Expert Opinion

Nanoparticle–aptamer bioconjugates for cancer targeting

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The combination of targeted drug delivery and controlled-release technology may pave the road for 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 tumours. Aptamers are short nucleic acid molecules with binding properties and biochemical characteristics that may make them suitable for use as targeting molecules. The goal of this review is to summarise the key components that are required for creating effective cancer targeting nanoparticle–aptamer bioconjugates. The field of controlled release and the structure and properties of aptamers, as well as the criteria for constructing effective conjugates, will be discussed.

Keywords: aptamer, cancer therapy, controlled release, *in vitro* selection, nanoparticles, SELEX, targeted drug delivery

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1. Introduction

With the advances in nanotechnology, it is now possible to combine specialised delivery vehicles and targeting approaches to develop highly selective and effective therapeutic and diagnostic modalities that may improve the outcome for a myriad of important diseases, including cancers [1,2]. Cancer-specific drug delivery may be achieved by both local and systemic administration of specially designed vehicles. These vehicles can be engineered to recognise biophysical characteristics that are unique to the cancer cells. Most commonly, this represents the 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 generalised effect on all of the cells of the targeted organ. In the case of cancer, the cytotoxic effects of a therapeutic agent would be directed to cancer cells whilst minimising harm to non-cancerous cells within and outside 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 [3]. This approach is particularly useful for primary tumours that have not yet metastasised, such as localised brain or prostate cancer. For metastatic cancer, the vehicle would ideally be delivered systemically as the location, abundance and size of tumour metastasis within the body limits its visualisation or accessibility, thus making local delivery approaches impractical.

Several classes of molecules have been used for targeting applications, including various forms of antibody-based molecules, such as chimeric human-murine antibodies, humanised 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 small molecules, carbohydrates and

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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 1980s 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 [4]. 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 [5,6].

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 targeting, in particular as it relates to the treatment of oncological diseases. The first monoclonal antibody to get FDA approval for the treatment of cancer was in 1997 when rituximab was approved for treating patients with relapsed or refractory low-grade or follicular, $CD20^+$, B-cell non-Hodgkin's lymphoma [7]. A wide variety of antibody-based drugs are now under clinical development or in clinical practice today. For example, denileukin diftitox is an FDA-approved immunotoxin for the treatment of cutaneous T-cell lymphoma [8]. Many other radioimmunoconjugates or chemoimmunoconjugates that are directed against cell-surface antigens are currently in various stages of clinical and preclinical 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 that is 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) [9,10]. 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, as with monoclonal antibodies, bind with high affinity and specificity to a target antigen, but overcome or ameliorate some of the problems that are associated with the use and production of monoclonal antibodies. Aptamers are a novel class of ligand [5,6] that are small, non-immunogenic, are easy to synthesise, characterise and modify, and exhibit a high specificity and affinity for their target antigen. In the short time since Szostak and Gold independently described the ground-breaking methodology for *in vitro* selection of aptamers, these ligands have emerged as an important class of molecules for therapeutic and diagnostic applications [11,12].

Aptamers are oligonucleotides that can bind to target antigens with a high affinity and specificity. Considering the many favourable characteristics of aptamers, which have resulted in their rapid progress into clinical practice, Farokhzad, Langer and colleagues have begun to exploit this class of molecules for targeted delivery of controlled-release polymer drug delivery vehicles. Recently, they described the first proof-of-concept drug delivery vehicles using aptamers for targeted delivery (Figure 1A) [13] and have gone on to show efficacy of these vehicles in tumour reduction *in vivo* (Figure 1B) [14].

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 $\sim 10^{14} - 10^{15}$ oligonucleotides for their ability to bind to a target molecule (this procedure is referred to as systemic evolution of ligands by exponential enrichment [SELEX]). Aptamers have a molecular weight (10 – 15 kDa) that is one order of magnitude lower than that of antibodies (150 kDa) [15] and derive their name from the Latin word 'aptus' meaning 'to fit'. Aptamers fold through intramolecular interactions 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 synthesised; a significant advantage for commercialising this class of molecule for drug development. Furthermore, due to their small size and similarity to endogenous molecules, aptamers exhibit superior tissue penetration [12] and are believed to be less immunogenic than antibodies [16]. They may be circularised, linked together in pairs or clustered onto a substrate, and aptamers against any target may be isolated, provided that a small quantity of the 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 hybridising to the mRNAs that encodes it, aptamers may inhibit a function of the protein through directly binding to it. Aptamers typically bind with an equilibrium dissociation constant (K_d) in the range of 10 pM – 10 µM [17] to a wide array of molecular targets [18] including other nucleic acids, proteins, peptides and small molecules. They can be described by a sequence of ~ 15 – 60 nucleotides (A, U, T, C and G). The conformation of the aptamer confers specificity for a target molecule through interaction 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 from theophylline only by the substitution of a methyl group at the nitrogen

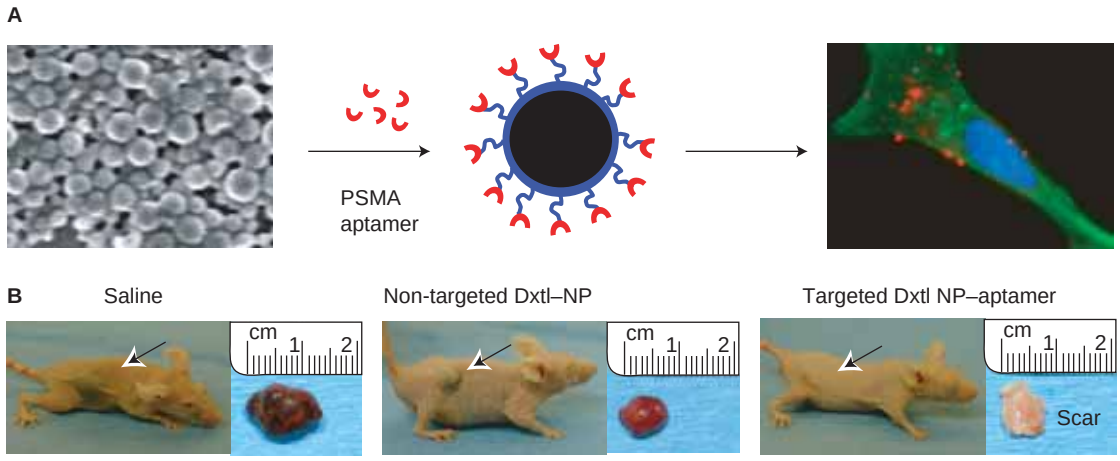

Figure 1. Development and evaluation of NP–aptamer bioconjugates. A) Rhodamine-labelled dextran was encapsulated within pegylated poly(lactic acid) NPs and these were conjugated to the A10 RNA aptamer [37] that recognises the PSMA on the surface of prostate cancer cells. These NP–aptamer bioconjugates were shown to effectively bind and get taken up by LNCaP prostate epithelial cells that express the PSMA antigen on their plasma membrane [13]. The actin cytoskeleton is labelled green with Alexa-Fluor Phalloidin and the nucleus is labelled blue with DAPI. **B)** Using the A10 PSMA aptamer and a similar conjugation approach, docetaxel-encapsulated pegylated PLGA NP–aptamer bioconjugates were developed and shown to be remarkably efficacious in tumour-reduction studies using LNCaP xenograft nude mice models of prostate cancer. In these studies, LNCaP prostate epithelial cells were implanted in the flanks of mice and the tumours were allowed to develop to ~ 300 mm^3, at which point seven animals per group were injected intratumourally with placebo (saline; left panel), Dtxl-encapsulated NPs without aptamer (non-targeted Dxtl–NP; middle panel), or similar NPs with PSMA aptamer (targeted Dxtl NP–aptamer, right panel). The image of the representative mice and the excised tumours from each mouse is shown at the study end point (day 109 or tumour size of 800 mm^3). In the case of the targeted NP–aptamer bioconjugates, the tumour was eliminated and the image represents skin, subcutaneous fat and scar tissue as determined by histological evaluation.
Dtxl: Docetaxel; NP: Nanoparticle; PSMA: Prostate-specific membrane antigen.

atom N7 position [19]. Based on their unique molecular recognition properties, aptamers have found great use for applications in areas such as *in vitro* and *in vivo* diagnostics, analytical techniques, imaging and therapeutics [15,20,21].

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* application. Several approaches have been adopted to optimise the properties of aptamers, including:

- capping their terminal ends,
- substituting naturally occurring nucleotides with unnatural nucleotides that are poor substrates for nuclease degradation (i.e., 2′-fluoropyrimidine, 2′-OCH_3 or 2′-NH_2 modified nucleotides),
- substituting naturally occurring nucleotides with hydrocarbon linkers,
- use of L-enantiomers of nucleotides to generate mirror-image aptamers, commonly referred to as spiegelmers [22-25].

Aptamers can also be stabilised by using locked nucleic acid modifications to reduce conformational flexibility [26]. Alternatively, a nuclease-resistant aptamer may be selected *de novo* using a pool of oligonucleotides with 2′-fluoropyrimidine or 2′-OCH_3 modified nucleotides. Through combining some of these strategies, the half-life of the aptamer can be prolonged from several minutes to many hours [15]. To prolong the rate of clearance of aptamers, their size may be increased by conjugation with polymers such as PEG [27,28].

The conjugation of aptamers to drug-encapsulated nanoparticles results in targeted-delivery vehicles for therapeutic application. These may include the delivery of small-molecule drugs, protein-based drugs, nucleic acid therapy (antisense oligonucleotide, RNA interference or gene therapy) and the targeted delivery of agents for neutron-capture therapy or photodynamic therapy. Aptamers may also be bound to imaging agents to facilitate the diagnosis and identification of tumour metastases. For example, it may be useful to bind aptamers to optical imaging agents including fluorophores [29] and quantum dots (nanocrystals) [30], or MRI agents such as magnetic nanoparticles [31,32] for the detection of small foci of cancer metastasis. Additional imaging agents that may make useful conjugates are reviewed elsewhere [33]. Multiplex systems that comprise of drug-laden nanoparticle–aptamer bioconjugates together with imaging agents represent a prospective avenue for 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.

Table 1. Aptamers for targeting cancer.

Aptamer	Specific target	Application	Ref.
A30	Human epidermal growth factor receptor-3	Binds to the cancer cell surface	[35]
A9, A10	Prostate-specific membrane antigen	Binds to the cancer cell surface	[37]
AS-1411	Nucleolin	Binds to the cancer cell surface	[40]
Clone 5	Sialyl Lewis X	Binds to the cancer cell surface	[43]
CTLA-4 aptamer	Cytotoxic T cell antigen-4	Binds to T cells	[44]
TTA1	Fibrinogen-like domain of tenascin-C	Binds to extracellular matrix proteins	[45]
PDGF-r aptamer	Platelet derived growth factor receptor	Binds to microvasculature	[46,47]
III.1	Pigpen	Binds to microvasculature	[48]

Alternatively, they may be delivered to extracellular matrix molecules that are expressed preferentially in tumours. So far, many aptamers have been isolated that bind specifically to receptors on cancer cells and these are outlined in Table 1 [34].

3.1 Listing of aptamers for targeted delivery

3.1.1 Human epidermal growth factor-3

Human epidermal growth factor (HER)-3 is a receptor tyrosine kinase that is overexpressed in several cancers. Overexpression of HER-3 is also associated with drug resistance in many HER-2 overexpressed tumours, making it a candidate target for drug delivery. A panel of RNA aptamers against the extracellular domain of HER-3 has been isolated and one, the A30 aptamer, can inhibit heregulin-dependent tyrosine phosphorylation of HER-2 and heregulin-induced growth response of MCF-7 cells at inhibition constants (K_i) of 10 and 1 nM, respectively [35]. The A30 aptamer is comprised of natural nucleotides and is thus susceptible to nuclease degradation. The above studies were carried out in the presence of Rnase inhibitors. The future use of A30 for *in vivo* application may require optimisation of this aptamer, including nuclease stabilisation and size minimisation.

3.1.2 Prostate-specific membrane antigen

Prostate-specific membrane antigen (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, which is of particular importance as its expression is tightly restricted to prostate acinar epithelium and its expression is increased in prostatic intraepithelial neoplasia, prostatic adenocarcinoma and in tumour-associated neovasculature. An immunoconjuagate of the J591 antibody that binds to the extracellular domain of the PSMA is currently in Phase I clinical trials [36]. Two 2′-fluoropyrimidine RNA aptamers against the extracellular domain of the PSMA were recently described [37]. The aptamer xPSM-A9 inhibits the enzymatic function of the PSMA non-competitively with a K_i of 2.1 nM and the aptamer xPSM-A10 inhibits the enzymatic function of PSMA competitively with a K_i of 11.9 nM. The aptamer xPSM-A10 has also been truncated from 71 nucleotides to its current size of 56 nucleotides (18 kDa). Farokhzad and colleagues recently used the xPSM-A10 aptamer to develop nanoparticle–aptamer bioconjugates for prostate cancer targeting and demonstrated that these bioconjugates preferentially bind to and are 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 of the PSMA protein [13].

3.1.3 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 [38,39]. Nucleolin has a multi-domain structure, which reflects its remarkably diverse functions. It is involved in the organisation of the nuclear chromatin, recombinant DNA 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 AGRO-100) is an aptamer that is capable of making G-quadruplexes that bind to nucleolin on the cell surface [40] and interact with the NFκB essential modulator inside the cell [41]. The cytosolic localisation 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 the company Antisoma is evaluating this aptamer in Phase I clinical trials [42]. The therapeutic benefit of AS-1411 is presumably attributed to the disruption of the NFκB signalling inside the cells.

3.1.4 Sialyl Lewis X

Sialyl Lewis X (sLe^x) is a tetrasaccharide glycoconjugate of transmembrane proteins that acts as a ligand for the selectin proteins during cell adhesion and inflammation. sLe^x is also abnormally 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 the clone 5 RNA aptamer was shown to have subnanomolar affinity of the sLe^x that was

capable of blocking the sLe^x/selectin-mediated cell adhesion of HL60 cells *in vitro* [43]. Considering the high level of sLe^x expression on the surface of cancer cells, it may be possible to use the clone 5 RNA aptamer for targeted nanoparticle delivery. The future use of clone 5 for *in vivo* applications will require post-SELEX optimisation of this aptamer, including nuclease stabilisation and size minimisation.

3.1.5 Cytotoxic T-cell antigen-4

Cytotoxic T-cell antigen-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 that is needed for T-cell activation. The *in vitro* selection against CTLA-4 resulted in isolation of six distinct 2′-flouropyrimidine RNA aptamers [44]. The most potent inhibitory aptamer, M9-9 (K_d = 10 nM), was truncated from its original length of 79 base pairs to 35 base pairs. This resulted in the aptamer D60 with a K_d of 33 nM for CTLA-4, which was shown to inhibit CTLA-4 function *in vitro* and enhance tumour immunity in mice.

3.1.6 Fibrinogen-like domain of tenascin-C

Tenascin-C is an extracellular matrix protein that is overexpressed during tissue-remodelling processes, such as foetal development and wound healing, as well as tumour growth. Due to its high expression in tumours, high-affinity tenascin-C ligands may be clinically useful tumour-targeting agents. TTA1 is an aptamer that has been generated against the fibrinogen-like domain of tenascin-C [45]. It has an equilibrium K_d of 5 nM. Thus, TTA1 is a potentially interesting target for various cancer diagnostic and therapeutic applications.

3.1.7 Platelet-derived growth factor receptor

Platelet-derived growth factor (PDGF) receptor is a tyrosine kinase that is a mediator of tumour hypertension. It is believed that lowering of the tumour interstitial hypertension, which acts as a barrier for tumour transvascular transport, is a potential strategy to enhance tumour uptake and therapeutic effects of anticancer drugs. Therefore, PDGF antagonists can be used to relieve tumour hypertension. For example, inhibitory PDGF aptamers have been shown to enhance the antitumour effect of paclitaxel in severe combined immunodeficient mice [46,47]. The use of this approach along with standard chemotherapy may be a potential mechanism of using aptamers for enhancing the effects of chemotherapeutic drugs.

3.1.8 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 microglial cells were used, respectively, in a selection and counter-selection in SELEX to isolate the III.1 DNA aptamer that preferentially binds to YPEN-1 cells [48]. The III.1 aptamer was also shown to selectively bind to the microvessels of experimental rat glioblastoma using histological specimens. The isolation and characterisation of the III.1 target identified pigpen as the target antigen. The use of the III.1 aptamer for targeting the microvasculature of tumours 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 [49] that is called *in vitro* selection [5] or SELEX [6] (Figure 2). Similar to phage display or other strategies that are 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 nM 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 hybridisation in a PCR. 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 plateau. 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 minimisation to truncate the nucleotides that are not necessary for binding characteristics, and nuclease stabilisation by replacing naturally occurring nucleotides with modified nucleotides (i.e., 2′-fluoropyrimidine, 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 [49]. The advantage of RNA SELEX, however, is that unnatural nucleotides such as 2′-fluoropyrimidine and 2′-OCH_3 nucleotides may be used in the transcription of the RNA pool, as these modified bases are used by RNA polymerase as a substrate. Furthermore, mutant RNA polymerases have also been described that are capable of improved incorporation of modified bases during transcription [50]. The resulting modified RNA pool can be used for the isolation of nuclease stable RNA aptamers. Recently, a fully 2′-OCH_3-modified VEGF aptamer was selected and when conjugated to 40-kDa PEG demonstrated a circulating half-life

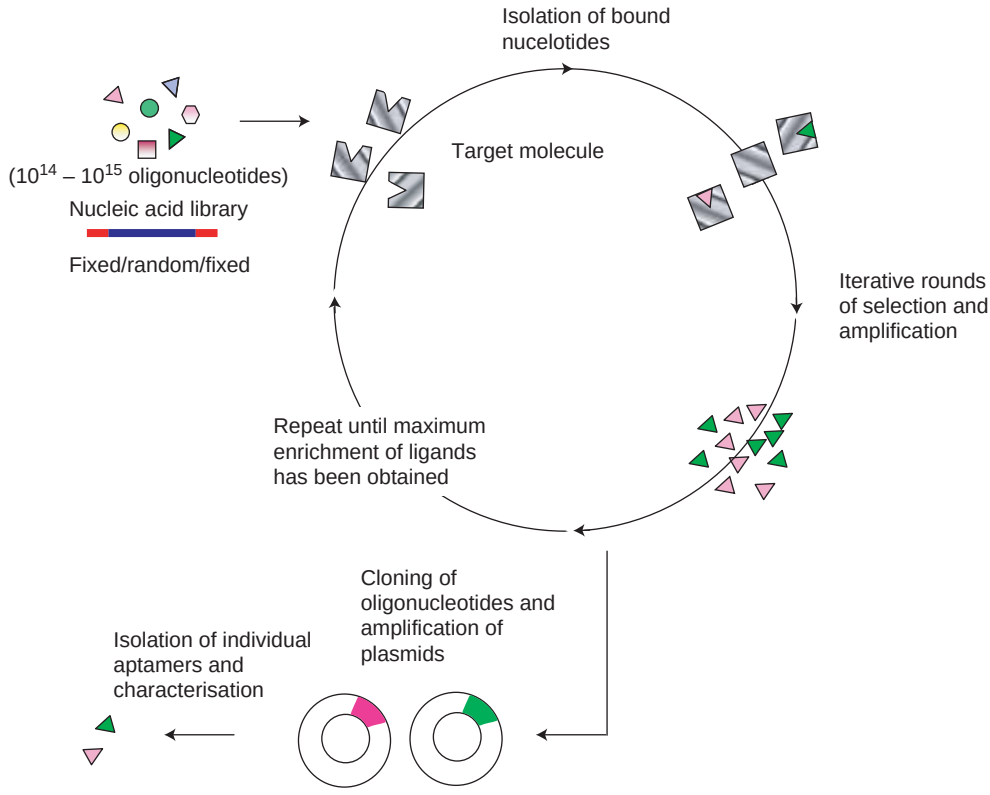

Figure 2. A schematic representation of systemic evolution of ligands by exponential enrichment. An oligonucleotide library is synthesised that contains random sequences that are flanked by fixed sequences, which facilitate polymerase chain reaction amplification. Target molecules are incubated with 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, which are isolated from a large library of random oligonucleotides.

of 23 h [51]. Conversely, a DNA polymerase that can incorporate unnatural bases such as 2′-fluoropyrimidine and 2′-OCH_3 has not been described and, consequently, DNA aptamers must be nuclease stabilised after the SELEX procedure.

5. Development of nanoparticles for conjugation to aptamers

During the past four decades [52-56], controlled drug delivery strategies have dramatically impacted nearly every branch of medicine, including: cardiology [57], ophthalmology [58], endocrinology [59], oncology [60], immunology [61] and orthopaedics [62]. 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 [2] such as changes in pH [63], light [64], temperature [65] or the presence of an analyte such as glucose [66]. In general, controlled-release polymer systems deliver drugs in the optimum dosage for long periods, thus increasing the efficacy of the drug, maximising 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 as they can be synthesised to recognise tumour-specific antigens and deliver drugs in a controlled manner [1,13]. The design of targeted drug delivery nanoparticles combines drug-encapsulated materials, such as biodegradable polymers, with a targeting moiety (Figure 3). Ideally, biodegradable nanoparticles should be designed with the following parameters [67]:

- small size (preferably between 50 – 150 nm)
- high drug loading and encapsulation efficiency
- low rate of aggregation
- slow rate of clearance from the bloodstream
- optimised targeting to the desired tissue with minimised uptake by other tissues.

The following sections will discuss the various parameters that must be considered for engineering nanoparticles for targeted drug delivery applications, including the development of

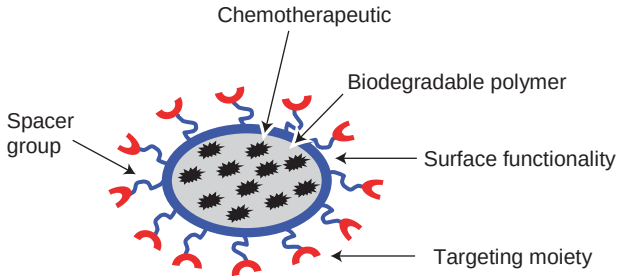

Figure 3. A schematic representation of a targeted drug delivery vehicle, which is composed of polymeric nanoparticles that are surface modified with targeting agents.

nanoparticle–aptamer bioconjugates. This will include the 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 [68-72].

5.1 Size of nanoparticles

The biodistribution and tumour targeting of nanoparticles (active nanoparticle targeting to tumour antigens and passive nanoparticle targeting by enhanced permeation and retention [EPR] [73]) are greatly affected by the size of the nanoparticle. Passive nanoparticle targeting occurs because the microvasculature of tumours are more 'leaky', thus permitting selective permeation of nanoparticles into the tumour tissue. This phenomenon has been exploited to target various forms of nanoparticles, quantum dots, liposomes and drug–ligand conjugates to cancer tissue for therapeutic and diagnostic applications (reviewed by Maeda [73]). The EPR phenomenon is greatly dependent on the size of the nanoparticle. Although larger particles are more effectively taken up by macrophages, smaller particles are better suited for permeating through the leaky microvasculature of the tumour 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 [74]. Biodistribution studies using liposomes have shown that although particles that are > 200 nm are largely taken up by the spleen, those that are < 70 nm are also efficiently cleared by the liver [75]. Taken together, the optimal nanoparticle size should be experimentally determined for each formulation, as 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. The authors' biodistribution studies using various sizes of poly(lactic-co-glycolic acid) (PLGA)-PEG nanoparticle–aptamer bioconjugates has suggested a linear relationship with regards to uptake by the liver and spleen, such that smaller particles (~ 80 nm) are better at avoiding uptake by these organs (B Teply, J Cheng, R Langer and O Farokhzad, unpublished observations).

5.2 Polymers for synthesis of nanoparticles

Controlled-release biodegradable nanoparticles for drug delivery applications can be made from a wide variety of polymers including poly(lactic acid) (PLA) [76], poly(glycolic acid) (PGA), PLGA [77], polyorthoesters [78], polycaprolactone [79], polybutyl cyanoacrylate [80], polyanhydrides [81] and poly(*N*-isopropylacrylamide) [82]. Although many fabrication methods exist, drug-encapsulated polymeric nanoparticles are frequently made using an oil-in-water emulsion (single emulsion) [83], 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 is evaporated, which forces the polymer to precipitate as nanoparticles in the aqueous phase. The particles are then recovered by centrifugation and lyophilisation. Other common methods of developing nanoparticles are w/o/w emulsion (double emulsion) [84] and nanoprecipitation [85,86].

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 Langer and colleagues, as well as 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 and clinical applications [87,88]. PGA and PLA are common biocompatible polymers that are used for many biomedical applications. PGA is more hydrophilic as 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 [89]. 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.

5.3 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 the particle charge may impact particle opsonisation and phagocytosis [90]. Cationic charge on liposomes has also been shown to reduce their circulating half-life in the blood and to affect their biodistribution between the tumour microvasculature and interstitium, without impacting overall tumour uptake [91]. Nanoparticles could be synthesised 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 the surface properties of nanoparticles, such as quantum dots, by depositing ionic polymers of interest on the charged nanoparticle surfaces [92]. Furthermore, the 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 whilst increasing their uptake by the liver. This results in an increase in the accumulation of liposomes in the tumour vasculature [91]. These experiments suggest that optimising surface physicochemical properties of nanoparticles to better match the biochemical and physiological features of tumours may enhance the intratumoural 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, Farokhzad and colleagues believe that direct immobilisation of aptamers on cationic nanoparticles made from PEI may result in the formation of an aptamer–PEI complex that renders the aptamer ineffective as a targeting molecule (O Farokhzad, S Jon and R Langer, unpublished observation). Therefore, neutral polymers such as PLA, PLGA or those with a more negative charge such as polyanhyrides may be the most suitable for conjugation to aptamers. PLA–PEG block copolymers have been used to generate aptamer–nanoparticles bioconjugates [13,93]. 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 a 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.

5.4 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 as intravenously applied nanoparticles may get captured by macrophages before ever reaching the target site. Therefore, by modifying the surface of the particles to render them 'invisible' to macrophages is essential to making long-circulating nanoparticles [67,94]. The ability to control the biodistribution of nanoparticles is particularly important for drug-carrying nanoparticles, as the delivery of drugs to the normal tissues can lead to toxicity [95,96].

Hydrophilic polymers such as PEG [67,94], polysaccharides [97,98] and small molecules [99] can be conjugated on the surface of nanoparticles to engineer particles with desirable biodistribution and characteristics. For example, to enhance the rate of circulation within the blood and to minimise uptake by non-desired cell types, nanoparticles may be coated with polymers such as PEG [67,94]. Various molecular weights and types of PEG (linear or branched) have been used to coat nanoparticles [100]. PEG coatings are also useful for minimising nanoparticle aggregation, which can be used to prevent the clogging of small vasculature and to improve size-based targeting. More recently, novel approaches that are aimed at conjugating small molecules on nanoparticles using high-throughput methods have yielded nanoparticle libraries that could be subsequently analysed for targeted delivery [99]. The use of similar high-throughput approaches has significant potential in optimising nanoparticle properties for cancer therapy.

Surface modification of nanoparticles can be achieved in a multistep 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 encapsulating drugs and eliminating the need for subsequent chemical modifications once the particle has been synthesised. This method may provide a more stable coating and better nanoparticle protection in contact with the blood. For example, PLA, polycaprolactone and polycyanoacrylate polymers have been chemically conjugated to PEG polymers [67,101,102]. Gref and colleagues have synthesised nanoparticles from amphiphilic copolymers that are 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 [67]. A similar approach has also been used to generate pegylated PLA nanoparticles using PLA–PEG block-copolymers [13,93]. These particles may be used to extend the nanoparticle residence times in the circulation and enhance accumulation in tumour 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 that results in a stable amide linkage [13,93] or through maleimide thiol chemistry. Potential non-covalent strategies include affinity interactions (i.e., streptavidin–biotin) and metal coordination (i.e., between polyhistidine tag at the end of the aptamer and Ni^{+2} chelates with immobilised nitrilotriacetic acid on the surface of the polymer particles). These covalent and non-covalent strategies have been used to immobilise a wide range of biomolecules, including proteins, enzymes, peptides and nucleic acids to delivery vehicles.

Farokhzad and colleagues believe that covalently linked bioconjugates may result in enhanced stability in physiological salt

and pH whilst avoiding the unnecessary addition of biological components (i.e., streptavidin); thus minimising immunological 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 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% [103]. One potential difficulty with maleimide thiol chemistry is the oxidation of the thiol group that is attached to aptamers during storage (formation of S-S bond between two thiol modified aptamers), resulting in dimers of aptamers that are not able to participate in the conjugation reaction with the malimide group on particles. This problem can be partially alleviated by using a reducing agent such as Tris (2-carboxyethyl) phosphine, β-mercaptoethanol or dithiothreitol during the conjugation reaction. Furthermore, a potential advantage of using *N*-hydroxysuccinimide amine chemistry is that the unreacted carboxylic acid groups on the particle surface make the particle surface charge (zeta potential) slightly negative, thus reducing nonspecific interactions 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 [13,93]. In this case, the hydrophilic PEG group facilitated the presentation of the carboxylic acid on the particle surface for conversion to activated carboxylic acid *N*-hydroxysuccinimide ester and conjugation to the primary amine-modified aptamers (Figure 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 isothiocyanate, which are conjugated directly to the aptamers or indirectly to complementary oligonucleotides that hybridise to the aptamers [13]. Alternatively, analytical approaches such as X-ray photoemission may be used for characterisation of the nanoparticle 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 by using an excess molar amount of aptamer relative to the reactive group on the nanoparticle surface during the conjugation reactions. However, the optimal density of the targeting molecule on the nanoparticle surface may need to be experimentally determined [104].

The covalent conjugation approach has been used 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 that express the PSMA protein specifically and efficiently [13]. The use of a microfluidic system has also demonstrated that these nanoparticles–aptamer bioconjugates are capable of binding to their target cells under flow

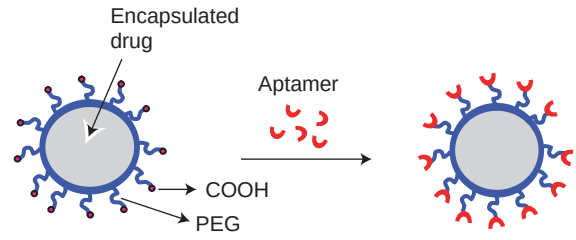

Figure 4. A schematic outlining a conjugation reaction between aptamers and polymer nanoparticles containing encapsulated drug. Through incorporating a carboxyl-terminated PEG functionalised surface on the nanoparticle, amide-modified aptamers can be easily conjugated using simple aqueous chemistry.
PEG: Poly(ethylene glycol).

conditions, suggesting their suitability for targeted drug delivery applications [93]. Most recently, the *in vivo* efficacy of docetaxel-encapsulated nanoparticle–aptamer bioconjugates using a xenograft prostate cancer nude-mouse model has been demonstrated (Figure 1B) [14]. These approaches have paved the way for the future use of aptamers for the targeted delivery of drug-encapsulated nanoparticles to a myriad of human cancers.

7. Challenges with systemic administration of targeted nanoparticles

A problem that needs to be overcome to realise the full potential of targeted cancer drug delivery vehicles after systemic administration is the nonspecific uptake of nanoparticles by the mononuclear phagocytic cells that are present in the liver, spleen, lungs and bone marrow [67,95,96,105]. This is due to the large percentage of cardiac output that is directed to these organs and 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 tumour and ultimately be capable of delivering therapeutically effective concentrations of the cancer drugs directly to the cancer cells.

The amount of the nanoparticle that reaches the tumour is dependent on a variety of factors, including those that are related to the biochemical and physical characteristics of the nanoparticles. These include the chemical properties of the controlled-release polymer system and the encapsulated drugs; the size of the particles; the surface charge and surface hydrophilicity of the nanoparticles; and the characteristics of the tumour microenvironment, such as the permeability of the vessel wall (which is determined by the number, size and distribution of transvascular pathways [106]). Tumour microvasculature is inhomogeneous in nature with areas of tumour necrosis, together with areas of high density of aberrant blood vessels. Indeed, compared with normal blood vessels, there is an elevated probability for extravasation of nanoparticles from blood vessels

in a tumour, 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, VEGF, prostaglandins, collagenase and peroxynitrite [107]. The correlation between the size of the nanoparticles and ease of extravasation is the function of the pore cut-off 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 cut-off size of these transport pathways has been estimated to be between 400 and 600 nm, and extravasation of liposomes into tumours *in vivo* suggests a cut-off size in the range of 400 nm [108]. As a general rule, particle extravasation is inversely proportional to size and small particles (< 150 nm size) should be the most effective for extravasating the tumour microvasculature [108-110]. The lack of normal functioning lymphatic vessels in the tumour also has broad implications for the delivery of nanoparticles. For example, as compared with most normal tissues where extravasated fluid and macromolecules are returned to the central circulation by the lymphatic vessels, abnormal lymphatics in tumours can lead to fluid retention [111]. The resulting increase in tumoural interstitial fluid pressure as compared with normal tissues may hinder the extravasation of nanoparticles from the microvasculature into the tumour interstitial space. Indeed, some of the particles that enter the tumour interstitum through leaky microvasculature and EPR effect may get pushed back into the microvasculature because of the outward fluid pressure within the tumour tissue. Targeted nanoparticles such as nanoparticle–aptamer bioconjugates tend to accumulate more efficiently within the tumour through the selective binding to receptors on the tumour cells when the particles enter the tumour interstitial space. The combined EPR and active targeting effects may result in a relatively higher intratumoural drug concentration over an extended period of time, translating into enhanced tumour cytotoxicity.

8. Conclusion

Bioconjugates that comprise of 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 tumour. The successful achievement of this goal requires the isolation of aptamers that bind to the extracellular domain of antigens that are expressed exclusively or preferentially on the plasma membrane of cancer cells or on the extracellular matrices of tumour tissue. In addition, nanoparticles would have to be designed with the optimised properties that facilitate targeting and delivery of the drugs to the desired tissues, whilst avoiding uptake by the mononuclear phagocytic system in the body.

9. Expert opinion

The targeted delivery of chemotherapeutic drugs for cancer therapy may minimise their side effects and enhance their cytotoxicity to cancer cells, resulting in better clinical outcome. Farokhzad and colleagues 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 delivery and biorecognition 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 advances in the laboratory. This is partly due to the nonspecific 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 nonspecific uptake after systemic administration and thus must be engineered with surface physiochemical characteristics to avoid toxicity to non-targeted cells. The authors of this review believe that optimal particle size and surface properties to sufficiently decrease the rate of nonspecific particle uptake whilst achieving successful targeting must be determined experimentally on a case-by-case basis, as this also depends on the polymer system, the drug being encapsulated and the tumour microenvironment including its vascularity.

These authors have demonstrated the proof-of-concept nanoparticle–aptamer bioconjugates and believe that, when appropriately optimised, these vehicles may be widely used for targeted drug delivery and treatment of a myriad of cancers. By addressing the challenges that are outlined in this article, the promise of nanotechnology-based cancer therapies may be realised.

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