a disulfide bond.

Although this reaction is fast and efficient, the disulfide bond is unstable over time under physiological conditions [307]. Nevertheless, a disulfide bond was used to conjugate anti-My9 mAbs onto stealth liposomes containing the cationic ionophore monesin. These antibody-liposomes conjugates bound to CD33 expressed on human HL-60 promyelocytic leukemia cells and potentiated the *in vitro* cytotoxicity of the anti-My9 immunotoxin by a factor of 2070 [308]. The reduction of cysteine residues is a common method used to conjugate thiol groups on biomolecules with maleimide functional groups on nanocarrier surfaces. These reactions are selective, produce good yields and are stable in human serum for over a day even in the presence of a reducing agent. This type of conjugation strategy has been intensively explored to link anti-HER2 mAbs to liposomes for breast cancer therapy [309–311].

4.1.2. Click Chemistry—In the last decade, the emergence of click chemistry introduced a new set of reactions to conjugate targeting moieties to nanoparticles (reviewed in [312] for liposome conjugation). These new reactions are particularly popular because they are highly specific, efficient, physiologically stable, generate a single reaction product, produce high yields and can be performed under mild reaction conditions in aqueous solutions. Moreover, unreacted functional groups do not result in non-specific binding compared to the amine or thiol group linkages detailed above. Three major classes of reactions are employed: Copper catalyzed Azide-Alkyne Click Chemistry (CuAAC) which involves the reaction between an azide and an alkyne under Cu(I) [313], Strain-promoted Azide - Alkyne Click Chemistry

reaction (SPAAC), commonly called Copper free click chemistry, which involves the same components but without a catalyst [314], and Tetrazine – trans-Cyclooctene (TCO) Ligation [315].

4.1.3. Histidine tag—The nitrilotriacetic acid (NTA)/Ni²⁺ complex was first used to affinity purify proteins containing a polyhistidine tag (His-Tag) of 6 histidine residues, and can comprise up to 14 residues, at either the N or C terminus with 100 fM affinity. Subsequently, the NTA/Ni²⁺ complex was used to link His₆-Tag-biomolecules to nanocarriers since their dissociation constant is stronger than most antibody interactions [316] without non-specific binding [317]. In 1999, Hainfeld et al. presented one of the first applications of His₆-Tag on gold nanoparticles in which the NTA/Ni²⁺ complex was introduced on the surface of gold nanoparticles prior to reaction with a His₆-tagged protein [318].

4.1.4. Coiled/Coil— α -helical coiled-coil interactions are naturally occurring tertiary structures in a wide variety of proteins, whereby oligomerization events are energetically favored and are key to many biological functions [319]. The typical primary structure is based on the $(a-b-c-d-e-f-g)_n$ repeated amino acid motif, where positions *a* and *d* are typically occupied by hydrophobic residues that mediate coil oligomerization, while positions *e* and *g* mediate interhelical electrostatic interactions.

Inspired by nature, a variety of coiled-coil pairs were designed and exploited as biosensors, and as protein expression and purification tags. The E and K heterodimer coil pair [320] and its variants [321] are two parallel coils composed of 5 repeats of 7 amino acids. Their interaction affinity is as low as 60 pM and they have been successfully used in a variety of applications, including flow cytometry-based high-throughput screens [322] to display of GFP and its variants on phage particles [323]. Due to the stability, strength and specificity of the coiled/coil interaction, we envision the use of E/K coils as a straightforward and versatile conjugation strategy to functionalize protocells. In the proposed functionalized protocell, the protocell is functionalized with the K-coil and the targeting moiety is expressed, chemically linked or synthesized as a fusion protein product containing the E-coil.

4.2. Multi-step conjugation strategies

4.2.1. Avidin, NeutrAvidin and Streptavidin—The avidin-biotin complex is one of the oldest crosslinker conjugation techniques [324] and also represents one of the strongest non-covalent bonds, with a $K_d \sim 10^{-15}$ M. The highly specific interaction between avidin and biotin is utilized to decorate avidin-containing liposomes with biotinylated antibodies. Other models have been developed based on the avidin/biotin complex [325] such as streptavidin and neutrAvidin. The characteristics of these proteins include lower molecular weight and the absence of carbohydrates, which decreases isoelectric points and in turn, non-specific binding. Although the avidin-biotin conjugation techniques are very easy to use and produce strong bonds, they have limited utility in *in vivo* targeting with nanocarriers due to their potential immunogenicity and this restricts their repeated use [326–328]. Research is currently underway to produce low immunogenicity variations of streptavidin to allow continual use of this strategy in therapeutics [326, 328].

4.2.2. Homobifunctional linkers—In the late seventies, homobifunctional crosslinkers such as glutaraldehyde and dimethyl suberimidate were used for amine-amine crosslinking [329, 330] to attach proteins or mannose ligands onto liposomes [331]. Currently, this type of crosslinking is not widely used due to possible homopolymerization during the reaction, which leads to aggregates [332]. Moreover, since a majority of biological ligands contain numerous amine groups, the use of homobifunctional linkers produces a variety of targeting moiety orientations, which may ultimately interfere with specific targeting [303].

Crosslinkers can be used to stabilize direct disulfide bonds formed between a targeting moiety and a variety of nanocarriers. Conjugation between two thiols can be performed by reagents carrying halogens such as bromobimane or bis-((N-iodoacetyl)piperazinyl)-sulfonerhodamine that undergo nucleophilic substitution with thiols. The use of these crosslinkers allows insertion of a fluorescent probe between two biological components [333, 334] but has not yet been used to label nanoparticles to our knowledge.

4.2.3. Heterobifunctional linkers—The use of heterobifunctional crosslinkers represents the future with regards to linking targeting moieties to nanoparticles. These crosslinkers facilitate conjugation reactions because they are available in a multitude of different functional groups. Additionally, they incorporate PEG chains that augment the solubility of nanocarriers in the physiological milieu, thereby increasing end product stability. Three classes of reactions are commonly used: amine to thiol, carboxylic acid to amine and click chemistry [335].

One of the most common uses of heterocrosslinkers is to link a carboxyl group to an amine. This reaction occurs in two stages, initially to create EDC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide) on the carboxyl group that is present on the nanoparticle surface to form an intermediate reactive species towards primary amines [336]. Recently, other conjugation methods introduced an N-hydroxysulfosuccinimide (NHS) linker in a second step such as sulfo-NHS to produce a more stable intermediate in order to improve reaction efficiency [337]. This approach was used to attach siRNA onto gold nanoparticles to silence the *c-myc* protooncogene *in vitro* and *in vivo* [338].

As described previously (see Section 4.1.1), cysteine residues at the C- or N-terminus in numerous biomolecules can be utilized as functional moieties to conjugate targeting peptides or antibodies. Since nanoparticles can be easily modified to incorporate amines on their surface, most heterobifunctional crosslinkers contain a NHS function on one end to bind to amines, and on the other end to maleimide to link to sulfhydryl groups. Heterobifunctional crosslinkers are water soluble, easy to use, and the reactions are specific and produce high yields. One example of this is the conjugation of the SP94 peptide to protocells containing a drug cocktail to human hepatoma 3B cells [38]. Importantly, these studies demonstrated specific delivery of SP94-protocells loaded with high concentration drug cocktails, and long-term stability with minimal non-specific binding and low toxicity to normal cells.

Finally, click chemistry can be employed with heterobifunctional crosslinkers as well, using the same reactions detailed earlier (see Section 4.1.2) namely, copper-catalyzed, copper-free, and tetrazine - trans-cyclooctene mediated ligation. Most commercially available

crosslinkers include a PEG chain in their backbone and allow the insertion of a new functional group by click chemistry. The newly inserted functional group reacts with biomolecules through maleimide (Methyltetrazine-PEG4-maleimide, TCO-PEG3-maleimide), carboxylic acid (DBCO-PEG4-Amine) or even amine groups (Alkyne-PEG4-NHS Ester, Azido-PEG4-NHS Ester). Among recent examples, liposomes containing DBCO labeled lipids were used to conjugate to tetraacetylated N-azidoacetyl-d-mannosamine (Ac4ManNAz) ligands, which resulted in specific binding to A549 cells *in vitro* and to tumors *in vivo* [339].

5. Conclusion

The technological advances in peptide phage display and antibody display, combined with the improved loading and biocompatibility of sophisticated nanocarriers, should facilitate the production of modular, targeted theranostic nanomedicines that specifically treat solid tumors in the near future (Fig. 4). Conjugation of tumor-specific peptide ligands or scFvs to the outer leaflet of the protocell lipid bilayer will depend on the available functional groups and may require the use of homo- or heterobifunctional crosslinkers. The orientation of the targeting moiety can be constrained by adding a His₆-Tag, a short α -helical E-coil or a biotin group to the C-terminus for non-covalent association with NTA, a short α -helical K-coil or streptavidin, respectively, present on the surface of the protocell. Additionally, the composition of the protocell lipid bilayer may be adjusted to control the concentration of the targeting moiety, increase its circulation retention time and promote endosomal escape. Each protocell can be loaded with different types of imaging or therapeutic agents depending on the clinical application. Compared to conventional systemic chemotherapy, functionalized protocells present a safe alternative that simultaneously permits real-time, non-invasive imaging to monitor tumor growth inhibition. Taken together, these advantages provide greater clinical flexibility to personalize treatment regimens as dictated by treatment outcomes.

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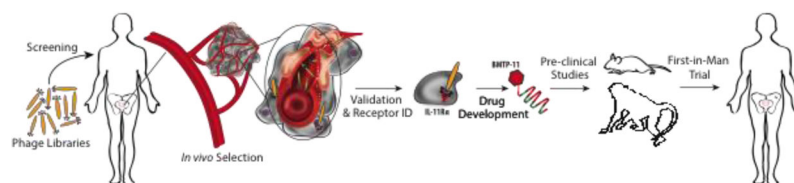
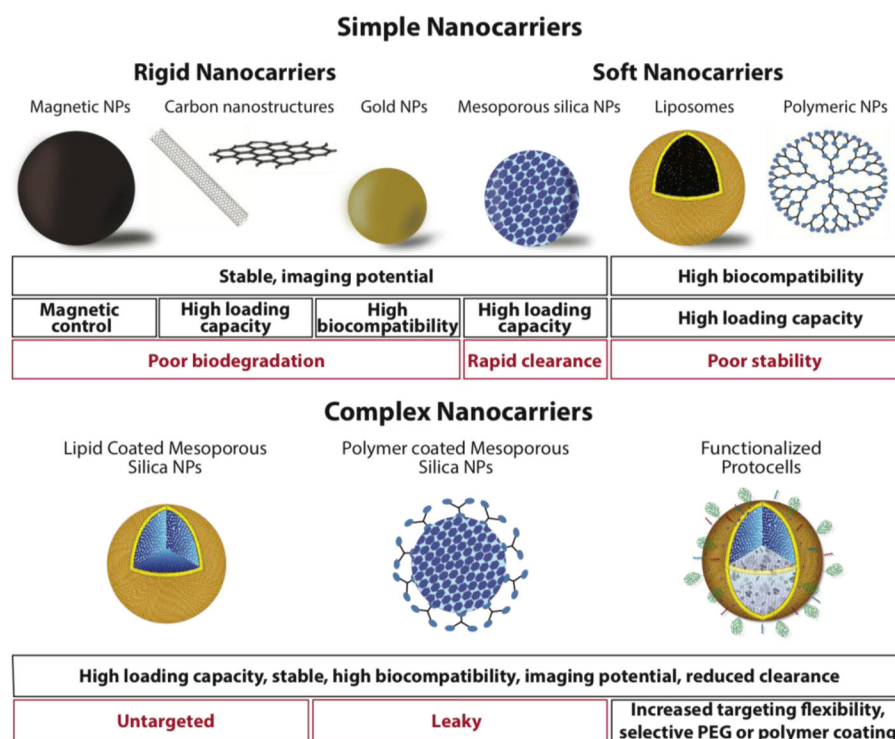


Fig. 1.

Drug development pipeline for BMTP-11. Development of a peptide-based therapeutic, BMTP-11, starting from *in vivo* phage display using a peptide combinatorial library in a terminal wean patient identified a prostate tumor-specific peptide ligand, which was followed by receptor identification and validation. Drug development of BMTP-11 included toxicological studies in mice and cynomolgus monkeys followed by a first-in-man phase 0 clinical trial, in which BMTP-11 localized and induced apoptosis of tumor cells at a secondary metastatic site [23].

**Fig. 2.**

Comparison of simple vs. complex nanocarriers. Complex nanocarriers incorporate high loading capacity of a variety of cargos, greater stability and high biocompatibility. The lipid bilayer of the functionalized protocell may contain selective polymers, such as PEG (green) or cholesterol (purple diamonds) to improve membrane fluidity and overall charge. Additional functional moieties for conjugating targeting peptides (red) or fusogenic peptides to promote endosomal escape (blue) may be added. These modifications optimize protocell retention, increase drug concentrations at the tumor site and allow protocells to target different tumors [38].

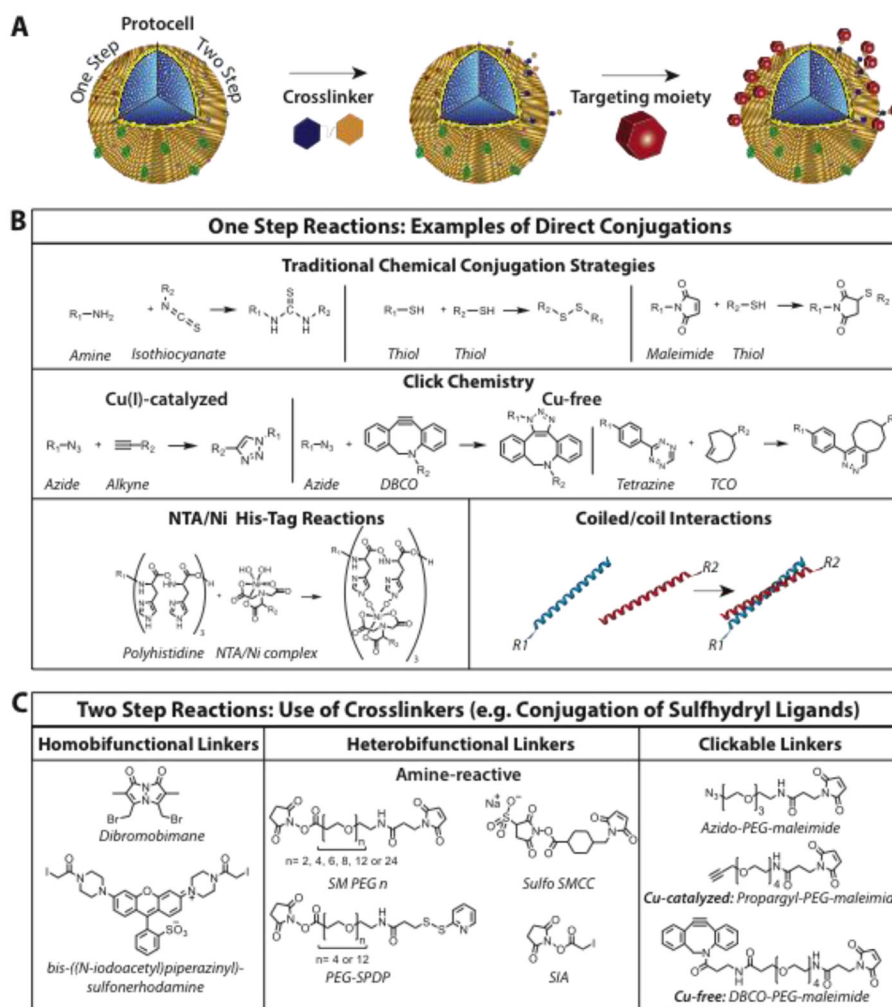


Fig. 3. Covalent and non-covalent conjugation strategies for nanocarriers. (A) Schematic representation of conjugating targeting moieties to resident functional groups (red or grey spheres) on the phospholipid head groups of the protocell outer lipid leaflet using a one- or two-step process. (B) Common single-step conjugation strategies including covalent traditional conjugation strategies, click chemistry, NTA/Ni²⁺-His₆ or hydrophobic coiled/coil interactions. (C) Linking targeting moieties that contain sulfhydryl groups utilize two-step reactions that require homo- or heterobifunctional crosslinkers or clickable linkers.

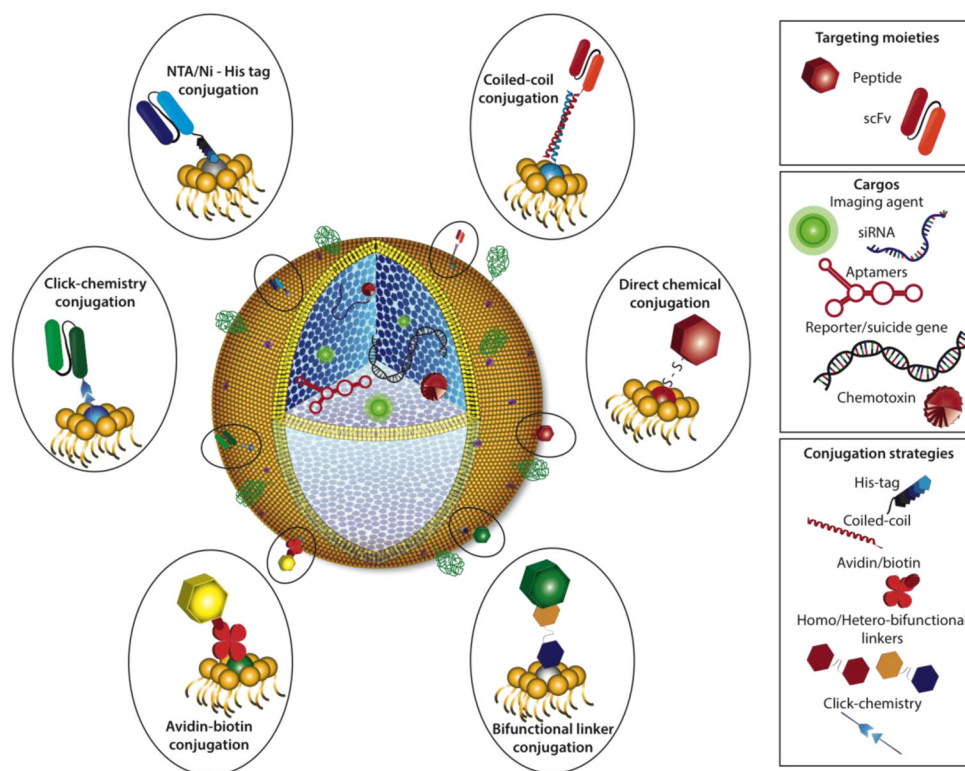


Fig. 4. Schematic design of a functionalized protocell. Tumor-targeting peptide ligands or recombinant human scFvs can be conjugated directly or indirectly to functional groups on the outer leaflet of the protocell lipid bilayer. Functionalized protocells can be loaded with a wide variety of cargos such as chemotoxins, genes, siRNA, aptamers or imaging agents. The composition of the lipid bilayer can be modified to regulate the concentration of bound peptide ligands or scFvs to minimize binding site inhibition and optimize therapeutic indices, and may also incorporate different polymer coatings (purple dots) to improve circulation retention times.

Table 1

Peptide ligand motifs and corresponding tumor receptors

Peptide Ligand Motif	Tumor Receptor	<i>In Vitro</i>	<i>In Vivo</i>	Reference
RGD-4C, VVISYSMPD	$\alpha v\beta 3$ & $\alpha v\beta 5$	Y	Y	[9, 340]
TAASGVRSMH, LTLRWVGLMS	NG2	Y	Y	[341]
CTTHWGFTLC	MMP-2, MMP-9	Y	Y	[342]
NGR	APN (CD13)	Y	Y	[74–76, 168, 172]
RPL, CTQYAMHLC, CSQYSFNWC, CGFYWLRSC	VEGFR-1, Neuropilin-1	Y	Y	[78, 80, 88, 343–345]
CGRRAGGSC	IL-11R α	Y	Y	[23, 40–42]
CNVSDKSC, WIFPWIQL, WDLAWMFRLPVG, SNTRVAP	GRP78	Y	Y	[43, 45, 46, 101]
CPRECESIC	APA	Y	Y	[84]
CKGGRAKDC	Prohibitin (receptor identified in normal adipose tissue)	Y	Y	[19, 346, 347]
CVPELGHEC	HSP90	Y	N	[348]
GFE	MDP (metastasis)	Y	Y	[349]
CVRAC	EGFR	Y	N	[77]
WXDDG	TWEAK	Y	N	[350]
CLFMRLAWC, HDERMFLCKS	MUC18	Y	Y	[351]
YRCTLNSPFFWEDMTHECHA	CRKL	Y	Y	[86]
CRTIGPSVC	Transferrin	Y	Y	[28]
GNFRYLAPP	DNA-PKcs	Y	N	[352]
CWKLGGGPC	Human leukocyte proteinase-3	Y	N	[27]
CSGIGSGGC, CRFESSGGC	EphA5	Y	Y	[18, 29]
GLTFKSL	PPP2R1A	Y	Y	[83]
CTFAGSSC	Fetulin-A	Y	N	[85]
HSYWLRs, YKSHSYWLRSGGGC	NPTX2/NPTXR	Y	N	[353]
YKWYYRGAA-pen	RPL29	Y	N	[354]
CGIYRLRSC	$\alpha 6$ integrin & E-cadherin complex	Y	N	[355]

Table 2

Advantages and disadvantages of various nanocarrier platforms for therapeutic delivery

Nanocarrier	Advantages	Disadvantages
Magnetic nanoparticles	Magnetic targeting [155–157] Imaging potential [158–161] Previous FDA approval [157, 162] Control of size and shape [155]	No internal loading capacity [155] Requires surface modification to achieve stability [155] Poor biodegradation [156, 157, 163–165]
Gold (Au) nanoparticles	Highly biocompatible [157, 163, 166–168] Imaging potential [356] Potential for photothermal therapy [157]	No internal loading capacity Poor biodegradation [157, 163, 166, 167]
Graphene Sheets	Very high loading capacity [159, 171] Stable in suspension [171]	Dose dependent toxicity <i>in vivo</i> [171] Non-biodegradable [171]
Single-walled carbon nanotubes	Very high loading capacity [159] Low synthesis cost [157]	Potential for pulmonary injury <i>in vivo</i> [157, 172] Immune suppression [172] Accumulation in organs [157, 172, 173]
Multi-walled carbon nanotubes	Very high loading capacity [159] Low synthesis cost [157]	Accumulation in organs [157, 159, 357] Potential for pulmonary damage [157, 175] Immune suppression [157, 174]
Mesoporous silica nanoparticles (MSNP)	Biocompatible [189] Biodegradable [178, 188, 190, 191] Very high loading capacity [176, 183, 185, 186]	Instability in physiological buffers [176] Poor circulation, rapid clearance [178, 192–194]
Liposomes	Biocompatible [33, 198–201] Ease of synthesis [33] Flexible formulation [33] FDA approved [195, 197, 198] Potential to add targeting moieties [33, 196, 202, 203]	Poor universal carrier [204, 205] Invariant size and shape [200, 219, 220] Poorly controllable drug release characteristics [198–201]
Polymeric nanoparticles	Biocompatible [157, 159] Easy to manufacture [159] High loading capacity [157] Potential to add targeting moieties [213]	Limited <i>in vivo</i> stability [159, 218] Poorly controllable drug release characteristics [198–201] Dose dependent toxicity [159, 218]