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Biofunctionalized Targeted Nanoparticles for Therapeutic Applications

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Abstract

Background—The development of nanoparticles for the delivery of therapeutic agents has introduced new opportunities for improvement in medical treatment. Recent efforts have focused on developing targeted nanoparticles for therapeutic delivery by functionalizing nanoparticle surfaces with targeting molecules, such as antibodies, peptides, small molecules and oligonucleotides.

Objectives—This paper will review the state of targeted nanoparticles development.

Methods—We will discuss nanoparticle platforms for therapeutic delivery, targeting molecules and the biofunctionalized targeted nanoparticles currently in development.

Results/Conclusions—Biofunctionalized targeted nanoparticles have demonstrated exciting data in preclinical studies. With continued improvements, they may fulfill their potential as therapeutics carriers that can truly treat target tissue without affecting normal cells.

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Keywords

biofunctionalized; nanoparticle; targeting; targeted nanoparticles; therapeutics delivery

1. Introduction

Advances in nanotechnology have significantly impacted the field of therapeutics delivery. This is evidenced by the increase in the number of nanoparticle-based therapeutic products in development over the last two decades. A 2006 global survey conducted by the European Science and Technology Observatory (ESTO) revealed that more than 150 companies are developing nanoscale therapeutics, and twenty-four nanoparticle therapeutics are currently in clinical use [1]. These drugs are being developed to treat a wide range of diseases, such as fungal or bacterial infections, HIV infections, diabetes and cancers. There are several advantages to using nanoparticles for therapeutics delivery. The use of materials on the nanoscale level provides the unprecedented freedom to modify some of the most fundamental properties of therapeutic carriers, such as solubility, diffusivity, biodistribution, release characteristics and immunogenicity. Precise nanoparticle engineering has yielded longer circulation half-lifes, superior bioavailability and lower toxicity [2, 3]. For example, the liposomal encapsulation of doxorubicin significantly reduces its most serious and dose-limiting side effect, cardiac toxicity [4, 5].

One strategy to further improve the therapeutic index of nanoparticle therapeutics is to functionalize nanoparticles with targeting ligands. The addition of targeting ligands allows the delivery of drug-encapsulated nanoparticles to uniquely identified sites while having minimal undesired effects elsewhere. Since biologically targeted nanoparticles have the potential to be the optimal drug delivery vehicle, there has been tremendous amount of interest in developing novel targeted nanoparticles for therapeutic applications. This paper will review recent advances in the development of biofunctionalized targeted nanoparticles. We will discuss the existing nanoparticle platforms for therapeutic applications, targeting ligands that can be used to functionalize the nanoparticles, and the various targeted nanoparticles in development.

2. Nanoparticle Platforms for Therapeutic Applications

Over the last several decades, numerous nanoparticle platforms have been studied for their use in therapeutic applications. These nanoparticle platforms include liposomes, polymer-therapeutic conjugates, polymeric micelles, dendrimers, nanoshells and nucleic acid-based nanoparticles. The two dominant classes of nanoparticles, liposomes and polymer-drug conjugates, account for more than 80% of the available nanoparticle therapeutics in clinical use.

2.1 Liposomes

Liposomes have been widely used as pharmaceutical carriers in the past decade, with eleven formulations approved for clinical use and many more in clinical development. Some of the commonly used therapeutics include liposomal amphotericin, liposomal doxorubicin, and liposomal daunorubicin. Liposomes are spherical vesicles that contain a bilayered membrane structure composed of natural or synthetic amphiphilic lipid molecules [6, 7]. Their biocompatible and biodegradable composition, as well as their unique ability to encapsulate both hydrophilic and hydrophobic therapeutic agents make liposomes excellent therapeutic carriers. Liposomes can also be coated with biocompatible and antibiofouling polymers, such as polyethylene glycol (PEG), to prolong their circulation half-life [7]. The polymer

coating of the liposomes can also be engineered to carry a functional group which can be used for targeting ligand conjugation.

2.2 Polymer-drug conjugates

Another nanoparticle drug delivery platform, polymer-drug conjugates, has been extensively studied [8]. Small molecule therapeutic agents and proteins usually have two unfavorable properties: short circulation half-life, leading to frequent administrations, and non-site-specific targeting, resulting in undesired systemic side effects. The conjugation of drugs to polymeric nanocarriers can reduce these undesirable adverse effects. Polymer-drug conjugates not only prolong *in vivo* circulation time from several minutes to several hours but also reduce cellular uptake along the endocytic route. This characteristic enhances the passive delivery of drugs to tissues with leaky blood vessels, such as tumors and atherosclerotic plaques [9, 10].

Many polymers have been proposed as drug delivery carriers but only a few of them with linear architecture have been used in clinic. The major challenges of most polymer-drug conjugates include polymer toxicity, immunogenicity, nonspecific biodistribution, *in vivo* circulation instability, low drug carrying capacity, rapid drug release and manufacturing challenges. Polyethylene glycol (PEG), which was first introduced into clinical use in the early 1990s, enhances plasma stability and drug solubility while reducing drug immunogenicity [11]. There are currently six examples of PEG-drug conjugates in clinical practice (Table 1). In addition to PEG, other linear polymers such as polyglutamic acid, N-(2-hydroxypropyl) methacrylamide (HPMA), polysaccharide and poly(allylamine hydrochloride), have been harnessed as polymeric drug delivery carriers. The polymers in the polymer-drug conjugates can be used for conjugation to a targeting ligand, in turn creating biologically targeted therapeutics.

2.3 Dendrimers

Dendrimers are well defined, regularly branched macromolecules that are 2.5–10 nm in size [12]. They are synthesized from either synthetic or natural building blocks such as amino acids, sugars, and nucleotides. The core of a dendrimer is denoted generation zero and each additional level of branching adds another generation. Dendrimers' characteristics as carriers of therapeutics include nanoscale spherical architecture, narrow polydispersity, multifunctional surface chemistry and large surface area. Many dendrimer families have been reported [13]. Among the families, the polyamidoamine (PAMAM) and poly(propylenemine) (PPI) have been most widely used for biomedical applications. The specific molecular structure of dendrimers enables them to carry various drugs through their multivalent surfaces by covalent conjugation or electrostatic adsorption. Alternatively, dendrimers can be loaded with drugs, by using the cavities in their cores through hydrophobic interaction, hydrogen bond or chemical linkage. Their surface can be engineered to provide precise spacing of surface molecules and to conjugate targeting molecules.

2.4 Polymeric Nanoparticles

Biodegradable polymer nanoparticles have been extensively investigated as therapeutic carriers [14, 15]. They are generally formed by the self-assembly of copolymers consisting of two or more polymer blocks with different hydrophobicity. These copolymers spontaneously assemble into a core-shell micellar structure in an aqueous environment. Specifically, the hydrophobic blocks form the core to minimize their exposure to aqueous surroundings while the hydrophilic blocks form the corona-like shell to stabilize the core [16]. This core-shell structure provides an ideal drug delivery nanocarrier. Its hydrophobic core is capable of carrying therapeutics with varying loading capacity (5–25% weight). The

hydrophilic shell not only provides a steric protection for the micelle but also provides functional groups for further particle surface modifications. Polymeric nanoparticles have been formulated to encapsulate either hydrophilic or hydrophobic small drug molecules, as well as macromolecules such as proteins and nucleic acids [17, 18]. The release of encapsulated drugs occurs at a controlled rate in a time or environment dependent manner. Furthermore, the rate of drug release can be controlled by modification of the polymer side chain, development of novel polymers, or synthesis of copolymers [19–23]. In general, these biodegradable polymer systems can provide drug levels at an optimum range over a longer period of time than other drug delivery methods, thus increasing the efficacy of the drug and maximizing patient compliance, while enhancing the ability to use highly toxic, poorly soluble, or relatively unstable drugs. Poly(D,L-lactic acid), poly(D,L-glycolic acid), poly(ε-caprolactone), and their copolymers at various molar ratios diblocked or multiblocked with PEG are the most commonly used biodegradable polymers, while PEG is the most commonly polymer used to engineer the polymeric micelle surface [16, 24, 25]. For example, poly lactide-co-glycolide (PLGA) encapsulated antibiotics have been investigated for the treatment of tuberculosis using murine models [26].

2.5 Metallic Nanoshells

Metallic nanoshells are characterized by a dielectric core coated with a thin metallic shell to improve their biocompatibility and optical absorption [27]. These particles possess a highly tunable plasmon resonance mediated by the size of the core and the thickness of the shell, which in turn determines their absorbing and scattering properties over a broad range on the spectrum from the near-ultraviolet to the mid-infrared. Gold nanoshells have been developed for *in vivo* photothermal therapy using near infrared light [28]. Similarly, thermally sensitive polymeric hydrogels and optically active nanoshells have been developed for the purpose of photothermally modulated drug delivery. Nanoshell particles with a magnetic core (carbonyl iron) and a biodegradable poly(butylcyanoacrylate) (PBCA) shell have also been developed for controlled release of 5-fluorouracil [29].

2.6 Nucleic acid-based nanoparticles

In nucleic acid based nanoparticles, DNA and RNA macromolecules can be used as substrates for developing therapeutic and imaging nanocarriers. By rationally construct nucleic acid chains that can lead to shapes others than the traditional linear or circular shapes, researchers have been able to formulate novel nanoparticles using nucleic acids as building blocks. A multivalent DNA delivery vehicle, with an average size of 100 nm, was recently reported for simultaneous targeted drug delivery, imaging and gene therapy [30]. Targeted multifunctional RNA nanoparticles (25–40 nm) have also been developed with a trivalent RNA core, RNA aptamers for targeting, and siRNAs for therapeutic effect [31].

3. Targeting ligands

3.1 Monoclonal antibodies

Monoclonal antibodies (mAb) have been the preferred class of targeting molecules for the last several decades. Artificially engineered mAbs have been commonly used for molecular targeting purposes. In order for the engineered antibodies to function in the human body, they have to evade the immune system. Current development of mAbs has thus been focused on chimeric, humanized and fully humanized derivatives to decrease their immunogenicity. The ability of engineered monoclonal antibodies to target disease processes has been demonstrated by the success of several monoclonal antibody therapeutics including rituximab, trastuzumab, cetuximab, and bevacizumab.

Despite the vast effort on their development, mAbs have their share of limitations. Monoclonal antibodies are large, complex molecules that require significant engineering at the molecular level to be effective [32, 33]. They are expensive to manufacture and there exists variation from batch to batch, limiting their efficiency as targeting molecules.

3.2 Aptamers

Aptamers are small nucleic acid ligands that can bind to targets with high sensitivity and specificity. Aptamers fold by intra-molecular interaction into unique conformations with ligand binding characteristics [34]. For a particular target, aptamers are selected through an *in vitro* process called Systemic Evolution of Ligands by Exponential Enrichment (SELEX) [35]. This process uses the principles of evolution, where a library of 10^{15} random oligonucleotides is enriched to identify those aptamers that can bind to the target with the highest affinity and specificity.

Aptamers have potential advantages as targeting ligands. They are small in size (~ 15 kD), and generally have less immunogenicity which leads to better biodistribution[36, 37]. Most importantly, SELEX is a chemical process which can be scaled up with ease, without batch-to-batch variations and with lower costs [38, 39]. More than 200 aptamers have been isolated [40, 41]. For example, RNA aptamers to the Vascular Endothelial Growth Factor $(VEGF)_{165}$ isoform with 2′-O-methylpurine and 2′-F pyrimidines have been reported [42, 43]. It was found that VEGF aptamers not only can lead to regression of tumor vessels but the aptamers also exhibited a remarkable stability in plasma in monkeys. Pegaptanib, an aptamer targeted against the $VEGF_{165}$, was approved by the FDA in December 2004 for the treatment of neovascular macular degeneration, underscoring the rapid progress of aptamers from its original conception to clinical application. Aptamers' major shortcomings are their low serum stability and their high production cost.

3.3 Peptide based targeting molecules

Peptides are an attractive alternative targeting molecule due to their smaller size, lower immunogenicity, higher stability and ease to manufacture. The development of peptide phage libraries (~ 10^{11} different peptide sequences), bacterial peptide display library, plasmid peptide library, and new screening technologies have made their selection much easier, contributing to their popularity as targeting ligands. Peptides can also bind to their targets with high specificity and affinity. For example, cilengitide is a cyclic peptide that binds to integrins, which is currently in Phase II clinical trials for the treatment of non-small cell lung cancer and pancreatic cancer [44].

There are a handful oligopeptides that are distinct in their characteristics. These include A-domain proteins, AdNectins, and affibodies. A-domain proteins are 40 amino acid oligopeptides that bind to cell surface through multiple points of attachment [45]. The first A domain protein was found in the low-density lipoprotein receptor (LDLR) by Tschopp and Mollnes [46]. AdNectins represent another distinct type of peptides. They are thermostable and protease resistant oligopeptides that were initially derived from the 10FN3 domain of human fibronectin. Each AdNectin typically has three distinct loop structures. A large library of AdNectins has been created by introducing diversity into these loops. Recently, an AdNectin for human vascular endothelial growth factor receptor 2 (VEGFR2) named Angiocept been isolated by AdNexus Pharmaceuticals, and entered Phase I clinical trials for treating advanced solid tumors and non-Hodgkin's lymphoma in 2006. Affibody, which are small polypeptides derived from an antibody binding domain of staphylococcal protein A [47]. Affibody targeted against a specific cell marker can be selected using phage display technology. For example, a 6kD affibody with selective binding to HER2 receptor was found to have subnanomolar affinity [48].

3.4 Antibody fragments

Due the limitations and challenges of using mAbs as discussed earlier, there is increasing interest in using antibody fragments as targeting molecules while retaining the high antigen binding specificity of antibodies. These include the fragment antigen binding (Fab) fragments, single chain variable fragments (scFV), minibodies, diabodies, and nanobodies. The Fab fragment is composed of one constant and one variable domain of each of the heavy and the light chain, where scFV is a fusion of the variable regions of the heavy and light chains. Minibodies are engineered antibody fragments that is a fusion between scFV and a C_H-3 domain that self- assembles into a bivalent dimer [49]. Diabodies are covalently linked dimers or non-covalent dimers of scFvs [50]. Nanobody, which are the smallest of all fully functional antigen-binding fragments, evolved from the variable domain of heavy-chain antibodies [51]. Nanobodies are typically evolved from single domain antibodies-antibodies carrying only a functional heavy chain without the light chain. These antibody fragments are engineered to retain high affinity for target antigens but have less immunogenicity and smaller size, thus better suited for molecular targeting.

Most recently, small antibody mimetics were formulated by Qiu *et al.* [52]. They fused two complementarity-determining regions that retained the antigen recognition of their parent molecules. These 3 kD mimetics showed better biodistribution than their parent molecules, suggesting their potential as a new class of targeting ligands.

3.5 Small molecules

Small molecules have shown great promise as a class of targeting molecules because of their small size and low cost of production. One of the most extensively studied small molecule targeting moieties in targeted drug delivery is folic acid (folate). The high affinity vitamin folate is a commonly used ligand for cancer targeting because folate receptors (FRs) are frequentlyoverexpressed on tumor cells [53]. Folate specifically binds to FRs with a high affinity (K_D = ~ 10^{-9} M), enabling a variety of folate derivatives and conjugates to deliver molecular complexes to cancer cells without causing harm to normal cells. It has been used as a targeting moiety combined with a wide array of drug delivery vehicles including liposomes, protein toxins, polymeric NPs, linear polymers, and dendrimers to selectively deliver drugs into cancer cells using FR-mediated endocytosis.

4. Targeted nanoparticle therapeutics

Molecular targeting has been a key concept in recent years. Drugs such as trastuzumab, bevacizumab, rituximab have achieved great results that have eluded conventional therapeutics [54–57]. Most of the success can be contributed to targeting, as targeted therapeutics can selectively treat diseases without affecting normal tissue. There has been increasing interest in applying molecular targeting to nanoparticle therapeutics and formulate biofunctionalized targeted nanoparticles. Targeted nanoparticles, when compared to non-targeted nanoparticles, have several potential advantages: the ability to partition more of the nanoparticles within target tissue, increased uptake into target cells, higher therapeutic efficacy and lower toxicity. Although there is no clinically approved targeted nanoparticle therapeutics yet, many are in preclinical and clinical development. Almost all the combinations between the nanoparticle platforms and targeting ligands mentioned in the previous sections have been formulated. Data obtained from these targeted nanoparticles so far have supported the theoretical advantages of targeted nanoparticles.

The most significant effect of functionalizing nanoparticles with targeting ligands is increased intracellular uptake by the target cells. Kim *et al.* showed that folate targeted polymeric nanoparticles had more than 6.7 times cell uptake than non-targeted nanoparticles [58]. Our own data showed that aptamer targeted nanoparticles had 77-fold increase in

intracellular uptake study by prostate cancer cells *in vitro* when compared to non-targeted nanoparticles [59]. In a separate study, we demonstrated that by varying the targeting ligand density on the nanoparticle surface (0–10%), targeted nanoparticles had more than 7 times the intracellular uptake as the non-targeted nanoparticles after two hours of incubation [60]. Oyewumi *et al.* also studied folate targeted polymeric nanoparticles' uptake into KB cells [61]. Folate-coated nanoparticles showed higher uptake in comparison to PEG-coated nanoparticles. At a nanoparticle concentration of 180 mg/ml, KB cell uptake of folate coated nanoparticles was 20-fold higher than non-targeted nanoparticles. Kirpotin *et al.* formulated anti-HER2 MAb-liposome conjugates to study tumor targeting [62]. In this study, targeted liposomes had 6-fold higher intracellular uptake when compared to non-targeted nanoparticles.

Biofunctionalized targeted nanoparticles also preferentially accumulate in the tumors when compared to non-targeted nanoparticles. Kukowska-Latallo *et al.* demonstrated that folate targeted PAMAM dendritic polymers concentrated in KB tumor xenograft in SCID mice over 4 days [63]. Our own experience also showed higher concentration of targeted nanoparticles in tumors when compared to that of non-targeted nanoparticles [60]. We studied the biodistribution of aptamer targeted polymeric nanoparticles and non-targeted nanoparticles. Tumor accumulation for targeted nanoparticles were 2.5 times higher than that of non-targeted nanoparticles. On the other hand, Kirpotin *et al.* showed that antibody-targeted lipidic nanoparticles did not increase tumor localization but did increase internationalization in tumor cells (6-fold) [62]. The tumor accumulation of targeted nanoparticles are highly dependent on the characteristics of the targeting ligands and the nanoparticles. As more *in vivo* studies on targeted nanoparticles become reported, we will obtain more information on the factors determining tumor localizations.

Targeted delivery of therapeutics has also been shown to achieve greater efficacy. Park *et al.* studied anti-HER2 immunoliposomes encapsulating doxorubicin in tumor xenograft models [64]. They demonstrated that in four different xenograft models, immunoliposome-dox was significantly superior to free dox, liposomal dox, and anti-HER2 monoclonal antibody. Bartlett *et al.* showed that transferrin targeted siRNA nanoparticle is more effective than non-targeted siRNA nanoparticles despite similar biodistribution and tumor accumulation of the two nanoparticles [65]. Using mouse xenograft tumors expressing luciferase and siRNA against luciferase, they showed that transferring targeted nanoparticles reduced luciferase activity to 50% of compared to non-targeted nanoparticles. Increased efficacy was also seen in the Kukowska-Latallo study. Folate targeted methotrexate (MTX) lead to statistically slower tumor growth compared to non-targeted MTX. In our own experience, we have developed aptamer targeted nanoparticles (NP-Apt) that target the prostate specific membrane antigen (PSMA) on prostate cancers [59]. Using NP-Apts encapsulating docetaxel and a murine xenograft model of prostate cancer, the targeted nanoparticles effectively decreased tumor size following a single intra-tumor injection while non-targeted nanoparticles did not.

5. Expert opinions and conclusions

The development of biofunctionalized targeted nanoparticles as therapeutic agents has generated great enthusiasm in both academia and industry. Targeted nanoparticles have shown exciting data in preclinical studies, demonstrating their potential as therapeutics carriers. However, several challenges remain in their development.

The first key challenge lies in balancing the targeting ligand density against the antibiofouling surface of nanoparticles. Therapeutic nanoparticles require an antibiofouling surface for increased circulation uptake and decreased non-specific interaction. The addition

of targeting ligands increases targeted delivery but also compromises the 'stealth' surface of nanoparticles. Therefore, targeted nanoparticles should be engineered and formulated with precise control of the targeting ligand density on their surfaces.

Another challenge in formulating the optimal therapeutic carrier depends on engineering small nanoparticles that can carry a high payload. The optimal therapeutic carriers' size should be around or below 150 nm to lower liver uptake. On the other hand, the size should not be too small (less than 5 nm) since the payload will be lower and the particles may be rapidly excreted by the renal system. One technique to lower nanoparticle size is the utilization of microfluidic devices in formulating nanoparticles.

Lastly, the discovery of the optimal targeting ligand for a specific disease process can be challenging. Folate, other small molecules or smaller macromolecules, like peptides, aptamers, Fab, and scFv have the most potential because of their smaller size. Targeting molecules that have purely chemical synthesis steps are more attractive to pharmaceutical industry as they are less expensive and have no batch to batch variation. These include small molecules, peptides and aptamers.

MCC-465, a immunoliposomal formulation of doxorubicin conjugated to the F(ab)2 fragment of the human monoclonal antibody GAH, was the first targeted nanoparticle entering clinical trials [66]. Another immunoliposome, HER2 targeted liposomal formulation of doxorubicin, is awaiting clinical trials [67]. In the coming years, many more biofunctionalized targeted nanoparticles including targeted polymeric nanoparticles will be entering clinical trials. These nanoparticles will likely have higher intracellular uptake, higher target tissue concentration, improved efficacy and lower toxicity compared to non-targeted nanoparticles, make them the 'ultimate' delivery vehicles of therapeutic agents. By perfecting the nanoparticle surface and size, as well as the targeting ligand, more and better targeted nanoparticle systems will be discovered as well. One day, we may finally formulate a therapeutics carrier that can truly treat target tissue without affecting normal cells.

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Table 1

PEG-drug conjugates in clinical practice

Composition	Trade name	Company	Indication
PEG-adenosine deaminase	Adagen	Enzon	Severe combined immunodeficiency(SCID) disease associated with adenosine deaminase deficiency
PEG-anti-VEGF aptamer	Macugen	OSI Pharmaceuticals	'Wet' form of macular degeneration
PEG-α-Interferon 2a	Pegasys	Nektar, Hoffmann-La Roche	Hepatitis B, Hepatitis C
PEG-GCSF	Neulasta	Amgen	Neutropenia associated with cancer chemotherapy
PEG-HGF	Somavert	Nektar, Pfizer	Acromegaly
PEG-L-asparaginase	Oncaspar	Enzon	Acute lymphoblastic leukemia