Nanoparticle-Aptamer Bioconjugates for Cancer Targeting

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Abstract

The combination of targeted drug delivery and controlled release technology may pave the road to more effective yet safer chemotherapeutic options for cancer therapy. Drug encapsulated polymeric nanoparticle-aptamer bioconjugates represent an emerging technology that can facilitate the delivery of chemotherapeutics to primary and metastatic tumors. Aptamers are short nucleic acid molecules with binding properties and biochemical characteristics that may make them superior as targeting molecules to current antibody approaches. The goal of this review is to summarize the key components required for creating effective cancer targeting nanoparticle-aptamer conjugates. The field of controlled release and the structure and properties of aptamers, as well as the criteria for constructing effective conjugates will be discussed.

1.   Introduction

With advances in nanotechnology it is now possible to combine specialized delivery vehicles and targeting approaches to develop highly selective and effective therapeutic and diagnostic modalities to improve the outcome for a myriad of important diseases, including cancers (40, 66). Cancer-specific drug delivery may be achieved by both local and systemic administration of specially designed vehicles. These vehicles can be engineered to recognize biophysical characteristics that are unique to the cancer cells. Most commonly this represents binding of vehicles to antigens that are expressed on the plasma membrane of the targeted cells.

In the case of local drug delivery such as through the injection of delivery vehicles within an organ, it is possible to achieve a desired effect within a subset of cells as opposed to a generalized effect on all the cells of the targeted organ. In the case of cancer, the cytotoxic effects of a therapeutic agent would be directed to cancer cells while minimizing harm to non-cancerous cells within and outside of the targeted organ. For example, suicide gene delivery has been demonstrated to be effective in killing prostate cancer but not healthy muscle cells in xenograft mouse models of prostate cancer (2). This approach is particularly useful for primary tumors that have not yet metastasized such as localized brain or prostate cancer. For metastatic cancer, the vehicle would ideally be delivered systemically since the location, abundance and size of tumor metastasis within the body limits its visualization or accessibility, thus making local delivery approaches impractical.

Several classes of molecules have been utilized for targeting applications including various forms of antibody based molecules such as chimeric human-murine antibodies, humanized antibodies, single chain Fv generated from murine hybridoma or phage display, and minibodies. Multivalent antibody based targeting structures such as multivalent minibodies, single-chain dimers and dibodies, and multispecific binding proteins including bispecific antibodies and antibody-based fusion proteins have all been evaluated. More recently, other classes of ligands such as carbohydrates and

nucleic acid ligands also called aptamers, have been used as escort molecules for targeted delivery applications.

The concept of nucleic acid molecules acting as ligands was first described in the 1980's when it was shown that some viruses encode small structured RNA that binds to viral and host proteins with high affinity and specificity. These RNA nucleic acid ligands had evolved over time to enhance the survival and propagation of the viruses. Subsequently, it was shown that these naturally occurring RNA ligands can inhibit the viral replication and have therapeutic benefits(97). More recently methods have been described to perform in vitro evolution and to isolate novel nucleic acid ligands that bind to a myriad of important molecules for diverse applications in research and clinical practice(36, 102).

2.   Aptamers an emerging class of ligands

Over the past decade, a large body of data has been generated that demonstrates the feasibility of antibodies for tissue targeting, in particular as it relates to treatment of oncologic diseases. The first FDA approval for therapeutic monoclonal antibodies for the treatment of cancer came in 1997 when rituximab (Rituxan) was approved for treating patients with relapsed or refractory low-grade or follicular, CD20 positive, B-cell non-Hodgkin's lymphoma(57). A wide variety of antibody-based drugs are now under clinical development or in clinical practice today. For example, denileukin diftitox (Ontak) is an FDA approved immunotoxin for the treatment of cutaneous T cell lymphoma(42). Many other radioimmunoconjugates or chemoimmunoconjugates directed against cell surface antigens are currently in various stages of clinical and pre-clinical development. Despite the recent success of monoclonal antibodies as targeting moieties, the use of antibodies for drug targeting may have a number of potential disadvantages. Foremost, the biological production of monoclonal antibodies can be difficult and unpredictable. For example, the target antigen may not be well tolerated by the animal used to produce the antibodies or the target molecules may be inherently less immunogenic making it difficult to raise antibodies against such targets (although this problem is overcome with the use of phage display libraries)(78, 79). In addition, the

performance of antibodies may vary from batch to batch, in particular when production is scaled up.

The ideal class of targeting molecule for the delivery of controlled release polymer systems should, like monoclonal antibodies, bind with high affinity and specificity to a target antigen, but overcome or ameliorate some of the problems associated with the use and production of monoclonal antibodies. Aptamers are a novel class of ligands(36, 102) that are small, non-immunogenic, easy to synthesize, characterize, modify, and exhibit high specificity and affinity for their target antigen. In the short time since Jack Szostak and Larry Gold independently described the ground breaking methodology for *in vitro* evolution of aptamers, these ligands have emerged as an important class of molecules for therapeutic and diagnostic applications(30, 54).

Aptamers are oligonucleotides that can bind to target antigens with high affinity and specificity. Considering the many favorable characteristics of aptamers, which have resulted in their rapid progress into clinical practice, we begin to exploit this class of molecules for targeted delivery of controlled release polymer drug delivery vehicles. Recently we described the first proof-of-concept drug delivery vehicles utilizing aptamers for targeted delivery (Fig 1A) (38) and have gone on to show efficacy of these vehicles in tumor reduction *in vivo* (Fig 1B).

3. Structure, Properties and Examples of Aptamers

Aptamers are single stranded DNA, RNA or unnatural oligonucleotides that have been selected *in vitro* from a pool of ($\sim 10^{14} - 10^{15}$) of the – the random oligonucleotides for their ability to bind to a target molecule. Aptamers have a molecular weight (10 – 15 kD) which is one order of magnitude lower than that of antibodes (150 kD)(105) and derive their name from the Latin word "aptus" meaning "to fit". Aptamers fold through intramolecular interaction to create tertiary conformations with specific binding pockets which bind to their target molecules with high specificity and affinity. This tertiary conformation is analogous to the globular shape of tRNA. For large scale production, aptamers unlike antibodies, can be chemically synthesized; a significant advantage for

commercializing this class of molecule for drug development. Furthermore, due to their small size and similarity to endogenous molecules, aptamers exhibit superior tissue penetration(54) and are believed to be less immunogenic than antibodies(31). Aptamers may be circularized, linked together in pairs, or clustered onto a substrate, and classically aptamers against any target may be isolated, provided that a small quantity of target is available in the screening process.

Unlike antisense compounds, which are single-stranded nucleic acids that affect the synthesis of a targeted protein by hybridizing to the mRNAs that encodes it, aptamers may inhibit a protein's function through directly binding to it. Aptamers typically bind with an equilibrium dissociation constant (Kd) in the range of 10 pM to 10 μM(52) to a wide array of molecular targets(106) including other nucleic acids, proteins, peptides and small molecules. Aptamers can be described by a sequence of approximately 15 – 60 nucleotides (A, U, T, C, and G). The conformation of the aptamer confers specificity for a target molecule through interacting with multiple domains, or a binding pocket. Small changes in the target molecule can foil interactions and thus aptamers can distinguish between closely related but non-identical targets. For example, specific RNAs were identified that have a high affinity for the bronchodilator theophylline (1,3-dimethylxanthine) yet exhibit a >10,000 times weaker binding affinity to caffeine (1,3,7-trimethylxanthine) which differs form theophylline only by the substitution of a methyl group at the nitrogen atom N7 position(58). Based on their unique molecular recognition properties, aptamers have found great utility for applications in areas such as *in vitro* and *in vivo* diagnostics, analytical techniques, imaging, and therapeutics(19, 101, 105).

Although aptamers are highly stable and may tolerate a wide range of temperature, pH (~4 – 9) and organic solvents without loss of activity, these molecules are susceptible to nuclease degradation or renal clearance *in vivo*. Therefore, their pharmacokinetic properties must be enhanced prior to *in vivo* applications. Several approaches have been adopted to optimize the properties of aptamers such as: 1) capping their terminal ends, 2) substituting naturally occurring nucleotides with

unnatural nucleotides that are poor substrates for nuclease degradation (i.e. 2'-F, 2'-OCH3 or 2'-NH2 modified nucleotides), 3) substituting naturally occurring nucleotides with hydrocarbon linkers, and 4) use of L-enantiomers of nucleotides to generate mirror image aptamers commonly referred to as spiegelmers(3, 9, 37, 87). Aptamers can also be stabilized using locked nucleic acid modifications to reduce conformational flexibility(94). Alternatively, a nuclease resistant aptamer may be selected *de novo* using a pool of oligonucleotides with 2'-F or 2'-OCH3 modified nucleotides. Through combining some of these strategies, an aptamer's half life can be prolonged from several minutes to many hours(105). To prolong the rate of clearance of aptamers, their size may be increased by conjugation with polymers such as polyethylene glycol (PEG)(14, 51).

The conjugation of aptamers to drug encapsulated nanoparticles results in targeted delivery vehicles for therapeutic application. These may include delivery of small molecule drugs, protein based drugs, nucleic acid therapy (anti-sense oligoneucleotide, RNAi or gene therapy) and targeted delivery of agents for neutron capture therapy or photodynamic therapy. Aptamers may also be bound to imaging agents to facilitate diagnosis and identification of tumor metastases. For example, it may be useful to bind aptamers to optical imaging agents including fluorophores(44) and quantum dots (nanocrystals)(18) or MRI imaging agents such as magnetic nanoparticles(13, 50) for detection of small foci of cancer metastasis. Additional imaging agents that may make useful conjugates are reviewed elsewhere(103). Multiplex systems comprising drug laden nanoparticle aptamer conjugates together with imaging agents represents a prospective avenue to future research.

In choosing aptamers for targeting cancer cells, the aptamer must be directed towards receptors that are preferentially or exclusively expressed on the plasma membrane of cancer cells. Alternatively, they may be delivered to extracellular matrix molecules that are expressed preferentially in tumors. To date, many aptamers have been isolated that bind specifically to receptors on cancer cells are these are outlined in Table 1. (reviewed by Pestourie et al.(86)).

Listing of Aptamers for Targeted Delivery:

Human Epidermal Growth Factor-3 (HER-3)

HER-3 is a receptor tyrosine kinase which is over-expressed in several cancers. Over expression of HER-3 is also associated with drug resistance in many HER-2 over-expressed tumors making HER-3 a candidate target for drug delivery. A panel of RNA aptamers against the extracellular domain of the HER-3 has been isolated and one, the A30, can inhibit heregluin dependent tyrosine phosphorylation of HER-2 and heregluin-induced growth response of MCF-7 cells at Ki = 10 nmol and 1 nmol, respectively(21). The A30 is comprised of natural nucleotides and thus susceptible to nuclease degradation. The above studies were carried out in the presence of RNAase inhibitors. The future use of A30 for *in vivo* application will require post-SELEX optimization of this aptamer including nuclease stabilization and size minimization.

Prostate Specific Membrane Antigen (PSMA)

PSMA exists as two splice variants, a transmembrane protein referred to as PSMA and an intracellular protein referred to as PSM'. PSMA encodes a folate carboxypeptidase and it is of particular importance since its expression is tightly restricted to prostate acinar epithelium and its expression is increased in prostatic intraepithelial neoplasia, prostatic adenocarcinoma, and in tumor-associated neovasculature. An immunoconjuagate of the J591 antibody which bind the extracellular domain of the PSMA is currently in phase I clinical trials(5) and two 2'-F pyridmidine RNA aptamers against the extracellular domain of the PSMA were recently described(74). The aptamer xPSM-A9 inhibits the enzymatic function of the PSMA non-competitively with a Ki = 2.1 nmol, and aptamer xPSM-A10 inhibits the enzymatic function of PSMA competitively with a Ki = 11.9 nmol. Aptamer xPSM-A10 has also been truncated from 71 nucleotides to its current size of 56 nucleotides (18 Kd). We recently utilized the xPSM-A10 aptamer to develop nanoparticle-aptamer bioconjugates for prostate cancer targeting and demonstrated that these bioconjugates preferentially bind to and get taken up by LNCaP prostate epithelial cells which express the PSMA

protein but not by PC3 prostate epithelial cells which do not express any detectable levels the PSMA protein.

Nucleolin

Nucleolin was originally described as a nuclear and cytoplasmic protein, however, a number of recent studies have shown that it can also be expressed at the cell surface(24, 28). Nucleolin has a multi-domain structure, which reflects its remarkably diverse functions. Nucleolin is involved in the organization of the nuclear chromatin, rDNA transcription, packaging of the pre-RNA, ribosome assembly, nucleocytoplasmic transport, cytokinesis, nucleogenesis and apoptosis. The presence of nucleolin at the surface of cancer cells suggests that it could be valuable as a marker for the diagnosis of cancer. AS-1411 (formerly AGRO100) is an aptamer capable of making G-quartdruplexes that bind to nucleolin on cell surface(25) and interact with the nuclear factor kappa B (NFκB) essential modulator (NEMO) inside the cell(6). The cytosolic localization of AS-1411 after binding to cell surface nucleolin may be exploited for the intracellular delivery of nanoparticles to cancer cells. The use of AS-1411 as a therapeutic modality has also shown promise for the treatment of cancer in humans and Antisoma of United Kingdom is evaluating this aptamer in phase I clinical trials(62). The therapeutic benefit of AS-1411 is presumably attributed to the disruption of the NFκB signaling inside the cells.

Sialyl Lewis X (sLe^x)

sLex is a tetra-saccharide glycoconjugate of transmembrane proteins which acts as a ligand for the selectin proteins during cell adhesion and inflammation. sLe^x is also abnorammly overexpressed on the surface of cancer cells and may play a role in cancer cell metastasis. RNA aptamers that bind to the sLe^x were isolated and clone 5 RNA aptamer was shown ot have sub-nanomolar affinity fo the sLe^x capable of blocking the sLe^x / selectin mediated cell adhesion of HL60 cells *in vitro*(59). Considering the high level of sLe^x expression on the surface of cancer cells it may be possible to utilize clone 5 RNA aptamer for targeted nanoparticle delivery. The future use of clone 5 for *in vivo*

applications will require post-SELEX optimization of this aptamer including nuclease stabilization and size minimization.

Cytotoxic T cell antigen-4 (CTLA-4)

CTLA-4 is a transmembrane protein that is expressed on the surface of activated but not resting T-cells. It functions to attenuate the T-cell response by raising the threshold response needed for T-cell activation. The *in vitro* selection against CTLA-4 resulted in isolation of 6 distinct 2'-flouropyrimidine RNA aptamers(93). The most potent inhibitory aptamer, M9-9 (Kd = 10 nmol) was truncated from its original length of 79 base pairs to 35 base pairs resulting in aptamer D60 with a Kd = 33 nmol for CTLA-4 and shown to inhibit CTLA-4 function *in vitro* and enhance tumor immunity in mice.

Fibrinogen-like domain of Tenascin-C

Tenascin-C is an extracellular matrix protein that is overexpressed during tissue remodeling processes, such as fetal development, wound healing as well as tumor growth. Due to its high expression in tumors, high-affinity Tenascin-C ligands may be clinically useful tumor-targeting agents. TTA1 is an aptamer that has been generated against the fibrinogen-like domain of Tenascin-C(53, 94). TTA1 has an equilibrium dissociation constant (Kd) of 5 nM. Thus, TTA1 is a potentially interesting target for various cancer diagnostic and therapeutic applications.

Platelet derived growth factor receptor (PDGF-r)

PDGF-r is a tyrosine kinase that is a mediator of tumor hypertension. It is believed that lowering of the tumor interstitial hypertension, which acts as a barrier for tumor transvascular transport, is a potential strategy to enhance tumor uptake and therapeutic effects of anticancer drugs. Therefore, PDGF antagonists can be used to relieve tumor hypertension. For example, inhibitory PDGF aptamers have been shown to enhance the antitumor effect of Taxol in SCID mice(88, 89). The use of this approach along with standard chemotherapy may be a potential mechanism of using aptamers for enhancing the effects of chemotherapeutic drugs.

Pigpen

Pigpen is an endothelial protein of the Ewing's sarcoma family that parallels the transition from quiescent to angiogenic phenotypes *in vitro*. Using a non-classical approach to aptamer isolation, YPEN-1 endothelial cells and N9 micorglial cells were used respectively, in a selection and counter-selection in SELEX to isolate the III.1 DNA aptamer that preferentially bound to YPEN-1 cells(12). The III.1 was also shown to selectively bind to the microvessels of experimental rat glioblastoma using histological specimens. The isolation and characterization of the III.1 target identified pigpen as the target antigen. The use of III.1 aptamer for targeting the microvasculature of tumors is a potentially powerful means of delivering drugs to the site of the cancer.

4. Isolation of Aptamers from Random Oligonucleotide Libraries

Aptamers are isolated using an iterative protocol(41) called *in vitro* selection(36) or Systematic Evolution of Ligands by Exponential Enrichment (SELEX(102) (Fig. 2). Similar to phage display or other strategies used to isolate ligands from random libraries, SELEX is essentially an iterative selection and amplification protocol to isolate single stranded nucleic acid ligands which bind to their target with high affinity and specificity. The complexity of the starting library is determined in part by the number of random nucleotides in the pool. For example, by using a library with 40 random nucleotides, a pool of 10^{24} distinct nucleotides can be generated. Practically speaking the number of ligands in the starting pool for *in vitro* selection is closer to 10^{15} representing 1 nmol of the library.

In the initial step a library of random nucleotides flanked by fixed nucleotides is generated by solid phase oligonucleotide synthesis. The oligonucleotide pool is incubated with the target of interest and the bound fragments are partitioned and amplified using the flanking sequences for primer hybridization in a PCR reaction. The resulting pool is used in a follow-up round of selection and amplification and the process is repeated until the affinity for the target antigen plateaus. Typically this will be achieved in 6 – 10 rounds of SELEX. After the last round of SELEX aptamers are cloned in plasmids, amplified, sequenced and their binding constants are determined.

These aptamers may be subject to additional modification such as size minimization to truncate the nucleotides not necessary for binding characteristics and nuclease stabilization by replacing naturally occurring nucleotides with modified nucleotides (i.e. 2'-F pyrimidines, 2'- OCH_3 nucleotides) that are poor substrates for endo- and exonuclease degradation.

In contrast to the isolation of DNA aptamers which require single step amplification after portioning, the selection of RNA ligands involves additional steps, including reverse transcription of the partitioned RNA pool to generate a cDNA fragment and subsequent amplification of DNA and transcription into RNA for the next round of selection[41]. The advantage of RNA SELEX however, is that unnatural nucleotides such as 2'F pyrimidines and 2'-OCH_3 nucleotides may be used in the transcription of the RNA pool since these modified bases are utilized by RNA polymerase as substrate. Furthermore, mutant RNA polymerases have also been described capable of improved incorporation of modified bases during transcription[20]. The resulting modified RNA pool can be used for isolation of nuclease stable RNA aptamers. Recently, a fully 2'-OCH_3 modified VEGF aptamer was selected and when conjugated to 40 kD PEG demonstrated a circulating half-life of 23 hours. Conversely, a DNA polymerase that can incorporate unnatural bases such as 2'-F and 2'-OCH_3 has not been described and consequently DNA aptamers must be nuclease stabilized after the SELEX procedure.

5. Development of Nanoparticles for Conjugation to Aptamers

During the past 4 decades[34, 35, 63, 67, 92], controlled drug delivery strategies have dramatically impacted nearly every branch of medicine including cardiology[100], ophthalmology[33], endocrinology[49], oncology,[48] immunology[60] and orthopedics[99]. Controlled release of drugs that are encapsulated within a material is achieved by the release of encapsulated drugs through surface or bulk erosion, diffusion, or swelling followed by diffusion, or triggered by the environment or other external events[66] such as changes in pH[75], light[70], temperature[71], or the presence of an analyte such as glucose[109]. In general, controlled-release polymer systems deliver drugs in the optimum dosage for long periods, thus increasing the

efficacy of the drug, maximizing patient compliance and enhancing the ability to use highly toxic, poorly soluble, or relatively unstable drugs.

Nanoparticles are a particularly attractive drug delivery vehicles for cancer therapeutics since they can be synthesized to recognize tumor-specific antigens and deliver drugs in a controlled manner(38, 40). The design of targeted drug delivery nanoparticles combines drug encapsulated materials, such as biodegradable polymers, with a targeting moiety (Fig. 3). Ideally, biodegradable nanoparticles should be designed with the following parameters(47):

1) Small size (preferably between 50 - 150 nm);
2) High drug loading and entrapment efficiency;
3) Low rate of aggregation;
4) Slow rate of clearance from the bloodstream;
5) Optimized targeting to the desired tissue with minimized uptake by other tissues.

The following sections will discuss the various parameters that must be considered for engineering of nanoparticles for targeted drug delivery applications, including the development of nanoparticle-aptamer bioconjugates. This will include discussion of nanoparticle biomaterial, size, charge, and surface modification schemes to achieve the desired design parameters. It is important to note that a detailed review is beyond the scope of this manuscript and the reader is referred to the following reviews for further information(4, 32, 81, 85, 90).

Size of Nanoparticles

The biodistribution pattern of nanoparticles; active nanoparticle targeting to tumor antigens; and passive nanoparticle targeting by enhanced permeation and retention (EPR)(77) are all greatly effected by the size of the nanoparticle. Passive nanoparticle occurs because microvasculature of tumors are more 'leaky' thus permitting selective permeation of nanoparticles into the desired tumor tissue. This phenomenon has been

exploited to target liposomes; therapeutic and diagnostic nanoparticles; and drug-polymer conjugates to cancer tissue (reviewed by Maeda(77)). The EPR phenomenon is greatly dependant on the size of the nanoparticle. While larger particles (>100 nm) are more effectively taken up by macrophages, smaller particles are better suited for permeating through the leaky microvasculature of the tumor cells. In the case of smaller particles, the high surface curvature can also reduce interaction with the receptors on the surface of macrophages and subsequent clearance of the particles(16). Biodistribution studies using liposomes have shown that although particles that are larger than 200 nm are largely taken up by the spleen, those less than 70 nm are also efficiently cleared by the liver(72) Taken together the optimal nanoparticle size should be experimentally determined for each formulation since the interplay of various parameters (polymer system, encapsulated drug, surface charge, surface modification) makes it difficult to extrapolate the ideal nanoparticle size from seemingly similar studies. Our biodistribution studies using various size of PLGA-PEG nanoparticle-aptamer bioconjugates has suggested a linear relationship with regards to uptake by liver and spleen such that smaller particles (~80 nm) are better at avoiding uptake by these organs (unpublished results).

Polymers for synthesis of nanoparticles:

Controlled release biodegradable nanoparticles for clinical applications can be made from a wide variety of polymers including, poly (lactic acid) (PLA)(1), poly (glycolic acid) (PGA), poly (lactic co-glycolic acid) (PLGA)(26), poly (orthoesters)(29), poly(caprolactone)(82), poly(butyl cyanoacrylate)(96), polyanhydrides(43) poly-N-isopropylacrylamide(55). Although many fabrication methods exist, drug encapsulated polymeric nanoparticles are frequently made using an oil-in-water emulsion (single emulsion)(91) which involves dissolving a polymer and drug in an organic solvent such as methylene chloride, ethyl acetate, or acetone. The organic phase is mixed with an aqueous phase by vortexing and sonicating and then evaporated which forces the polymer to precipitate as nanoparticles in the aqueous phase. The particles are then recovered by centrifugation and lyophilization. Other common methods of developing

nanoparticles are water-in-oil-in water emulsion (double emulsion)(11) and nanoprecipitation(10, 23).

One of the considerations with respect to the material used for drug delivery is its ability to encapsulate drugs as well as degrade over the appropriate times. This subject has been an active area of investigation by our group and other investigators in academic and industry laboratories for several decades. The result has been an increasing arsenal of polymers with distinct encapsulation and release characteristics for a myriad of research, industrial ad clinical applications(64, 65). PGA and PLA are common biocompatible polymers that are used for many biomedical applications. PGA is hydrophilic since it lacks a methyl group and is more susceptible to hydrolysis making this polymer easily degradable. Alternatively, PLA is relatively more stable in the body(80). Through these unique properties polymers such as PLGA have been derived that are made from both glycolic acid and lactic acid components. The ability to change the ratio of these two components of the polymer can then be used to dramatically alter the rate of degradation. Therefore, by choosing the desired polymer system for the synthesis of nanoparticles the rate of degradation and subsequent release of the molecule may be tuned for the intended application.

Charge of nanoparticles:

Nanoparticle charge has been shown to be important for regulating its pharmacokinetic properties. For example, It has been shown that anionic and cationic liposomes activate the complement system through distinct pathways suggesting that particle charge may impact particle opsonization and phagocytosis(22). Cationic charge on liposomes has also been shown to reduce their circulating half-life in blood, and to affect their biodistribution between the tumor microvasculature and interstitium without impacting overall tumor uptake(17). Nanoparticles could be synthesized with charged surfaces either by using charged polymers such as poly-L-lysine, polyethylenimine (PEI) or polysaccharides or through surface modification approaches. For example, the layer-by-layer deposition of ionic polymers have been used to change surface properties of nanoparticles, such as quantum dots, by depositing ionic polymers of interest on the

charged nanoparticle surfaces(56). Furthermore, surface charge of nanoparticles has been shown to regulate their biodistribution. For example, increasing the charge of cationic pegylated liposomes decreases their accumulation in the spleen and blood while increasing their uptake by the liver and an increasing in the accumulation of liposomes in tumor vessels(17). These experiments suggest that optimizing surface physicochemical properties of nanoparticles to better match the biochemical and physiological features of tumors may enhance the intratumoral delivery of nanoparticles for systemic therapeutic approaches.

For conjugation of the negatively charged aptamers to nanoparticles, the surface charge of the nanoparticle may be important. For example, we believe that direct immobilization of aptamers on cationic nanoparticles made from PEI may result in formation of aptamer-PEI complex that render the aptamer ineffective as a targeting molecule (unpublished observation). Therefore, neutral polymers such as PLA, PLGA or those with a more negative charge such as polyanhyrides may be most suitable for conjugation to aptamers. We have used a PLA-PEG block copolymers to generate aptamer-nanoparticles bioconjugates(38, 39). One approach that may facilitate the use of a wider array of biomaterials for aptamer targeted drug delivery is through methods of 'masking' the surface charge of the particles. For example, the addition of neutrally charged hydrophilic layer of PEG on the surface of the nanoparticles may facilitate the use of positively charged materials for the synthesis of nanoparticles. These cationic nanoparticles are particularly useful for gene delivery applications and thus may enable efficient targeted gene delivery using aptamers.

Surface modification of nanoparticles:

Nanoparticle surface modification may also be used to engineer its interaction with the surrounding tissue. These interactions could be positive (i.e. targeting molecules) or negative (i.e. non-adhesive coatings). The surface modification of nanoparticles is particularly important since intravenously applied nanoparticles may get captured by macrophages before ever reaching the target site. Therefore, surface modifying particles to render them invisible to macrophages is essential to making long-

circulating nanoparticles(46, 47). The ability to control the biodistribution of nanoparticles is particularly important for drug carrying nanoparticles since the delivery of drugs to the normal tissues can lead to toxicity(27, 45).

Hydrophilic polymers such as PEG(46, 47), polysaccharides(68, 69) and small molecules(104) can be conjugated on the surface of nanoparticles to engineer particles with desirable biodistribtion and characteristic. For example, to enhance the rate of circulation within the blood and minimize uptake by non-desired cell types, nanoparticles may be coated with polymers such as PEG(46, 47). Various molecular weights and types of PEG (linear or branched) have been used to coat nanoparticles(84). PEG coatings are also useful for minimizing nanoparticle aggregation which can be used to prevent clogging of small vasculature and improve size-based targeting. More recently, novel approaches aimed at conjugating small molecules on nanoparticles using high-throughput methods have yielded nanoparticle libraries that could be subsequently analyzed for targeted delivery(104). The use of similar high-throughput approaches has significant potential in optimizing nanoparticle properties for cancer therapy.

Surface modification of nanoparticles can be achieved in a multi-step approach by first generating nanoparticles and subsequently modifying the surface of particles to achieve the desired characteristics. Alternatively, amphiphilic polymers may be covalently linked prior to generating nanoparticles to simultaneously control the surface chemistry as well as encapsulate drugs and eliminate the need for subsequent chemical modifications once the particle has been synthesized. This method may provide a more stable coating and better nanoparticle protection in contact with blood. For example, PLA, poly(caprolactone) and poly(cyanoacrylate) polymers, have been chemically conjugated to PEG polymers(8, 47, 73). We have synthesized nanoparticles from amphiphilic copolymers composed of lipophilic (i.e. PLGA) and hydrophilic (i.e. PEG) polymers where the PEG migrates to the surface of the nanoparticles in the presence of an aqueous solution(47). A similar approach has also been used to generate pegylated PLA nanoparticles using PLA-PEG block-copolymers(38, 39). These particles may be

used to extend the nanoparticle residence times in circulation and enhance accumulation in tumor tissue through "passive targeting" and EPR effect.

In the case of engineering nanoparticles for active targeting, the polymer and its coating should have functional groups for the attachment of targeting moieties (which may be bound directly to the nanoparticle surface or though a spacer group). The targeting molecules can enhance the molecular interaction of the nanoparticles with a subset of cells or tissue.

6.   Conjugation of nanoparticles to aptamers

Covalent conjugation of aptamers to substrates or drug delivery vehicles can be achieved most commonly through succinimidyl ester – amine chemistry which results in a stable amide likage(38, 39) or through maleimide – thiol chemistry. Potential non-covalent strategies include affinity interactions (i.e. streptavidan-biotin) and metal coordination (i.e. between polyhistidine tag at the end of the aptamer and Ni^{+2} chelates with immobilized nitrilotriacetic acid on the surface of the polymer particles). These covalent and non-covalent strategies have been used to immobilize a wide range of biomolecules including proteins, enzymes, peptides and nucleic acids to delivery vehicles.

We believe that covalently linked bioconjugates may result in enhanced stability in physiologic salt and pH while avoiding the unnecessary addition of biological components (i.e. streptavidin) thus minimizing immunologic reactions and potential toxicity. For covalent conjugation, the aptamer is typically modified to carry a terminal primary amine or thiol group which is in turn conjugated, respectively, to activated carboxylic acid N-hydroxysuccinimide (NHS) ester or maleimide functional groups present on the surface of drug delivery vehicles. These reactions are carried out under aqueous conditions with a product yield of 80 – 90%(95). One potential difficulty with maleimide – thiol chemistry is the oxidation of the thiol group attached to aptamers during storage (formation of S – S bond between two thiol modified aptamers), resulting in dimers of aptamers which are not able to participate in the conjugation reaction with

the malimide group on particles. This problem can be partially alleviate by using a reducing agent such as Tris (2-Carboxyethyl) Phosphine (TCEP), beta-mercaptoethanol or dithiothreitol (DTT) during the conjugation reaction. Furthermore, a potential advantage of using NHS – amine chemistry is that the unreacted carboxylic acid groups on the particle surface make the particle surface charge (ζ potential) slightly negative thus reducing non-specific interaction between the negatively charged aptamers and the negative particle surface. Recently, controlled release nanoparticles generated from PLA- PEG block copolymer with a terminal carboxylic acid group attached to the PEG were conjugated with primary amine terminated aptamers (38, 39). In this case the hydrophilic PEG group facilitated the presentation of the carboxylic acid on particle surface for conversion to activated carboxylic acid NHS ester and conjugation to the primary amine modified aptamers (Fig 4).

The conjugation of aptamers to nanoparticles can be qualitively confirmed by fluorescent microscopy or flow cytometry through the use of fluorescent probes such as Fluorescein iso-thiocyanate (FITC) that are conjugated directly to the aptamers or indirectly to complemantary oligonucleotides that hybridize to the aptamers(38). Alternatively analytical approaches such as X-ray photoemission (XPS) may be used for characterization of the nanoparticles surface to confirm the extent of conjugation. The presence of a hydrocarbon spacer group between the nanoparticle surface and the aptamer should improve the probability of interaction between the aptamer and its target. Furthermore, a consistent density of the aptamer on the surface of nanoparticles can potentially be achieved through utilizing an excess molar amount of aptamer relative to the reactive group on the nanoparticle surface during the conjugation reactions. However, the optimal density of targeting molecule on nanoparticle surface may need to be experimentally determined(98).

We have used the covalent conjugation approach to demonstrate a proof-of-concept for nanoparticle-aptamer bioconjugates which target the PSMA on the surface of prostate cancer cells and get taken up by cells which express the PSMA protein specifically and efficiently(38). We have also shown using a microfluidic system that

these nanoparticles-aptamer conjugates are capable of binding to their target cells under flow conditions suggestion their suitability for targeted drug delivery applications(39) Most recently we have demonstrated the in vivo efficacy of docetaxel encapsulated nanoparticle-aptamer conjugates using a xenograft prostate cancer nude mouse model (Fig 1B). These approaches have paved the way for future use of aptamers for targeted delivery of drug encapsulated nanoparticles to a myriad of human cancers.

7.   Challenges with Systemic Administration of Targeted Nanoparticles

A problem which needs to be overcome to realize the full potential of targeted cancer drug delivery vehicles after systemic administration is the non-specific uptake of nanoparticles by the mononuclear phagocytic cells present in the liver, spleen, lung and bone marrow(15, 27, 45, 47). This is in part due to the large percentage of cardiac output which is directed to these organs and in part due to the dense population of macrophages and monocytes present in these organs, which engulf these particles through receptor mediated endocytosis and phagocytosis. In addition to their clearance by the phagocytic cells, systemically administered nanoparticles must overcome many additional barriers to reach the tumor and ultimately be capable of delivering therapeutically effective concentrations of the cancer drugs directly to the cancer cells.

The amount of nanoparticle that reaches the tumor is dependent on a variety of factors including those related to the biochemical and physical characteristics of the nanoparticles, such as the chemical properties of the controlled release polymer system and the encapsulated drugs; the size of the particles; surface charge and surface hydrophilicity of nanoparticles; and characteristics of the tumor micoenvironment such as the permeability of the vessel wall, which is determined by the number, size and distribution of transvascular pathways(83). Tumor microvasculature is inhomogenous in nature with areas of tumor necrosis together with areas of high density of aberrant blood vessels. Indeed, compared to normal blood vessels, there is an elevated probability for extravasation of nanoparticles from blood vessels in a tumor, leading to an accumulation, due to the EPR effect. Multiple factors influence the EPR including active

angiogenesis and high vascularity, defective vascular architecture, impaired lymphatic clearance, and extensive production of vascular mediators such as bradykinin, nitric oxide, vascular endothelial growth factor (VEGF), prostaglandins, collagenase, and peroxynitrite(76). The correlation between the size of the nanoparticles and ease of extravasation is function of the pore cutoff size, which is a functional measure of the maximum size of the transvascular transport pathways, and is determined mainly through the size of open interendothelial gap junctions and trans-endothelial channels. The pore cutoff size of these transport pathways has been estimated between 400 – 600 nm and extravasation of liposomes into tumors *in vivo* suggests a cutoff size in the range of 400 nm(107). As a general rule, particle extravasation is inversely proportional to size and small particles (<150 nm size) should be most effective for extravasating the tumor microvasculature(61, 107, 108). The lack of normal functioning lymphatic vessels in the tumor also has broad implications for delivery of nanoparticles. For example, as compared to most normal tissues where extravasated fluid and macromolecules are returned to central circulation by the lymphatics vessels, abnormal lymphatics in tumors can lead to fluid retention(7). The resulting increase in tumoral interstitial fluid pressure as compared to normal tissues may hinder the extravasation of nanoparticles from the microvasculature into the tumor interstitial space. Indeed some of the particles that enter the tumor interstitum through leaky microvasculature and EPR effect may get pushed back into the microvasculature because of the outward fluid pressure within the tumor tissue. Targeted nanoparticles such as nanoparticle-aptamer conjugates tend to accumulate more efficiently within the tumor through the selective binding to receptors on the tumor cells when the particles enter the tumor interstitial space. The combined EPR and active targeting effects may result in a relatively higher intra-tumoral drug concentration over an extended period of time translating into enhanced tumor cytotoxicity.

8. Conclusion

Bioconjugates comprising nanoparticles and aptamers represent a potentially powerful tool for developing novel diagnostic and therapeutic modalities for cancer detection and treatment. As drug delivery vehicles for cancer therapy, nanoparticle-

aptamer bioconjugates can be designed to target and get taken up by cancer cells for targeted delivery and controlled release of chemotherapeutic drugs over an extended time directly at the site of tumor. The successful achievement of this goal requires the isolation of aptamers that bind to the extracellular domain of antigens expressed exclusively or preferentially on the plasma membrane of cancer cells or on the extra-cellular matrices of tumor tissue. In addition, nanoparticles would have to be designed with the optimized properties that facilitate targeting and delivery of the drugs to the desired tissues while avoiding uptake by the mononuclear phagocytic system in the body.

9. Expert Opinion

The targeted delivery of chemotherapeutic drugs for cancer therapy may minimize their side effects and enhance their cytotoxicity to cancer cells resulting in better clinical outcome. We anticipate that the combination of controlled release technology and targeted approaches may represent a viable approach for achieving this goal. One major clinical advantage of targeted drug encapsulated nanoparticle conjugates over drugs that are directly linked to a targeting moiety is that large amounts of chemotherapeutic drug may be delivered to cancer cells per each delivery and bio-recognition event. Another advantage would be the ability to simultaneously deliver two or more chemotherapeutic drugs and release each in a predetermined manner thus resulting in effective combination chemotherapy which is common for the management of many cancers. Antibodies and peptides have been widely used for the targeted delivery of drug encapsulated nanoparticles; however, the translation of these vehicles into clinical practice has lagged behind our advances in the laboratory. This is in large part due to the non-specific uptake of nanoparticle-antibody bioconjugates by non-targeted cells and tissues resulting in toxicity or poor efficacy. Nanoparticle-aptamer bioconjugates represent a novel approach for facilitating the delivery of nanoparticles to the target cell. The advantage of these bioconjugates lies largely in the ease of aptamer synthesis and development which can facilitate their translation into clinical practice. Nanoparticle-aptamer bioconjugates however, face the same challenge of non-specific uptake after systemic administration and thus must be engineered with surface

physiochemical characteristics to avoid toxicity to non-targeted cells. We believe that optimal particle size and surface properties to sufficiently decrease the rate of non-specific particle uptake while achieving successful targeting must be determined experimentally on case by case basis, as this also depends on the polymer system, the drug being encapsulated and the tumor microenvironment including its vascularity.

We have demonstrated the proof-of-concept nanoparticle-aptamer bioconjugates and believe that when appropriately optimized, these vehicles may be widely utilized for targeted drug delivery and treatment of a myriad of cancers. By addressing the challenges outlined in this article the promise of nanotechnology-based cancer therapies may be realized.

10. Acknowledgements

Table 1: Aptamers for Targeting Cancer

| Aptamer	Specific Target	Application	Ref.
A30	Human epidermal growth factor receptor-3 (HER-3)	Binds to the cancer cell surface	(21)
A9, A10	Prostate-Specific Membrane Antigen (PSMA)		(74)
AS-1411	Nucleolin		(6)
Clone 5	Sialyl Lewis X		(59)
CTLA-4 aptamer	Cytotoxic T cell antigen-4 (CTLA-4)	Binds to T cells	(93)
TTA1	Fibrinogen-like domain of Tenascin-C	Binds to extracellular matrix proteins	(53, 94)
PDGF-r aptamer	Platelet derived growth factor receptor (PDGF-r)	Binds to microvasculature	(88, 89)
III.1	Pigpen		(12)
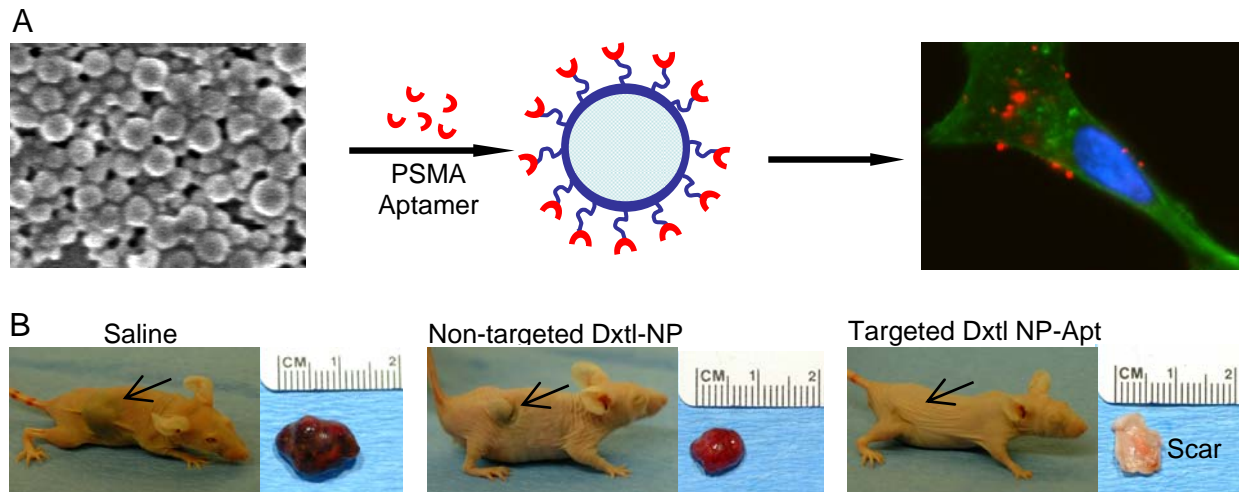
Figrue 1. Development and evaluation of nanoparticle-aptamer bioconjugates. **A.** Rohadmine-labeled dextran was encapsulated within pegylated PLA nanoparticles and these were conjugated to the A10 RNA aptamer (74) that recognizes the Prostate Specific Membrane Antigen (PSMA) on the surface of prostate cancer cells. These nanoparticle-aptamer bioconjugates were shown to effectively bind and get taken up by LNCaP prostate epithelial cells which express the PSMA antigen on their plasma membrane (38). The actin cytoskelatal is labeled green with Alexa-Flour Phalloidin and the nucleus is labeled blue with Dapi. **B.** Using the A10 PSMA aptamer and a similar conjugation approach, docetaxel encapsulated pegylated PLGA nanoparticle-aptamer bioconjugates were developed and shown to be remarkably efficacious in tumor reduction studies using LNCaP xenograft nude mice models of prostate cancer. In these studies mouse were implanted in their flank with LNCaP epithelial cells and the tumors were allowed to develop to ~300 mm^3, at which point 7 animals per group were injected intra-tumorally with placebo (saline; left panel), docetaxel encapsulated nanoparticles without aptamer (non-targeted Dxtl-NP; middle panel), or similar nanoparticles with PSMA aptamer (targeted Dxtl NP-Apt, right panel). The image of the median mice and the respective image of the excised tumor in each group are shown at the study end point (day 109 or tumor size of 800 mm^3). In the case of the targeted nanoparticle-aptamer bioconjugates the tumor was eliminated, and the mage represents skin, subcutaneous fat, and scar tissue as determined by histological evaluation.
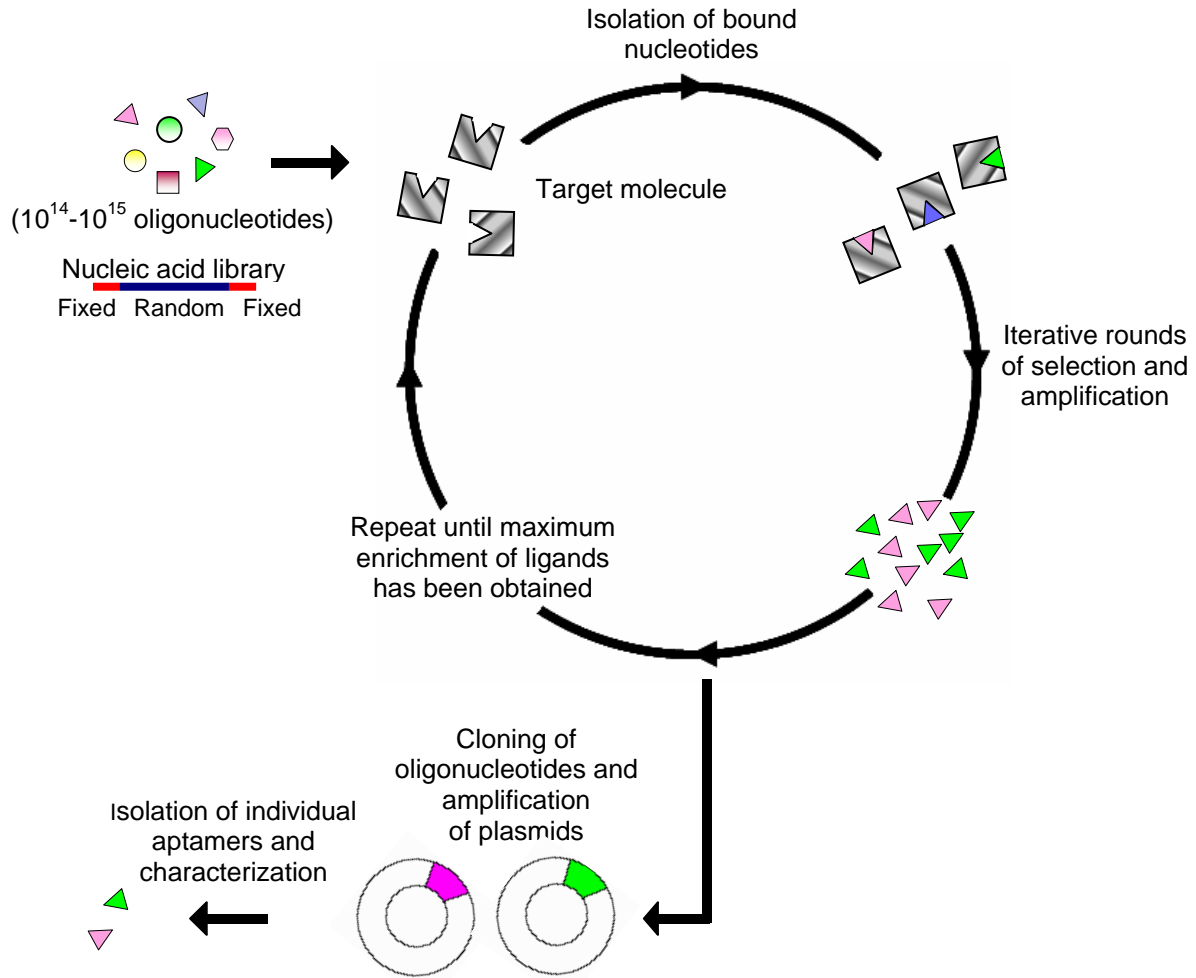
Figure 2: Schematic representation of SELEX. An oligonucleotide library is synthesized containing random sequences that are flanked by fixed sequences which facilitate PCR amplification. Target molecules are incubated with this pool of oligonucleotides and bound and unbound oligonucleotides are partitioned. Bound oligonucleotides are isolated and iterative rounds of selection and amplification are performed with increased stringency to isolate aptamers with high specificity and affinity for the target molecule. Oligonucleotide ligands representing the aptamers are subsequently cloned in plasmids, amplified, and sequenced. The net result of this enrichment process is a small number of highly specific aptamers that are isolated from a large library of random oligonucleotides.
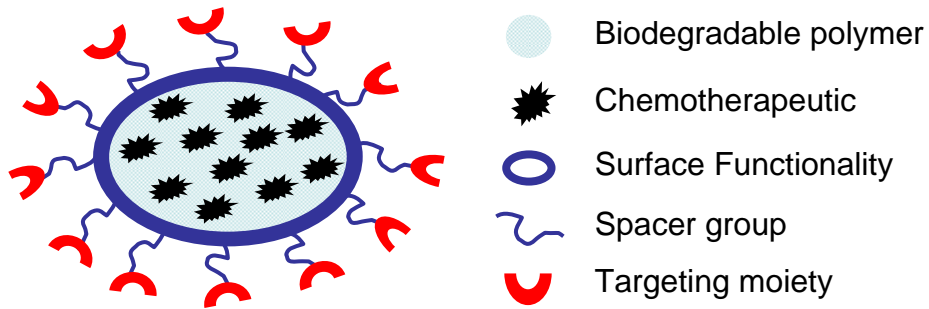
Figure 3: Schematic representation of targeted drug delivery vehicle composed of polymeric nanoparticles that are surface modified with targeting agents.
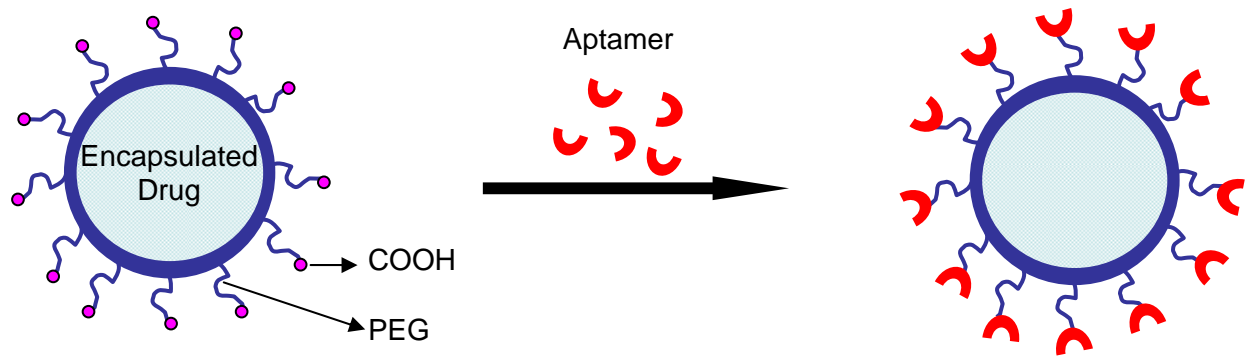
Figure 4: A schematic outlining a conjugation reaction between aptamers and polymer nanoparticles containing encapsulated drug. Through incorporating a COOH terminated PEG functionalized surface on the nanoparticle, NH_2 modified aptamers can be easily conjugated using simple aqueous chemistry.

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